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Primary Hyperoxaluria Type 1: Genotypic and Phenotypic Heterogeneity

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Summary: Primary hyperoxaluria type 1 (PH1) is an autosomal recessive disease caused by a deficiency of the liver-specific peroxisomal enzyme alanine: glyoxylate aminotransferase (AGT). The disease is notable for its extensive heterogeneity at the clinical, biochemical, enzymic and molecular genetic levels. A study of 116 PH1 patients over the past 8 years has revealed four main enzymic phenotypes: (1) absence of both AGT catalytic activity and immunoreactive AGT protein (~40% of patients); (2) absence of AGT catalytic activity but presence of immunoreactive protein (~16% of patients); (3) presence of both AGT catalytic activity and immunoreactive protein (~41% of patients), in most of which cases the AGT is mistargeted to the mitochondria instead of the peroxisomes; and (4) a variation of the mistargeting phenotype in which AGT is equally distributed between peroxisomes and mitochondria, but in which that in the peroxisomes is aggregated into matrical core-like structures (~3% of patients). Various point mutations, all occurring at conserved positions in the coding regions of the AGT gene, have been identified in these patients. The five mutations discussed in the present study, which have been found in individuals manifesting all of the four major enzymic phenotypes, account for the expressed alleles in about half of all Caucasian PH1 patients. The most common mutation found so far leads to a Gly170 → Arg amino acid substitution. This mutation, in combination with a normally occurring Pro11 → Leu polymorphism, appears to be responsible for the unprecedented peroxisome-to-mitochondrion mistargeting phenotype.

PH1 AND AGT DEFICIENCY

Primary hyperoxaluria type 1 (PH1, McKusick 259900) is a rare autosomal recessive inborn error of glyoxylate metabolism, characterized biochemically by increased synthesis and excretion of oxalate and glycolate, and clinically by the deposition of insoluble calcium oxalate, initially in the kidneys as urolithiasis and/or nephrocalcinosis, but, following renal failure, also throughout the body as systemic oxalosis (Williams and Smith 1983). The increased synthesis of oxalate and glycolate is caused by a deficiency of the liver-specific peroxisomal enzyme alanine: glyoxylate aminotransferase (AGT, EC 2.6.1.44), the normal role of which is to catalyse the

transamination (detoxification) of glyoxylate to glycine, using alanine as the amino donor (Danpure and Jennings 1986). AGT deficiency in PH1 allows the glyoxylate to be oxidized to oxalate within the peroxisome, catalysed by glycolate oxidase (GO, EC 1.1.3.15), or to diffuse through the peroxisomal membrane into the cytosol, where it is oxidized to oxalate, catalysed by lactate dehydrogenase (LDH, EC 1.1.1.27) and reduced to glycolate, catalysed by glyoxylate reductase (GR, EC 1.1.1.26/79) and possibly also LDH (Danpure and Jennings 1986; Danpure 1989).

CLINICAL HETEROGENEITY

The clinical manifestations of PH1 are quantitatively and qualitatively heterogeneous with respect to the timing, rate of progression and relative contribution made by each of the pathological sequelae (Danpure 1991). In most patients, the first symptoms occur in early childhood due to the presence of urolithiasis. However, there is an enormous spread in the ages at which the disease first becomes apparent; it can be as early as the first few months of life or as late as the seventh decade. Typically, PH1 is a progressive disease; renal deposition of calcium oxalate continues inexorably, accompanied by a gradual deterioration of renal function, until end-stage renal failure in late childhood or early adulthood. Before the introduction of modern treatment methodologies (e.g. hepatorenal transplantation; Watts et al 1987, 1991), it has been estimated that 80% of PH1 patients died from renal failure before the age of 20 years (Williams and Smith 1983).

A minority (probably less than 10%) of patients have a much more severe form of the disease, sometimes called acute neonatal PH1. These patients typically present in the first few months of life with renal failure due to nephrocalcinosis without urolithiasis (Leumann 1985). Progression of the disease is very rapid and such patients frequently die before the end of the first year of life.

Although concomitant hyperoxaluria and hyperglycolic aciduria are the biochemical hallmarks of PH1, the relative proportions of these glyoxylate metabolites found in the body fluids of patients vary over a wide range. Some patients with proven AGT deficiency have isolated hyperoxaluria with no evidence of elevated glycolate synthesis (Danpure 1991), whereas others have marked hyperglycolic aciduria with only mild hyperoxaluria (Watts et al 1983).

Pyridoxal phosphate is an essential cofactor for AGT. Some PH1 patients (10–30%) are pyridoxine-responsive insofar as their oxalate excretion can be significantly lowered and their clinical symptoms can be markedly improved by the chronic administration of pharmacological doses of pyridoxine (Ludwig 1963; Gibbs and Watts 1970).

ENZYMIC HETEROGENEITY

AGT expression

The wide clinical heterogeneity of PH1 is matched by its equally wide enzymic heterogeneity (Danpure 1991; Danpure and Jennings 1988). In the present study, 49 of the 116 patients were found to have undetectable levels of immunoreactive AGT protein (CRM^-) (Figures 1 and 2). Of the remaining 67, who did possess

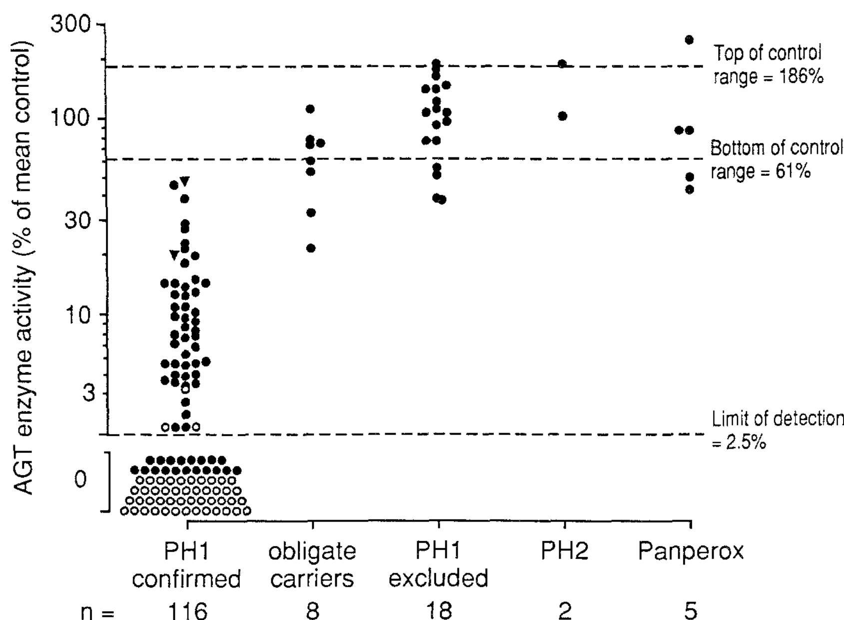

Figure 1 Hepatic AGT enzymic activity in PH1 patients, carriers and other individuals. Over a period of 8 years, 134 liver samples were assayed for AGT catalytic activity (Danpure and Jennings 1988; Allsop et al 1987) and immunoreactivity (Wise et al 1987) in order to obtain a definitive diagnosis for PH1. In 116 cases the diagnosis was confirmed and in 18 it was excluded. The results from these diagnoses are compared with the AGT activities in 8 asymptomatic carriers (parents of PH1 patients), 2 patients with primary hyperoxaluria type 2 (PH2, glyoxylate reductase/D-glycerate dehydrogenase deficiency) and 5 panperoxisomal disease patients. Open circles, CRM^-; closed circles, CRM^+; triangles, patients with some clinical characteristics of PH1 (together with the existence of affected siblings) but in whom the diagnosis is in doubt owing to the presence of significant AGT catalytic activity but the absence of any subcellular distribution data. Control range is taken from Danpure and Jennings (1988). Note the overlap between the AGT activities found in some of the patients and those found in some of the asymptomatic carriers, and that the levels of AGT are normal in PH2 and in various patients with aberrant peroxisomal biogenesis

immunoreactive AGT protein (CRM^+), nearly three-quarters also had detectable levels of AGT catalytic activity, which varied from only just above the lower limit of detection (2.5%) up to nearly 50% of the mean normal value (Figure 1). Some of the patients in the latter category could not be distinguished from some of the asymptomatic obligate carriers on the basis of enzyme activity alone (Figure 1). Although there was an approximate relationship between hepatic AGT activity and clinical severity, it was not clear enough to be prognostically useful (Danpure 1991).

Subcellular distribution of AGT

Normal distribution: In most normal individuals, AGT is localized exclusively in the peroxisomes of liver parenchymal cells (Figure 3A) (Noguchi and Takada 1979; Yokota et al 1987; Cooper et al 1988). However, in some people, although most of

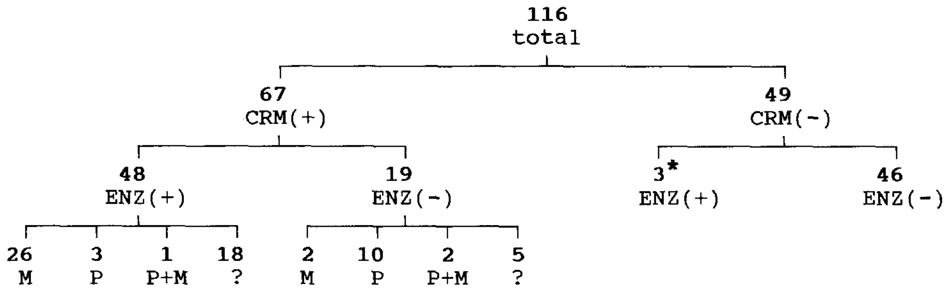

Figure 2 Summary of the AGT phenotypes found in 116 PH1 patients. CRM, presence (+) or absence (−) of imunoreactive AGT protein; ENZ, presence (+) or absence (−) of AGT catalytic activity; M, mainly (~90%) mitochondrial AGT; P, entirely peroxisomal AGT. P + M, peroxisomal and mitochondrial AGT, that in the peroxisomes being aggregated into cores; ?, unknown subcellular distribution of AGT. Theoretically, it should not be possible for CRM^- livers to possess AGT catalytic activity. However three CRM^- liver samples were ENZ^+ (*), albeit with low levels (only just above the limit of detection in the case of two of them). Unfortunately, owing to the very small size of these particular samples (< 2 mg), the assay could not be repeated. Note that the subcellular distribution of AGT can only be determined by protein A–gold immunoelectron microscopy in CRM^+ samples. When AGT catalytic activity is present in PH1 (i.e. ENZ^+) the AGT is almost always mitochondrial; when AGT is peroxisomal it is almost always catalytically inactive

the AGT remains located within the peroxisomal matrix, a small, but significant, amount (~5%) is also found located in the mitochondria (Figure 3B) (Purdue et al 1990).

Distribution in PH1: In most of the PH1 patients who were CRM^+/ENZ^- in the present study (16% of the total), the immunoreactive but catalytically defunct AGT protein was also localized totally within the peroxisomes (Figure 3C) (Cooper et al 1988). However, in most of the CRM^+/ENZ^+ patients, who comprised about 41% of the total, the intracellular distribution of AGT was found to be very different. In these patients, about 90% of the AGT was localized in the mitochondria and only 10% in the peroxisomes (Figures 3E and 3F) (Danpure et al 1989). This intracellular protein trafficking defect, which is without precedent in human genetic disease, was specific for AGT as the subcellular distribution of other peroxisomal enzymes remained unaltered. It would appear that AGT in human liver is unable to carry out its metabolic function (i.e. glyoxylate detoxification) properly when located within the mitochondria instead of the peroxisomes.

A small proportion of PH1 patients (3 so far identified) expressed a variation of the mistargeting phenotype. These $CRM^+/ENZ^\pm$ patients had low, but detectable, levels of immunoreactive AGT protein but little or no AGT catalytic activity. AGT in these individuals was more or less equally divided between peroxisomes and mitochondria (Danpure et al 1993). However, the AGT in the peroxisomes appeared to be aggregated into matrical core-like structures (Figure 3G) that did not contain any of the other peroxisomal enzymes tested.

Although most patients with mistargeted AGT fell into the CRM^+/ENZ^+ category,

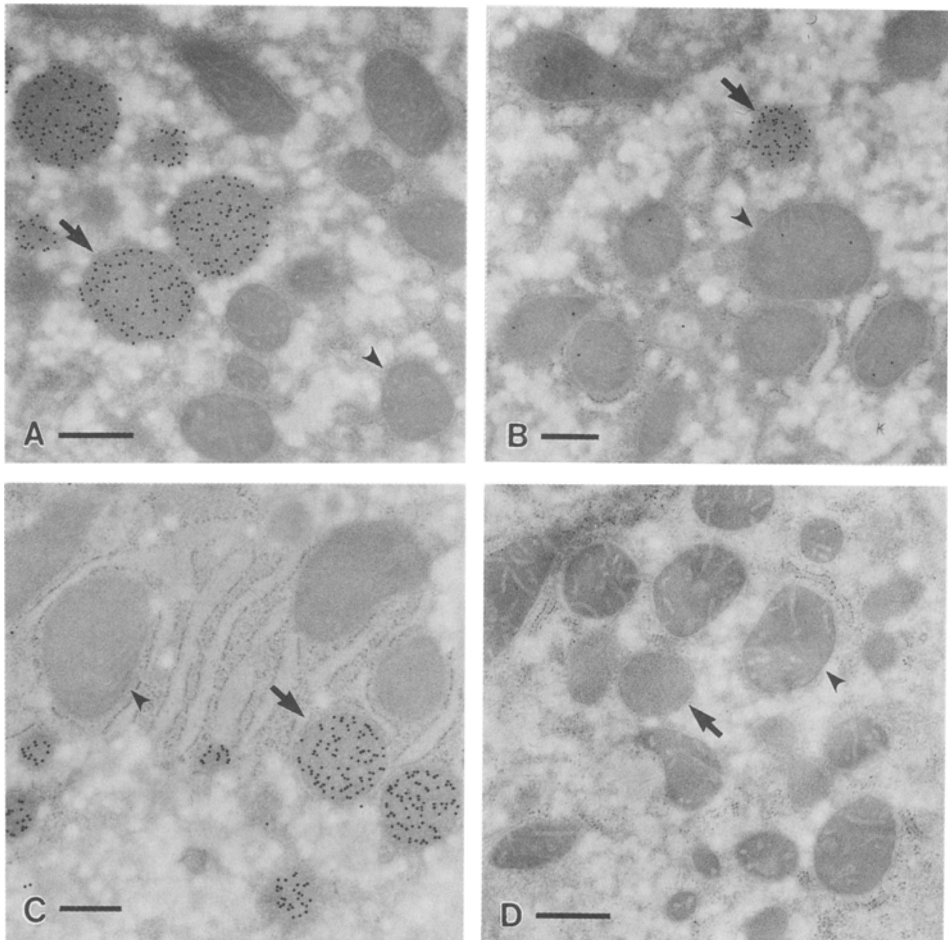

Figure 3 Subcellular distribution of immunoreactive AGT by protein A–gold immunoelectron microscopy. Liver sections were analysed for immunoreactive AGT protein by post-embedding protein A–gold (20 nm) immunoelectron microscopy (Cooper et al 1988; Danpure et al 1989). Arrows, peroxisomes; arrow heads, mitochondria; bars = 0.5 μm. (A) Normal control homozygous for the major AGT allele (100% peroxisomal AGT). (B) Normal control homozygous for the minor AGT allele, encoding the Pro11 → Leu polymorphism (95% peroxisomal + 5% mitochondrial AGT). (C) CRM^+/ENZ^- PH1 patient homozygous for the Gly82 → Glu mutation (100% peroxisomal AGT). (D) CRM^-/ENZ^- PH1 patient (no labelling).

two patients who were $CRM^{\pm}/ENZ^-$ also had detectable, albeit very low, levels of mitochondrial AGT (Figure 3H) (Danpure et al 1993).

MUTATIONS AND POLYMORPHISMS IN THE AGT GENE

The normal AGT gene

The human AGT gene, which maps to chromosome 2q36-37, consists of 11 exons spanning about 10 kb (Purdue et al 1991c). It encodes a 392 amino acid protein of

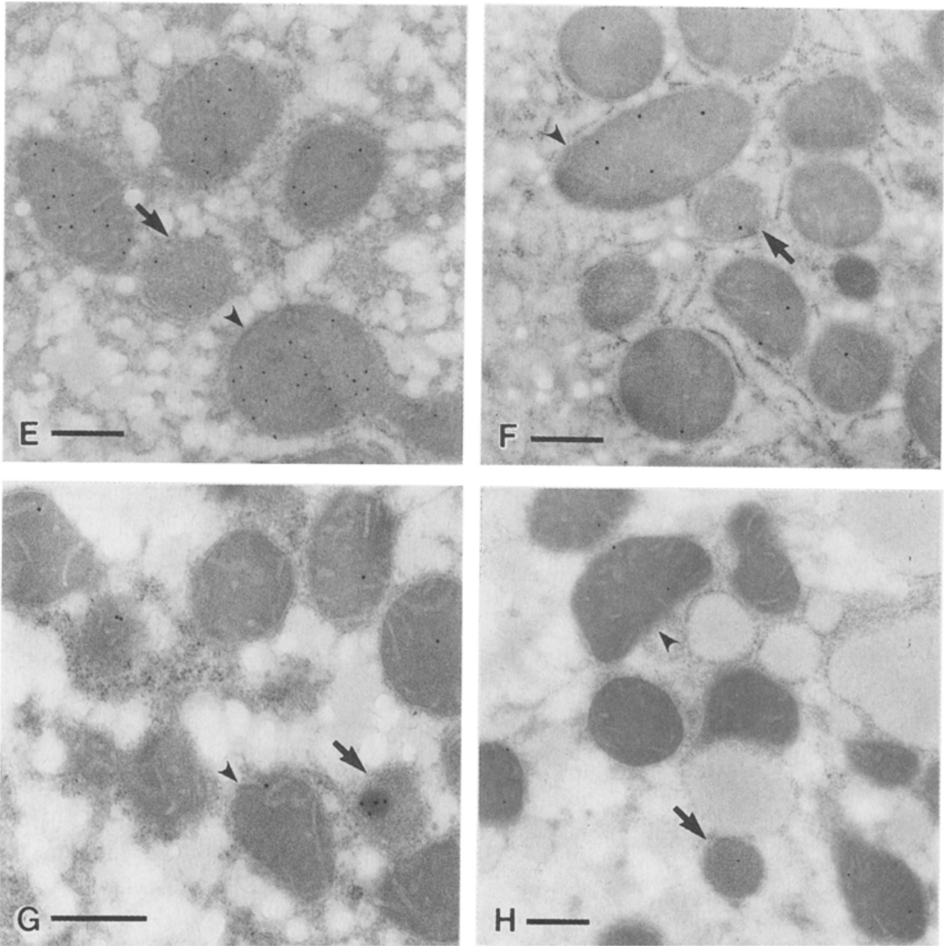

Figure 3 *continued* (E) CRM$^+$/ENZ$^+$ PH1 patient homozygous for the Gly170 → Arg mutation and the Pro11 → Leu polymorphism (10% peroxisomal + 90% mitochondrial AGT). (F) CRM$^+$/ENZ$^+$ PH1 patient heterozygous for the Gly170 → Arg mutation and the Pro11 → Leu polymorphism (10% peroxisomal + 90% mitochondrial AGT). (G) CRM$^+$/ENZ$^\pm$ PH1 patient heterozygous (compound) for the Phe152 → Ile and the Gly41 → Arg mutations and homozygous for the Pro11 → Leu polymorphism (50% peroxisomal + 50% mitochondrial AGT, peroxisomal labelling confined to core-like structures). (H) CRM$^\pm$/ENZ$^-$ PH1 patient heterozygous for the Phe152 → Ile mutation and the Pro11 → Leu polymorphism (labelling too low to quantitate, probably > 75% mitochondrial)

43 kDa (Takada et al 1990). Two different AGT alleles have been identified in normal individuals. The less common (minor) allele differs from the more common (major) allele in at least three positions, two of which (i.e. C154 → T and A1142 → G point base substitutions) lead to single amino acid alterations (i.e. Pro11 → Leu and Ile340 → Met substitutions, respectively) (Figure 4) (Purdue et al 1990). Although

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               (L)                               R
                ↑                                ↑
1     MASHKLLVTPPKALLKPLSIPNQLLLGPGPSNLPPRIMAAGGLQMIGSMS   50

                         Ter             E
                          ↑              ↑
51    KDMYQIMDEIKEGIQYVFQTRNPLTLVISGSGHCALEAALVNVLEPGDSF  100


101   LVGANGIWGQRAVDIGERIGARVHPMTKDPGGHYTLQEVEEGLAQHKPVL  150

      I                    R                    F
      ↑                    ↑                    ↑
151   LFLTHGESSTGVLQPLDGFGELCHRYKCLLLVDSVASLGGTPLYMDRQGI  200

         P
         ↑   *
201   DILYSGSQKALNAPPGTSLISFSDKAKKKMYSRKTKPFSFYLDIKWLANF  250


251   WGCDDQPRMYHHTIPVISLYSLRESLALIAEQGLENSWRQHREAAAYLHG  300

                                             (M)
                                              ↑
301   RLQALGLQLFVKDPALRLPTVTTVAVPAGYDWRDIVSYVIDHFDIEIMGG  350


351   LGPSTGKVLRIGLLGCNATRENVDRVTEALRAALQHCPKKKL  392
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Figure 4 Amino acid sequence of human AGT showing the PH1-specific mutations and the normal polymorphisms. The sequence, as deduced from the cDNA sequence, has 392 amino acid residues and a calculated relative molecular mass of 43 046 (Takada et al 1990). The residues double underlined are those conserved between the human, marmoset, rat and rabbit (Oda et al 1987; Takada et al 1990; Purdue et al 1992b). Various mutations found in PH1 patients are indicated. The amino acid substituions in brackets are the normal polymorphic variations encoded by the minor AGT allele. The asterisk marks the putative pyridoxal phosphate cofactor binding site (Oda et al 1987)

the frequency of the minor AGT allele was initially estimated to be 5–10% (Purdue et al 1990), further studies have led to a re-estimation of its frequency to be closer to 20% in Caucasian populations (unpublished observations).

Ile340 is not evolutionarily conserved (Oda et al 1987; Takada et al 1990; Purdue et al 1992b) and therefore its replacement by Met probably has no functional significance. However, Pro11 is conserved and its substitution by Leu is predicted, and has been shown, to have a major effect on the functional properties and behaviour of AGT. This substitution enables the N-terminus of the protein to fold into an amphiphilic α-helix (Purdue et al 1990). Such structures are typical of mitochondrial targeting sequences (MTSs) (von Heijne 1986). The existence of the minor AGT allele in the normal population explains the presence of small amounts of AGT localized

in the mitochondria in a minority of normal individuals (see above). Although the MTS encoded by the minor allele appears to work reasonably well *in vitro* (Purdue et al 1991a), it is much less efficient *in vivo*, as shown by the fact that in a normal individual homozygous for the minor AGT allele only ~5% of the AGT is targeted and imported into the mitochondria (see Figure 3B) (Purdue et al 1990). Presumably, in the presence of components of the peroxisomal import machinery (i.e. *in vivo*), the MTS of AGT is unable to compete successfully with the peroxisomal targeting sequence.

Mutations in the AGT gene in PH1

So far six mutations, including one identified by another laboratory, have been found in the coding regions of the AGT gene in patients with PH1, including individuals with all of the well-characterized enzymic phenotypes. These are outlined in Figure 4 and Table 1.

Peroxisome-to-mitochondrion mislocalization of AGT: All of the 15 CRM^+/ENZ^+ patients so far studied, who were proved to possess the AGT mistargeting phenotype, and 4/6 of those CRM^+/ENZ^+ patients with unknown AGT intracellular distribution, were shown to express at least one minor AGT allele (i.e. that encoding the Pro11 → Leu polymorphism). In addition, these patients also expressed at least one allele containing a G630 → A point mutation, which was predicted to lead to a Gly170 → Arg amino acid substitution (see Table 1) (Purdue et al 1990). In all cases, the G630 → A mutation segregated with the minor AGT allele. The three patients homozygous for the mutation (and the minor allele) (see Table 1) had higher levels of AGT catalytic activity (27–39% of the mean normal value) compared with those who were heterozygous (5–15% of the mean normal level). In at least three of the heterozygous patients, the apparently 'normal' AGT allele (i.e. that not possessing the G630 → A mutation) appeared not to be expressed, at least in terms of stable mRNA.

It had been shown previously (see above) that the Pro11 → Leu polymorphism (encoded by the minor allele) generated a novel N-terminal MTS, which was only able to target about 5% of the AGT protein to the mitochondria when present homozygously (Purdue et al 1990). The additional presence of the Gly170 → Arg mutation in PH1 patients with the mistargeting phenotype increased the proportion of AGT targeted and imported into the mitochondria to about 90%. Gly170 is evolutionarily conserved, but its substitution by Arg in PH1 had no direct effect on the mitochondrial import of AGT *in vitro* (Purdue et al 1991a). Its effect *in vivo* appeared to be due to the inhibition of the peroxisomal targeting and/or import of AGT by an, as yet, undetermined mechanism.

Partial peroxisome-to-mitochondrion mislocalization and intraperoxisomal aggregation of AGT: Analysis of three unrelated $CRM^+/ENZ^\pm$ patients, characterized by the presence of low levels of mitochondrial immunoreactive AGT and AGT aggregated into peroxisomal cores, demonstrated the presence of a complex genotype (Danpure et al 1993). All patients were homozygous for the minor AGT allele and heterozygous

Table 1 AGT genotypes and their associated phenotypes found in PH1

				Normal poly	*PH1-specific mutations*					
Base substitutions				C 154 T	G 243 A	G 367 A	T 576 A	G 630 A	C 682 T	
AA substitutions				Pro 11 Leu	Gly 41 Arg	Gly 82 Glu	Phe 152 Ile	Gly 170 Arg	Ser 187 Phe	
Phenotype			N	Genotype						Genotype → Phenotype
ENZ	CRM	SUB								
+	+	M/m	12*	+ −	− −	− −	− −	+ −	− −	√
			3	+ +	− −	− −	− −	+ +	− −	√
−	±	m	1	+ +	− −	− −	+ −	− −	− −	√
			1	+ −	− −	− −	+ −	− −	− −	√
±	+	p+m	2	+ +	+ −	− −	− +	− −	− −	√
			1	+ +	+ −	− −	− −	− +	− −	√
−	+	P	3	− −	− −	+ +	− −	− −	− −	√
			3	− −	− −	− −	− −	− −	− −	x
			2	+ +	− −	− −	− −	− −	− −	x
+	+	?	2	+ +	− −	− −	− −	+ −	− −	√?
			2	+ −	− −	− −	− −	+ −	− −	√?
			2	− −	− −	− −	− −	− −	− −	x
−	+	?	1	− −	− −	− −	− −	− −	− −	x
−	−	−	7	− −	− −	− −	− −	− −	− −	x
			3	+ −	− −	− −	− −	− −	− −	x
			1	+ +	− −	− −	− −	− −	− −	x
			1*	− −	− −	− −	− −	− −	+ −	√

Normal poly: normal polymorphic variation found in the less-common minor AGT allele.
PH1-specific mutations: other base/amino acid (AA) substitutions so far only found in PH1 patients and their families.
Phenotype: presence (+) or absence (−) or AGT catalytic activity (ENZ) or immunoreactivity (CRM), and subcellular distribution (SUB) of the immunoreactive AGT protein. M/m, mainly mitochondrial P, entirely peroxisomal; p + m, equally peroxisomal and mitochondrial, that in the peroxisomes being aggregated into cores; ?, unknown; upper case, easily detectable levels of labelling; lower case, low levels of labelling.
N: number of patients in each category examined at the DNA level.
Genotype: allelic distribution of mutations/polymorphism (+, present; −, absent).
Genotype → Phenotype: those individuals in whom the genotype explains, at least partially, the enzymic phenotype observed (√ = does explain, x = does not explain, √? = would explain the phenotype if these CRM^+/ENZ^+ patients turned out to be mitochondrial variants).
*In at least 3/12 of the patients heterozygous for the G630 → A mutation and the 1 patient heterozygous for the C682 → T mutation, the apparently normal allele (i.e. that with '−' throughout) is not expressed. Not included in this table is the C320 → G (Tyr66 → Ter) mutation in the non-expressed allele in one of the patients heterozygous for the G630 → A mutation (Purdue et al 1991b) because there is no reason to attribute to it any functional significance, and neither is the T735 → C (Ser205 → Pro) mutation found homozygously in an isolated CRM^-/ENZ^- Japanese patient (Nishiyama et al 1991)

for a novel G243 → A point mutation, which would lead to a Gly41 → Arg amino acid replacement. In addition, two patients were heterozygous for a T576 → A (Phe 152 → Ile) mutation and one was heterozygous for the previously recognized G630 → A (Gly170 → Arg) mutation (see above) (Table 1). In all cases, the G243 → A mutation was present on a different allele to the T576 → A or G630 → A mutations. All of these mutations occurred at positions of evolutionary conservation, Phe152 and Gly170 being only 18 residues apart in the same internal 58-amino-acid region of exceptional conservation (residues 133–190, see Figure 4). Studies on the families of these patients indicated that the Phe152 → Ile mutation appeared to have the same effect as the Gly170 → Arg mutation (i.e. it inhibited the peroxisomal targeting and/or import of AGT) and, together with the Pro11 → Leu polymorphism encoded by the minor AGT allele, was responsible for the presence of mitochondrial AGT in these patients. In addition, it was shown that the Gly41 → Arg mutation was likely to be responsible for the intraperoxisomal aggregation of AGT, either on its own or in combination with the Pro11 → Leu polymorphism. Such an interpretation was confirmed when it was found that two $CRM^{\pm}/ENZ^{-}$ patients who were heterozygous for the Phe152 → Ile mutation, but not possessing the Gly41 → Arg mutation, also had low levels of mitochondrial AGT, but not peroxisomal cores.

Normal localization of catalytically inactive AGT: Three unrelated CRM^{+}/ENZ^{-} patients were shown to be homozygous for the same G367 → A point mutation, which would lead to a Gly82 → Glu amino acid substitution (Table 1) (Purdue et al 1992a). All three patients were also homozygous for the more common major AGT allele. Another patient with the same enzymic phenotype did not possess this mutation. Gly82 is evolutionarily conserved (Figure 4) and its substitution by Glu is predicted to cause considerable local structural alterations. Although this substitution is at some distance, at least in linear terms, from the putative cofactor binding site at Lys209 (Oda et al 1987) (Figure 4), it might come into the proximity of the active site in the correctly folded protein.

Absence of AGT catalytic activity and immunoreactivity: The overt phenotypic homogeneity of the large group of CRM^{-}/ENZ^{-} PH1 patients is probably more apparent than real. At the molecular level, disease in these patients is likely to be the result of numerous different mutational events. Only two such patients have been studied at the DNA level, one from the presently studied group of 116 patients (Minatogawa et al 1992) and one (a Japanese patient) from another laboratory (Nishiyama et al 1991). Both patients expressed relatively normal levels of AGT mRNA, but were completely, or nearly completely, deficient in immunoreactive AGT protein. The first patient was heterozygous for a C682 → T point mutation, which is predicted to cause a Ser187 → Phe amino acid substitution (Table 1) (Minatogawa et al 1992). The other apparently 'normal' allele appeared not to be expressed. The second patient was homozygous for a T735 → C point mutation, which would specify a Ser205 → Pro amino acid replacement (Nishiyama et al 1991). Both patients were homozygous for the major AGT allele.

Ser187 and Ser205 are evolutionarily conserved and only 18 residues apart (Figure

4). Although there is at present no evidence to support such a contention, their substitution by Phe and Pro, respectively, might lead to a decrease in protein stability and rapid degradation, thus explaining the CRM^- phenotype. A number of other CRM^-/ENZ^- patients in the presently studied group were screened for both of these mutations but no further examples were found (Table 1).

Miscellaneous: It is probable that, in most CRM^+/ENZ^+ PH1 patients with the AGT peroxisome-to-mitochondrion mistargeting phenotype who are heterozygous for Pro11 → Leu/Gly170 → Arg, the apparently 'normal' allele is not expressed, at least in terms of stable mRNA species. Nevertheless, the non-expressed allele was studied in one such patient and a C320 → G mutation was found, which would alter Tyr66 into a stop codon (Figure 4) (Purdue et al 1991b). However, the significance of this finding is not clear.

CONCLUSIONS

There are few, if any, monogenic diseases that can claim such phenotypic heterogeneity as that found in PH1. Although our understanding of the causal relationship between genotype and enzymic phenotype in PH1 is beginning to be elucidated, there is still much that is unknown. For example, the molecular basis of the frequent occurrence of apparent allelic non-expression is not understood, and neither is the molecular basis of pyridoxine responsiveness. Even when a particular mutation has been identified, a full understanding of its mechanism of action will have to wait for the determination of the three-dimensional structure of AGT. The relationship between enzymic heterogeneity and clinical heterogeneity is even less well understood. Our ignorance in this respect probably reflects the importance of other, as yet undetermined, genetic and environmental factors that contribute to the severity of the disease sequelae.

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