Immunocytochemical Localization of Human Hepatic Alanine:Glyoxylate Aminotransferase in Control Subjects and Patients with Primary Hyperoxaluria Type 1

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Primary hyperoxaluria type 1 (PH1) is an inherited disorder of glyoxylate metabolism caused by a deficiency of the hepatic peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44) [FEBS Lett (1986) 201:20]. The aim of the present study was to investigate the intracellular distribution of immunoreactive AGT protein, using protein A-gold immunocytochemistry, in normal human liver and in livers of PH1 patients with (CRM+ or without (CRM-)) immunologically crossreacting enzyme protein. In all CRM+ individuals, which included three controls, a PH1 heterozygote and a PH1 homozygote immunoreactive AGT protein was confined to peroxisomes, where it was randomly dispersed throughout the peroxisomal matrix with no obvious associations with the peroxisomal membrane. No AGT protein could be detected in the peroxisomes or other cytoplasmic compartments in the livers of CRM+ PH1 patients (homozygotes). The peroxisomal labeling density in the CRM+ PH1 patient, who was completely deficient in AGT enzyme activity, was similar to that of the controls. In addition, in the PH1 heterozygote, who had one third normal AGT enzyme activity, peroxisomal labeling density was reduced to 50% of normal. (J Histochem Cytochem 36:1285–1294, 1988)

KEY WORDS: Alanine:glyoxylate aminotransferase; Primary hyperoxaluria type 1; Immunocytochemistry; Protein A-gold; Peroxisomes; Human liver.

Introduction

The autosomal recessive disease primary hyperoxaluria type 1 (PH1) is caused by a deficiency of peroxisomal alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44) activity in the liver (Danpure and Jennings, 1986). Heterogeneity in PH1 is manifested clinically (Williams and Smith, 1983), enzymologically (Danpure et al., 1987), and immunologically (Wise et al., 1987). In the latter case, nine out of 12 AGT-deficient PH1 patients had no detectable immunoreactive AGT protein (CRM+). Of the remaining three CRM+ patients, two were completely deficient in AGT enzyme activity and one was partly deficient. Human liver AGT activity is mainly peroxisomal (Noguchi and Takada, 1979). In experimental animals, AGT is either partly peroxisomal and partly mitochondrial (Noguchi et al., 1978a, 1978b, 1979) or entirely mitochondrial (Okuno et al., 1979), depending on the species. Ultrastructural observations in PH1 are scarce. However, unlike some other peroxisomal diseases, such as Zellweger’s syndrome (Goldfischer et al., 1973), peroxisomes are present in the livers of PH1 patients, although they may be somewhat smaller and less numerous than normal (Iancu and Danpure, 1987).

In this study we have investigated, using the protein A-gold technique, the intracellular distribution of immunoreactive AGT protein in hepatocytes of normal individuals and individuals heterozygous and homozygous for PH1.

Materials and Methods

Livers. Samples were obtained from three control livers by percutaneous needle biopsy. All were biopsied for suspected liver disease but two were subsequently diagnosed as normal (nos. 7 and 8), and the third was diagnosed as having Gilbert’s syndrome (no. 6) (see McGee et al., 1975). All three had histologically normal livers. The liver sample from the PH1 heterozygote (no. 4) was obtained by open biopsy while the patient was under general anesthesia for other surgery. Three pyridoxine-unresponsive PH1 patients (nos. 1, 2, and 5) had undergone total hepatectomy prior to combined liver and kidney transplantation (see Watts et al., 1987). The liver sample for patient no. 3 was obtained by percutaneous needle biopsy.

Informed consent was given in each case, and these studies were approved by the Ethical Committee of the Harrow Health Authority.

Tissue Preparation. Liver samples were cut into 1-mm cubes and fixed in either 1 or 3% glutaraldehyde in 100 mM phosphate buffer, pH 7.4,
for 1–2 h at room temperature. For routine morphological observations, tissues were post-fixed in 1% osmium tetroxide in 100 mM phosphate buffer, pH 7.4, for 1 h at room temperature. Tissues were then dehydrated in a graded acetone series and infiltrated with Spurr resin (Agar Scientific; Stanstead, UK)/acetone mixture (1:1) for 2 h, followed by three 180-min changes of absolute resin before embedding in Beem capsules and polymerizing at 65°C for 20 h. However, Spurr resin is unsuitable for immunocytochemical studies of peroxisomal proteins, whereas methacrylate resins have been used successfully (Bendayan et al., 1987; Yokota, 1986; Yokota and Oda, 1984). In the present study, methacrylate (Lowicryl K4M) embedding was also necessary for successful localization of human liver AGT protein, since preliminary experiments on exchanted OsO₄-fixed Spurr-embedded liver (see Bendayan and Zollinger, 1983) failed to reveal any specific labeling. For localization of AGT protein by immunocytochemistry, slices of liver tissue were first quenched for 60 min in 50 mM ammonium chloride in 10 mM PBS, then dehydrated in a graded methanol series and embedded in Lowicryl K4M (Agar Scientific). The following method, adapted from Roth et al. (1981) and Carlemalm et al. (1982), produced optimal results: 20 min in 30% (v/v) methanol at 4°C, 20 min in 50% methanol at 4°C, 20 min in 80% methanol at 20°C, 20 min in 90% methanol at 20°C. Tissues were then progressively infiltrated at −20°C with a 1:1 mixture of 90% methanol and freshly mixed de-gassed Lowicryl K4M resin for 40 min, a 1:2 mixture of 90% methanol and resin for 40 min, followed by five or more hourly changes of pure resin before embedding in Beem capsules. Polymerization with UV light was performed at −20°C for 24–36 hr, the capsules being inverted after 12–24 hr to enhance complete polymerization. Thin sections were cut on a diamond knife and collected onto formvar-coated grids. For morphological examination, Spurr-embedded sections were contrasted with Reynold’s lead citrate for 2 min. After the immunocytochemical procedures, Lowicryl-embedded sections were stained for 7 min in 4% aqueous uranyl acetate and 2 min in Reynold’s lead citrate. All electron microscopic examinations were performed using a Phillips EM 200 at 60 kV.

Antiserum Preparation. AGT was purified from normal human liver by a modification of the method of Thompson and Richardson (1967), yielding a single silver-staining band of molecular weight 40 kD on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Antiserum to the purified AGT were raised in New Zealand White rabbits. AGT protein (100 μg) was injected subcutaneously in Freund’s complete adjuvant, followed by three further injections of 100 μg in incomplete adjuvant at three-week intervals. The rabbits were bled out three weeks after the last injection. Immunoblotting against SDS–PAGE-fractionated normal liver proteins demonstrated that the antiserum detected the 40 kD AGT subunit and a contaminant of molecular weight 55 kD (Wise et al., 1987). In normal liver this contaminant was spread through a variety of subcellular compartments and was not peroxisomal, whereas the 40 kD band was only peroxisomal (Wise et al., 1987).

For some experiments, IgG was affinity purified from the antiserum using protein A-Sepharose (Goudswaard et al., 1978) and absorbed against human liver proteins from a CRM–PHI liver (Wise et al., 1987). The latter was carried out by sonication of a sample of a PHI liver that was CRM– for AGT (i.e., did not contain any detectable immunoreactive AGT protein) to give a 10% suspension in PBS, pH 7.2. After removing any fibrous material by low-speed centrifugation (1000 × g for 10 min), the liver proteins were denatured by heating at 100°C for 10 min. The insoluble material was collected by centrifugation (1000 × g for 10 min) and washed once with PBS, pH 7.2. Protein A-Sepharose-purified IgG was added at a ratio of about 1 mg IgG to 25 mg denatured liver proteins and placed on a rotary mixer overnight at 4°C. Insoluble liver protein–IgG complexes were removed by centrifugation (1000 × g for 10 min). Immunoblotting of SDS–PAGE-fractionated normal liver proteins, using the affinity-absorbed IgG, showed a single immunoreactive band of 40 kD (AGT) without detectable impurities.

Preliminary immunolabeling experiments indicated that the crude antiserum and the affinity-absorbed IgG fraction showed similar labeling patterns for AGT in human hepatocytes, including low-background labeling. Therefore, the non-absorbed antiserum was used for the quantitative results of peroxisomal labeling densities.

Fixation Stability of AGT. The stability of AGT to aldehyde fixation was tested by dot-blotting (Moeremans et al., 1984). Briefly, 1-μl aliquots of serially diluted (20–1 ng/μl) AGT in 10 mM PBS were spotted onto nylon membranes (Hybond-N; Amersham International, Amersham, UK), allowed to dry for 20 min, and then left unfixed or fixed in 1% or 3% glutaraldehyde in 10 mM PBS (Dubelcoo’s formula) for 10 min at room temperature. Protein blocking was carried out with 10% bovine serum albumin (BSA) in PBS, containing 20 mM sodium azide, for 16 hr at 37°C. The membranes were then incubated with anti-AGT antiserum, diluted 1:1000 in PBS containing 1% (v/v) normal goat serum (Janssen Life Sciences; Wannage, UK), for 2 hr at room temperature in sealed polythene bags while rotating at 60 rpm. The membranes were then rinsed in three 100-ml volumes of 20 mM Tris-saline buffer, pH 8.2, containing 0.1% BSA, each for 5 min. The antigen–antibody complex was visualized by incubation for 2 hr at room temperature with goat anti-rabbit IgG conjugated to colloidal gold (Auroprobe BL, 15 nm; Janssen Life Sciences). The IgG–gold complex was diluted 1:100 in 20 mM Tris–saline buffer, pH 8.2, containing 0.1% BSA and 5% gelatin (Janssen Life Sciences). The membranes were then washed in two 100-ml volumes of Tris–saline, each for 5 min, and two 100 ml volumes of distilled water, each for 1 min. For silver enhancement, they were then incubated in freshly prepared Intense II solution (Janssen Life Sciences) for 10 min at room temperature and finally rinsed in distilled water for 5 min before air-drying.

Dot-blotting showed that fixation with glutaraldehyde (1 or 3%) did not affect the immunoreactivity of the AGT protein. In addition, preliminary experiments in control liver showed that the labeling densities were similar whether the tissue was fixed in 1% or 3% glutaraldehyde. Other immunocytochemical studies on the localization of various peroxisomal and mitochondrial proteins have reported a similar resistance to fixation (Bendayan et al., 1987; Yokota et al., 1987).

Preparation of Protein A–Gold Complex. Colloidal gold was prepared by reduction of tetrachloroauric acid (BDH Ltd.; London, UK) with sodium citrate (Taab Laboratories; Reading, UK), according to the method of Frens (1973) as described in detail by Bendayan (1984). The pH of the gold colloid was adjusted to 6.5 with 200 mM potassium carbonate solution. The amount of protein A (Pharmacia Fine Chemicals; Uppsala, Sweden) needed to stabilize the gold sol was determined as described by Horisberger and Rosset (1977), and the resulting complex was centrifuged and recovered as described by Slot and Geuze (1981). Before use, the diluted protein A–gold complex (in 1.5% BSA–PBS) was spun for 5 min in an Eppendorf centrifuge to remove any aggregates.

Immunocytochemical Procedures. Ultra-thin sections of liver embedded in Lowicryl resin were pre-incubated for 30 min in 3% BSA in PBS by floating the grids onto 50-μl drops of buffer. Grids were then transferred to drops of the antibody solution, optimally diluted 1:500 for non-absorbed antiserum or 10 μg IgG/ml for the absorbed affinity-purified antibody in the same buffer, for 2 hr at room temperature. Grids were rinsed in 5 drops of 3% BSA in PBS before incubating in drops of a 1:10 dilution of protein A–gold in 1.5% BSA–PBS for 30 min at room temperature. After the protein A–gold incubation, grids were rinsed in PBS and stream-washed in distilled water before counterstaining and examination by electron microscopy.

Control sections were incubated in a 1:500 dilution of pre-immune rabbit serum or with antiserum that had been adsorbed with an excess of AGT protein for 12 hr at 4°C before use, followed by the protein A–gold step.

Quantitative Evaluation. The density of gold labeling, indicating the
Figure 1. Electron micrographs of human liver embedded in Spurr resin. (a) control (CRM⁺, no. 8); (b) PH1 homozygote (CRM⁺, no. 5); (c) PH1 heterozygote (CRM⁺, no. 4); (d) PH1 homozygote (CRM⁺, no. 1). ←, peroxisomes; →, mitochondria. Bars = 0.5 μm.
presence of immunoreactive AGT protein, was evaluated as follows. From four replicate experiments for each individual, a total of 20 micrographs of randomly selected hepatocyte fields containing peroxisomes were taken and printed at a final magnification of × 25,630. All liver tissues sampled were labeled at the same time under identical conditions. Peroxisome profile areas were calculated using a "Kontron Moppe" image analyzer (Kontron Electronic; Slough, Bucks, UK) interfaced to a Commodore "PET" microcomputer. The number of gold particles overlaying each peroxisome profile was counted and expressed as particles per μm² (Aberne and Dunnill, 1982). The extent of non-peroxisomal labeling was similarly assessed (number of gold particles per μm² area of non-peroxisomal cytoplasm) to determine (a) the degree of nonspecific background staining, and (b) whether AGT was present at very low levels in the cytosol (or in other subcellular compartments), particularly in the PHI patients showing crossreacting protein by immunoblotting (Wise et al., 1987).

Results are expressed as the mean peroxisomal and non-peroxisomal labeling density for each individual. Nonspecific background labeling densities were also estimated from four individuals where the liver sections were incubated in pre-immune rabbit serum. It should be noted that the mean peroxisomal areas shown represent mean profile and not absolute areas, and size comparisons are therefore relative but not absolute (see Aberne and Dunnill, 1982 and Weibel, 1969 for a more detailed discussion).

Results

Gross liver pathology was normal in the PHI patients, apart from increased amounts of lipofuscin and hemosiderin resulting from frequent blood transfusions as reported previously (Iancu and Danpure, 1987). The peroxisomal morphology, whether embedded in Spurr resin (Figure 1) or Lowicryl K4M (Figures 2–4), was similar to that observed in control subjects. Peroxisomes were identified by their near-circular profile, moderately electron-opaque heterogeneous matrix, and absence of both crystallloid inclusions and cristae (Novikoff et al., 1973). Marginal plates were recognized as peripheral electron-dense bands (Sternlieb and Quintana, 1977) and were seen in specimens from both controls (Figure 2a) and PHI subjects (Figure 3c).

The mean peroxisomal profile areas and form factors were calculated from micrographs of randomly selected peroxisomal fields taken from Lowicryl-embedded tissue, primarily to calculate the labeling density for AGT. However, these data also provided information about the size of peroxisomes in controls and PHI patients (Table 1). Form factors were similar for both patients and controls. The mean peroxisomal area in the controls was 0.215 ± 0.01 μm², and in the CRM⁺ PHI homozygotes (livers 1, 2, and 3) was 0.165 ± 0.02 μm², values which were not significantly different (Student’s t-test).

These values are in good agreement with previous estimates of normal human hepatocyte peroxisome size (Sternlieb and Quintana, 1977; Novikoff et al., 1973). However, we were unable to confirm any significant reduction in the size of PHI peroxisomes as suggested earlier (Iancu and Danpure, 1987). This discrepancy may be due to the different resins used (see Bendayan et al., 1987) or to different methods of calculating peroxisomal size.

Although no attempt was made in the present study to quantify peroxisome frequency, in general they appeared to be less numerous in PHI patients than normal, as had been found previously (Iancu and Danpure, 1987). However, patient no. 5, who was unusual in that he possessed AGT protein (i.e., CRM⁺), was an exception, as he appeared to have more peroxisomes than normal.

In control livers, gold particles indicating the presence of immunoreactive AGT protein were found to be exclusively located in the peroxisomes (Figure 2). AGT protein was randomly distributed throughout the peroxisomal matrix. No evidence was seen for any preferential membrane binding or association with filamentous cores (see Yokota et al., 1987). Although the quantitation of membrane-associated antigens is difficult to assess because of the variable degree of membrane exposed during cleavage (Kellenberger et al., 1987; Griffiths and Hoppeler, 1986), it is clear that the vast majority of the gold particles were more than 23 nm (approximate length of IgG + protein A + 20 nm gold sol) from the peroxisomal membrane, where visible. Background labeling over the non-peroxisomal cytoplasm and nuclei was negligible in comparison to the specific labeling for AGT in the peroxisomes, and was similar to that observed in control incubations with pre-immune serum (Figure 4; Table 1).

In control livers, the mean peroxisomal labeling densities for AGT ranged from 81–144 gold particles per μm² (Table 1). A similar spread of values has been found previously for AGT enzyme activity in normal human livers (see Table 1 and Danpure and Jennings, in press).

Relative frequency histograms of the peroxisomal labeling densities for control and other CRM⁺ livers showed the distribution to be approximately Gaussian. In addition, a good correlation (p < 0.001) was found between peroxisomal profile area and the number of gold particles counted per peroxisomal profile (Figure 5). This latter finding is interesting, as it indicates that the intraperoxisomal concentration of AGT protein is independent of peroxisomal size (i.e., the amount of AGT protein per peroxisome is proportional to peroxisomal size).

In an individual who was heterozygous for PHI (no. 4), the distribution of immunoreactive AGT protein was found to be similar to that in the controls. Again, gold labeling was restricted to the peroxisomal matrix, no specific labeling being observed in other

Table 1. Labeling densities and other characteristics of livers used in the present study

<table>
<thead>
<tr>
<th>Liver no.</th>
<th>Type</th>
<th>AGT</th>
<th>Perox Area</th>
<th>Labeling density</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PH1</td>
<td>0.2</td>
<td>0.195</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>PH1</td>
<td>0.8</td>
<td>0.135</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>PH1</td>
<td>12.7</td>
<td>0.165</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Heterozygote</td>
<td>30.9</td>
<td>0.175</td>
<td>59</td>
</tr>
<tr>
<td>5</td>
<td>PH1</td>
<td>1.8</td>
<td>0.215</td>
<td>140</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>...</td>
<td>0.235</td>
<td>81</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>...</td>
<td>0.205</td>
<td>139</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>...</td>
<td>0.205</td>
<td>144</td>
</tr>
</tbody>
</table>

Abbreviations: CRM, presence or absence of immunologically crossreacting material, as determined by immunoblotting (see Wise et al., 1987); AGT, alanine-glyoxylate aminotransferase activity, expressed as a percentage of the mean control activity (normal range: 45.8–139.7 nmol/min/mg protein; mean = 75.0, n = 12) (see Danpure and Jennings, in press); Perox Area, peroxisomal profile area in μm². Labeling densities are expressed as the number of gold particles per μm² in peroxisomes (Perox) and non-peroxisomal cytoplasm or background (BG). The peroxisomal form factors were all between 0.925 and 0.955. For the area, form factor, and labeling density determinations 123–283 peroxisomes were measured. For the peroxisomal profile areas the SEM were all between 1–5%, while for the peroxisomal labeling densities the SEM were 2–4% and for BG labeling were 8–20%. Control incubations using pre-immune rabbit serum gave a BG labeling of 0.12 ± 0.02 gold particles per μm².
Figure 2. Electron micrographs of Lowicryl K4M-embedded control and PH1 heterozygote livers after immunogold labeling. (a) Control CRM⁺ liver (no. 6); (b) control CRM⁺ liver (no. 7); (c) PH1 heterozygote CRM⁺ liver (no. 4). Note that immunoreactive AGT protein is confined to the peroxisomes in all CRM⁺ individuals. Liver no. 4 (c) illustrates the reduced density of peroxisomal labeling in the PH1 heterozygote compared with the controls (a, b). , peroxisomes; , mitochondria; ---, marginal plate. Bars = 0.5 μm.
subcellular compartments (Figure 2c). Quantitation of the peroxisomal labeling density for this individual (Table 1) showed a reduction to 49% of the mean control value. This finding is compatible with the biochemical finding (see Table 1 and Danpure and Jennings, in press) that there is also a marked reduction of AGT enzyme activity to 31% of the mean control value.

The liver of PH1 patient no. 5 had been shown previously to be completely deficient in AGT enzyme activity yet to contain large amounts of immunoreactive AGT protein (i.e., CRM⁺) (Wise et al., 1987; Danpure and Jennings, in press). The immunolabeling results for this patient (Figure 3a) demonstrated that the inactive AGT protein was located in the peroxisomal matrix, as was the active AGT protein in the controls. In addition, the peroxisomal labeling density was similar to that of the controls and, as with the controls, there was no indication of any non-peroxisomal labeling (Table 1).

Most PH1 patients studied so far are CRM⁺ (Wise et al., 1987). In the two CRM⁺ PH1 patients examined in the present study (nos. 1 and 2), no labeling was seen in either peroxisomes or any other subcellular compartment (Figure 3b; Table 1). The background (peroxisomal and non-peroxisomal) gold labeling density was similar to the control incubations using pre-immune serum (Figure 4).

PH1 patient no. 3 has been shown previously to be only slightly CRM⁺ (Wise et al., 1987). We were unable to demonstrate any specific peroxisomal labeling in this patient (Figure 3c), a result that may reflect the lower sensitivity of protein A-gold immunocytochemistry in detecting very low antigen concentrations (Kellenberger et al., 1987).

Discussion

The protein A-colloidal gold technique has been used successfully for immunocytochemical localization of a variety of peroxisomal enzyme proteins in microorganisms (Kunau et al., 1987), experimental animals (Yokota et al., 1987), and humans (Litwin et al., 1987; Reddy et al., 1987). Protein A-gold labeling in conjunction with Lowicryl K4M embedding is particularly useful for quantitative
Figure 5. Correlation between gold particles and peroxisome profile area in CRM* subjects. (A) Control (no. 7), r = 0.763; (B) control (no. 6), r = 0.762; (C) PH1 heterozygote (no. 4), r = 0.634; (D) CRM* PH1 homozygote (no. 5), r = 0.845. These graphs illustrate that for each individual CRM* liver the concentration of AGT protein is not dependent on peroxisome size.

Using this technique, we have now demonstrated the exclusive peroxisomal localization of human liver AGT protein. This finding is compatible with the original observation of Noguchi and Takada (1979) that human liver AGT enzyme activity is also peroxisomal. It is also consistent with our previous demonstration, based on the classic homogenization and sucrose density gradient centrifugation, that immunoreactive AGT protein is peroxisomal in normal human liver, as it was in the livers of a PH1 heterozygote and a CRM* PH1 homozygote (Wise et al., 1987). The present study also demonstrates that, despite the AGT in the CRM* PH1 patient being completely inactive, its intracellular distribution is normal. In addition, this study has shown that AGT protein is found in similar concentrations in all hepatocyte peroxisomes from the same pa-
tient, even in the CRM heterozygote with less than half the normal AGT activity and crosreactivity.

Yokota and colleagues (Yokota, 1986; Yokota and Oda, 1983, 1984) have examined immunocytochemically the intracellular distribution of serine:pyruvate aminotransferase (SPT; EC 2.6.1.51) in rat eosinophil leukocytes and liver cells, where it was found to be concentrated mainly in the mitochondria and peroxisomes. Rat liver SPT is identical to AGT1 (Noguchi and Takada, 1978), which is equivalent to human liver AGT. However, in the rat the enzyme activity is found not only in peroxisomes but also in mitochondria (Noguchi et al., 1978a, 1978b, 1979).

In the present study, the gold particles appeared to be distributed randomly across the matrix of the peroxisomes, with no apparent specific association with the peroxisomal membrane. This observation appears to be at variance with that of Chandoga et al. (1986), who, using homogenization and subcellular fractionation techniques, found that most AGT enzyme activity was bound to the peroxisomal membrane in human liver. Whether this is an artifact resulting from nonspecific artifact is not clear. In rat kidney peroxisomes, Yokota et al. (1987) found that over 90% of the immunoreactive SPT protein was located in the peripheral matrix. However, in the mitochondria there was some preferential localization in the cristae.

A number of previous studies on the intracellular localization of AGT/SPT, using both immunocytochemical techniques (Yokota and Oda, 1984) and subcellular fractionation techniques (Takada and Noguchi, 1982), have reported the presence of substantial amounts of cytosolic enzyme protein or activity. The former may be due to the low level of specific labeling, and more recent works (Yokota et al., 1987; Yokota, 1986) have failed to report the presence of cytosolic immunoreactive SPT protein. Apparent cytosolic AGT enzyme activity after homogenization and subcellular fractionation is probably due to peroxisomal breakage (Takada and Noguchi, 1981). Our finding that there was negligible extraperoxisomal gold labeling supports our previous suggestion that the cytosolic AGT enzyme activity observed after homogenization and sucrose gradient centrifugation is due to a different enzyme, i.e., glutamate:glyoxylate aminotransferase (Danpure and Jennings, 1986).

As is probably the case for most other inherited metabolic diseases, many different mutations affecting the AGT gene will lead to the clinical condition characteristic of PH1. Therefore, it is not surprising to find heterogeneous phenotypic expression both in molecular terms (e.g., presence or absence of immunoreactive AGT protein and AGT enzyme activity) and clinical terms (e.g., age of onset, severity, and pyridoxine responsiveness). However, the relationship between the two is not always clear. Whereas the absence of immunoreactive AGT protein inevitably leads to the conclusion that the patient will have no AGT enzyme activity and will therefore be unresponsive to pharmacological doses of pyridoxine, the reverse does not apply. For example, patient no. 5 looks completely normal immunocytochemically, yet he has no enzyme activity and was severely affected enough to require a combined liver/kidney transplant. His condition could be explained by a point mutation that irrevocably affected the substrate or co-factor binding sites, with transcription, translation, translocation, and degradation being unaffected.

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Literature Cited
Bendayan M (1984): Concentration of amylase along its secretory pathway in the pancreatic acinar cells as revealed by high resolution immunocytochemistry. Histochem J 16:85
McGeer JOD, Allan JG, Russel RI, Patrick RS (1975): Liver ultrastructure
in Gilbert's disease. Gut 16:220


Sterlieb I, Quintana N (1977): The peroxisomes of human hepatocytes. Lab Invest 36:140


