Correction of an enzyme trafficking defect in hereditary kidney stone disease *in vitro*

Michael J. LUMB, Graeme M. BIRDSEY and Christopher J. DANPURE¹

Department of Biology, University College London, Gower Street, London WC1E 6BT, U.K.

In normal human hepatocytes, the intermediary-metabolic enzyme alanine:glyoxylate aminotransferase (AGT) is located within the peroxisomes. However, in approx. one-third of patients suffering from the hereditary kidney stone disease primary hyperoxaluria type 1, AGT is mistargeted to the mitochondria. AGT mistargeting results from the synergistic interaction between a common P11L (Pro¹¹ → Leu) polymorphism and a disease-specific G170R mutation. The polymorphism generates a functionally weak mitochondrial targeting sequence, the efficiency of which is increased by the mutation. The two substitutions together, but not in isolation, inhibit AGT dimerization, highlighting the different structural requirements of the peroxisomal and mitochondrial protein-import machineries. In the present study, we show that treatments known to increase the stability of proteins non-specifically (i.e. lowering the temperature from 37 to 30 °C or by

the addition of glycerol) completely normalize the intracellular targeting of mutant AGT expressed in transfected COS cells. On the other hand, treatments known to decrease protein stability (e.g. increasing the temperature from 37 to 42 °C) exacerbate the targeting defect. Neither of the treatments affects the relative efficiencies of the peroxisomal and mitochondrial protein-import pathways intrinsically. Results are discussed in the light of the known structural requirements of the two protein trafficking pathways and the formulation of possible treatment strategies for primary hyperoxaluria type 1.

Key words: alanine:glyoxylate aminotransferase, enzyme trafficking defect, kidney stone disease, mitochondrial protein targeting, peroxisomal protein targeting, primary hyperoxaluria type 1.

INTRODUCTION

The import of at least some proteins into mitochondria and peroxisomes is dependent not only on the presence of adequate topogenic information (i.e. the presence of targeting sequences within the polypeptide chain) but also on the ability of the cargo to adopt the correct conformation [1]. Most mitochondrial matrix proteins are targeted by poorly conserved basic N-terminal leader sequences, which have the ability to adopt basic amphiphilic α -helical structures [2]. On the other hand, most peroxisomal matrix proteins are targeted by a highly conserved C-terminal tripeptide based on the SKL motif [3,4]. In addition to the very different nature of the two types of targeting sequence, the structural requirements for import are also very different for the two organelles. Whereas mitochondrial proteins can only be imported as unfolded or loosely folded monomers [5–7], peroxisomal proteins can be imported as fully folded, cofactor-bound, functionally active oligomers [1,8–12].

Although the mechanisms behind these differences are not completely understood, their functional importance is shown clearly by the autosomal recessive disorder primary hyperoxaluria type 1 (PH1; MIM 259900) [13]. PH1 is characterized by increased synthesis and excretion of the metabolic end product oxalate and by the deposition of insoluble calcium oxalate in the kidney and urinary tract. It is caused by a deficiency of the enzyme alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44), which normally catalyses the conversion (detoxification) of the intermediary metabolite glyoxylate into glycine. AGT deficiency allows glyoxylate to be oxidized to oxalate instead. Although most patients have no AGT enzyme activity or immunoreactive AGT

protein, approx. one-third do have significant amounts of both. Disease in these patients occurs because their AGT is mistargeted from its normal location in the peroxisomes to the mitochondria [14]. Although AGT is still catalytically active in mitochondria, it appears to be unable to fulfil its metabolic function of glyoxylate transamination/detoxification efficiently in this location. The mistargeting of AGT is a result of the presence on the same allele of two amino acid substitutions, one a common P11L (Pro¹¹ → Leu) polymorphism with an allelic frequency of approx. 20 % in European and North American populations [13,15] and the other a rare disease-specific G170R mutation [16]. The polymorphism generates a cryptic mitochondrial targeting sequence (MTS), which, despite being sufficient to target the reporter protein green fluorescent protein (GFP) to mitochondria, is insufficient to do the same for AGT [17]. However, the polymorphic MTS is capable of targeting AGT to mitochondria if the protein also possesses the G170R mutation. This mutation by itself has no effect on AGT targeting [18,19]. Neither of the amino acid replacements has any direct effect on peroxisomal targeting, which is mediated, at least in part, by the atypical C-terminal type 1 peroxisomal targeting sequence (PTS1) KKL [19]. The functional synergism between the P11L polymorphism and the G170R mutation appears to be related to the fact that the two substitutions together, but not in isolation, inhibit AGT dimerization partially, a necessary prerequisite for its mitochondrial import [1,17].

For a multi-subunit protein, attainment of the correct quaternary structure is dependent, at least in part, on the attainment of the correct tertiary structure (i.e. correct folding). Incorrect folding, as well as the consequent decreased protein stability, aggregation, accelerated degradation and/or aberrant trafficking, appears to

Abbreviations used: AGT, alanine:glyoxylate aminotransferase; CFTR, cystic fibrosis transmembrane conductance regulator; GFP, green fluorescent protein; MTS, mitochondrial targeting sequence; PH1, primary hyperoxaluria type 1; PTS1, peroxisomal targeting sequence type 1; for brevity the one-letter system for amino acids has been used: P11L, for example, means Pro¹¹ → Leu.

¹ To whom correspondence should be addressed (e-mail c.danpure@ucl.ac.uk).

Table 1 Description of expression constructs used in the present study

Construct	Description
AGT	Normal AGT encoded by the more common major <i>AGXT</i> allele [41]
AGT[L ₁₁]	AGT containing the P11L polymorphism
AGT[L ₁₁ ,M ₃₄₀]	Normal AGT encoded by the less common polymorphic minor <i>AGXT</i> allele (i.e. containing the P11L and I340M polymorphisms) [16]
AGT[L ₁₁ ,M ₃₄₀ ,Δ _{390–392}]	AGT[L ₁₁ ,M ₃₄₀] with the C-terminal KKL deleted
AGT[L ₁₁ ,R ₁₇₀ ,M ₃₄₀]	PH1 mutant AGT that is mistargeted from the peroxisomes to the mitochondria [16]
AGT[L ₁₁ ,R ₁₇₀ ,M ₃₄₀ ,Δ _{390–392}]	AGT[L ₁₁ ,R ₁₇₀ ,M ₃₄₀] with the C-terminal tripeptide KKL deleted
AGT[L ₁₁ ,R ₁₇₀ ,M ₃₄₀ ,S ₃₉₀]	AGT[L ₁₁ ,R ₁₇₀ ,M ₃₄₀] with the C-terminal tripeptide KKL replaced by the canonical PTS1 SKL
AGT[L ₁₁] _{1–20} -GFP-SKL	GFP with the first 20 residues of polymorphic AGT fused to its N-terminus and the canonical PTS1 tripeptide fused to its C-terminus
Pre-mitoAGT	AGT possessing the ancestral N-terminal 22-amino-acid leader MTS (MFQALAKASAAPGSRAAGWVRT) [18]

be a common mechanism by which disease-causing mutations in protein-encoding genes achieve their pathological effects [20]. At least *in vitro*, there are many ways in which protein stability can be increased. Some are specific, such as the addition of inhibitors, cofactors and their analogues, whereas others are non-specific, such as decreasing the temperature or the addition of 'chemical chaperones', such as poly(ethylene glycol), sugars or glycerol [21]. In a number of cases, the effects of these treatments *in vitro* can be translated to effects *in vivo* [22–27]. To gain further insights into the effect of protein conformation and stability on mitochondrial and peroxisomal protein import at the cellular level, and to identify strategies that might be of use in the treatment of AGT mistargeting in PH1, we have studied the effects of temperature and glycerol on the targeting of various normal, polymorphic and mutant AGT constructs in transiently transfected COS cells.

EXPERIMENTAL

Expression constructs

The constructs used in the present study are described in Table 1. AGT (normal AGT encoded by the more common major *AGXT* allele), AGT[L₁₁], AGT[L₁₁,M₃₄₀] (polymorphic AGT encoded by the less common minor *AGXT* allele), AGT[L₁₁,M₃₄₀,Δ_{390–392}], AGT[L₁₁,R₁₇₀,M₃₄₀], AGT[L₁₁,R₁₇₀,M₃₄₀,S₃₉₀] and AGT[L₁₁,R₁₇₀,M₃₄₀,Δ_{390–392}] were prepared as described previously [19], as were pre-mitoAGT [28] and AGT[L₁₁]_{1–20}-GFP-SKL [17]. The expression vector pHYK [19] was used for all constructs except AGT[L₁₁]_{1–20}-GFP-SKL, which was cloned into pcDNA3 (Invitrogen).

Cell culture and transfection

COS-1a cells grown to 40–80% confluency were trypsinized and added to 13 mm coverslips in a 4-well plate at a concentration of 30000 cells/well. After 24 h incubation at 37 °C, the cells were transfected using Superfect transfection reagent according to the manufacturer's instructions (Qiagen, Crawley, West Sussex, U.K.). Serum- and antibiotic-free Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) was mixed with 1 μg of DNA to a final volume of 60 μl. Superfect (5 μl) was added and incubated at room temperature (22–24 °C) for 5–10 min, after which 350 μl of the complete medium was added [containing 10% (v/v) foetal calf serum and antibiotics] and the mixture was placed on the PBS-washed coverslips and incubated at 37 °C for 2–3 h. The cells were then transferred to the appropriate temperature and maintained for 24 or 48 h (see the Results section). In some experiments, glycerol was added to the medium to a concentration of 5% (w/v).

Fluorescence microscopy

After incubation, the cells were fixed in 4% (w/v) paraformaldehyde, permeabilized in 1.0% (w/v) Triton X-100 and immunolabelled as described previously [1,17,19]. In some experiments, Triton X-100 was replaced by 25 μg/ml digitonin. AGT was visualized using rabbit anti-human AGT antiserum and FITC-conjugated goat anti-rabbit IgG (Sigma). The peroxisomal marker catalase was visualized using guinea-pig anti-human catalase antiserum followed by biotinylated goat anti-guinea-pig IgG and Texas Red-avidin D (Vector Laboratories, Peterborough, U.K.). Mitochondria were visualized using the auto-fluorescent vital stain MitoTracker (Molecular Probes; Cambridge Bioscience, Cambridge, U.K.). The fluorescence of fluorescein and GFP was observed using excitation and emission wavelengths of 488 and 522 nm respectively. For Texas Red and MitoTracker, excitation and emission wavelengths of 568 and 605 nm were used. The fluorescent images were captured using a confocal laser-scanning MRC 1000 microscope (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.).

RESULTS

Polymorphic AGT is mistargeted from peroxisomes to mitochondria at high temperatures

Previous studies have shown that polymorphic AGT encoded by the minor *AGXT* allele (i.e. AGT[L₁₁,M₃₄₀]) is entirely peroxisomal when expressed in COS cells or human fibroblasts at normal body temperature (i.e. 37 °C) [1,19], although approx. 5% is mitochondrial in human hepatocytes *in situ* [16]. To determine whether temperature affects its distribution, AGT[L₁₁,M₃₄₀] was expressed in COS cells for 24 h at 30, 34, 37, 40 and 42 °C; results from the studies at 30, 37 and 42 °C are shown in Figure 1. At temperatures between 30 and 37 °C, AGT[L₁₁,M₃₄₀] was targeted only to peroxisomes (Figures 1a, 1b, 1e and 1f), but at 40 and 42 °C some of it was also targeted to mitochondria. The temperature-induced shift in distribution was gradual, so that only a small proportion was mitochondrial at 40 °C, whereas a significant proportion was mitochondrial at 42 °C (Figures 1i and 1j).

It was difficult to visualize adequately the degree of peroxisome-to-mitochondrion mistargeting of polymorphic AGT at higher temperatures, because significant amounts also remained in the peroxisomes. To overcome this observational problem, the experiment was repeated using a polymorphic AGT construct in which peroxisomal targeting was blocked by deletion of the C-terminal KKL. The resulting construct (AGT[L₁₁,M₃₄₀,Δ_{390–392}]) was completely cytosolic at 37 °C (Figures 2a and 2b), but a significant shift to mitochondria could be seen clearly at 42 °C (Figures 2c and 2d).

Figure 1 Effect of temperature on the distribution of normal and polymorphic variants of AGT

AGT[L₁₁,M₃₄₀] (**a, b, e, f, i, j**), AGT (**c, g, k**) and AGT[L₁₁] (**d, h, l**) were expressed in COS cells for 24 h at 30 °C (**a–d**), 37 °C (**e–h**) or 42 °C (**i–l**). In addition, AGT[L₁₁,M₃₄₀] was expressed for 24 h at 37 °C in COS cells that had been heat-shocked at 42 °C for 24 h (**m**). Cells were then dual-labelled for AGT (green) and either the peroxisomal marker catalase (red) (**a, e, i**) or the mitochondrial marker MitoTracker (red) (**b–d, f–h, j–m**). At 30 and 37 °C, there was no association between any of the constructs and the mitochondria. However, at 42 °C significant amounts of both AGT[L₁₁,M₃₄₀] and AGT[L₁₁], but not AGT, were co-localized with MitoTracker. Pre-heat-shock was not in itself sufficient to redirect AGT[L₁₁,M₃₄₀] to the mitochondria. Scale bar, 10 μ m.

To check whether the mistargeted polymorphic AGT was imported actually into mitochondria or was non-specifically attached to the mitochondrial outer membrane, the Triton X-100 permeabilization step in the immunofluorescence analysis was replaced by digitonin, a procedure that breaks the plasma membrane but leaves internal membranes intact. When treated in this way, mitochondrial AGT[L₁₁,M₃₄₀, Δ _{390–392}] could not be detected (Figures 2e and 2f), implying that it had been imported. Confirmation of this was also shown by the fact that the proton iono-

phore, carbonyl cyanide *m*-chlorophenylhydrazone, blocked the mistargeting at 42 °C (results not shown).

Temperature dependence of the import of polymorphic AGT is due to the presence of the P11L substitution

To identify the sequence determinants of AGT[L₁₁,M₃₄₀] mistargeting at high temperatures, the distribution of normal AGT

Figure 2 Effect of temperature on the distribution of polymorphic AGT lacking its C-terminal PTS1

AGT[L₁₁,M₃₄₀,Δ₃₉₀₋₃₉₂] was expressed in COS cells for 24 h at 37 °C (**a, b**) or 42 °C (**c-f**). Cells were then dual-labelled for AGT (**a, c, e**) and MitoTracker (**b, d, f**). The cells in (**a-d**) were permeabilized as usual with Triton X-100 before immunolabelling, whereas the cells in (**e, f**) were permeabilized with digitonin (see the Experimental section). AGT[L₁₁,M₃₄₀,Δ₃₉₀₋₃₉₂] was cytosolic at 37 °C (**a, b**), but significantly mitochondrial at 42 °C (**c, d**). After digitonin permeabilization (**e, f**), no mitochondrial immunolabelling could be detected, suggesting that the antigen was not accessible to the antibody. Scale bar, 10 μm.

encoded by the major *AGXT* allele and a derivative containing only the P11L substitution (i.e. AGT[L₁₁]) was studied. The peroxisomal targeting of AGT at 37 °C is well-established, but its targeting at other temperatures has not been studied previously. At all temperatures studied between 30 and 42 °C, AGT was peroxisomal, with none being directed to the mitochondria (Figures 1c, 1g and 1k). However, when the temperature dependence of AGT[L₁₁] targeting was investigated, it was found to be indistinguishable from that of AGT[L₁₁,M₃₄₀] (i.e. peroxisomal at 30 and 37 °C, but mitochondrial and peroxisomal at 42 °C; Figures 1d, 1h and 1l). This strongly suggests that the mitochondrial mistargeting of AGT[L₁₁,M₃₄₀] at high temperatures is due to P11L polymorphism, and not due to I340M polymorphism.

Targeting of PH1 mutant AGT is normalized at low temperatures

PH1 mutant AGT (i.e. AGT[L₁₁,R₁₇₀,M₃₄₀]) is mistargeted to mitochondria in the liver cells of PH1 patients [14] and when expressed in COS cells or human fibroblasts [1,19]. To test the effect of temperature on its distribution, AGT[L₁₁,R₁₇₀,M₃₄₀] was expressed in COS cells for 24 h at 30, 34, 37, 40 and 42 °C; the results from the studies at 30, 37 and 42 °C are shown in Figure 3. At 37 °C, AGT[L₁₁,R₁₇₀,M₃₄₀] was mainly mitochondrial with a minority peroxisomal (Figures 3c and 3d), but at 42 °C it was entirely mitochondrial (Figures 3e and 3f) and at 30 °C it was

Figure 3 Effect of temperature on the distribution of PH1 mutant AGT

AGT[L₁₁,R₁₇₀,M₃₄₀] was expressed in COS cells for 24 h at 30 °C (**a, b, g, h**), 37 °C (**c, d**) or 42 °C (**e, f**). Cells were then dual-labelled for AGT (green) and either the peroxisomal marker catalase (red) (**a, c, e, h**) or the mitochondrial marker MitoTracker (red) (**b, d, f**). The cells in (**a-f**) were permeabilized as usual with Triton X-100 before immunolabelling, whereas the cells in (**g, h**) were permeabilized with digitonin (see the Experimental section). At 30 °C, AGT[L₁₁,R₁₇₀,M₃₄₀] was co-localized with catalase (**a**), but not with MitoTracker (**b**). At 37 °C, AGT[L₁₁,R₁₇₀,M₃₄₀] was co-localized predominantly with MitoTracker (**d**) and much less so with catalase (**c**). At 42 °C, AGT[L₁₁,R₁₇₀,M₃₄₀] was co-localized with MitoTracker (**f**), but not with catalase (**e**). After digitonin permeabilization (**g, h**), no peroxisomal immunolabelling could be detected, suggesting that the antigen was not accessible to the antibody (colour intensity boosted for visibility). Scale bar, 10 μm.

entirely peroxisomal (Figures 3a and 3b). At intermediate temperatures, there were intermediate distributions (results not shown), so that a certain amount of mitochondrial AGT[L₁₁,R₁₇₀,M₃₄₀] was still detectable at 34 °C, but it was much less when compared with that at 37 °C. At 40 °C, although most of it was mitochondrial, some peroxisomal labelling was also present, but it was less when compared with that at 37 °C.

To check that the peroxisomal distribution of AGT[L₁₁,R₁₇₀,M₃₄₀] at 30 °C truly reflected peroxisomal import as opposed to non-specific adherence to the outer face of the peroxisomal membrane, the Triton X-100 permeabilization step in the immunofluorescence assay was replaced by digitonin, a procedure that

Figure 4 Effect of temperature on the distribution of other peroxisomal and mitochondrial dual-targeted proteins

Pre-mitoAGT (**a, c, e**) or AGT[L₁₁]₁₋₂₀-GFP-SKL (**b, d, f**) were expressed in COS cells for 24 h at 30 °C (**a, b**), 37 °C (**c, d**) or 42 °C (**e, f**), and then dual-labelled for AGT (green) (**a, c, e**) and MitoTracker (red), or GFP autofluorescence (green) (**b, d, f**) and MitoTracker (red). The distribution of both constructs was the same at all temperatures studied, most of it being mitochondrial and a small proportion peroxisomal. Scale bar, 10 μm.

is known to permeabilize the plasma membrane but not the peroxisomal membrane [29]. Under such conditions, the punctate (peroxisomal) immunolabelling of AGT[L₁₁,R₁₇₀,M₃₄₀] was lost (Figures 3g and 3h), showing that it had been imported.

Distribution of two other dual-targeted proteins is unaffected by temperature

To check whether the temperature effects observed for AGT[L₁₁], AGT[L₁₁,M₃₄₀] and AGT[L₁₁,R₁₇₀,M₃₄₀] were due to changes in the protein itself or changes in the peroxisomal and mitochondrial import machineries, we studied the effect of temperature on the distribution of two other proteins shown previously to be targeted to both organelles at normal body temperature [17,28]. Pre-mitoAGT, which is a construct in which the putative ancestral 22-amino-acid MTS of human AGT is attached to the N-terminus of normal human AGT, was distributed in both peroxisomes and mitochondria in similar proportions at all temperatures studied (i.e. 30, 37 and 42 °C; Figures 4a, 4c and 4e). Similarly, AGT[L₁₁]₁₋₂₀-GFP-SKL, which is a construct in which the 20 N-terminal amino acids from polymorphic human AGT (i.e. containing the P11L substitution) were attached to the N-terminus of a GFP construct containing the canonical PTS1 SKL at

Figure 5 Distribution of various constructs derived from PH1 mutant AGT

AGT[L₁₁,R₁₇₀,M₃₄₀] (**a, b**) and AGT[L₁₁,R₁₇₀,M₃₄₀,Δ₃₉₀₋₃₉₂] (**c**) were expressed in COS cells for 48 h (rather than 24 h) at 30 °C, or AGT[L₁₁,R₁₇₀,M₃₄₀,S₃₉₀] was expressed in COS cells for 24 h at 30 °C (**d**) or 37 °C (**e**). Cells were then labelled for AGT (green) and either MitoTracker (red) (**b-e**) or catalase (red) (**a**). AGT[L₁₁,R₁₇₀,M₃₄₀] (**a, b**) co-localized with catalase, but not with MitoTracker, when cells were cultured for 48 h at 30 °C, just as it does after 24 h (cf. Figures 3a and 3b). On the other hand, AGT[L₁₁,R₁₇₀,M₃₄₀,Δ₃₉₀₋₃₉₂] (**c**) was clearly mitochondrial under the same conditions. After 24 h at 30 and 37 °C, AGT[L₁₁,R₁₇₀,M₃₄₀,S₃₉₀] behaved in an identical manner to AGT[L₁₁,R₁₇₀,M₃₄₀] (see Figures 3a-3d), so that it was peroxisomal at 30 °C (**d**) and mainly mitochondrial with some of it peroxisomal at 37 °C (**e**). Scale bar, 10 μm.

its C-terminus, was also distributed in both peroxisomes and mitochondria in similar proportions at all temperatures studied (Figures 4b, 4d and 4f). Because the relative distributions of peroxisomes and mitochondria in these constructs did not change with temperature, it is probable that the effects observed with AGT[L₁₁], AGT[L₁₁,M₃₄₀] and AGT[L₁₁,R₁₇₀,M₃₄₀] (see above) were due to changes intrinsic to the constructs themselves and not due to changes in the peroxisomal and mitochondrial targeting pathways intrinsically.

PH1 mutant AGT retains the potential for mitochondrial import at 30 °C after removal of its PTS1

To determine whether the normalization of AGT[L₁₁,R₁₇₀,M₃₄₀] targeting at 30 °C (see above) was due to a complete block of its mitochondrial import or whether it was due to a decrease in the efficiency of its mitochondrial import compared with its peroxisomal import, the distribution of a PTS1-deficient derivative (AGT[L₁₁,R₁₇₀,M₃₄₀,Δ₃₉₀₋₃₉₂]) was studied. When expressed for 24 h at 30 °C, labelling seemed to be diffuse and was very difficult to detect (results not shown). However, when expressed for 48 h at 30 °C, AGT[L₁₁,R₁₇₀,M₃₄₀,Δ₃₉₀₋₃₉₂] was easily visualized as being entirely mitochondrial (Figure 5c), as we have found it to be at 37 °C in a previous study [19]. This stands in marked contrast with the distribution of AGT[L₁₁,R₁₇₀,M₃₄₀] at 30 °C, which was entirely peroxisomal even after 48 h (Figures 5a and 5b). On the assumption that the C-terminal KKL does not influence the overall conformation of AGT[L₁₁,R₁₇₀,M₃₄₀] [1], this result suggests that its loss of mitochondrial import competence at 30 °C

is not absolute, so that mitochondrial import is possible, but only if its peroxisomal import is prevented.

Pre-heat-shock does not lead to subsequent mistargeting of polymorphic AGT

Incubating mammalian cells at 42 °C for 24 h is almost certain to lead to a heat-shock response, particularly the increased synthesis of molecular chaperones, which might affect the folding patterns of the various AGT constructs used in the present study. To test this, COS cells were heat-shocked at 42 °C for 24 h before the expression of AGT[L₁₁,M₃₄₀] at 37 °C for a further 24 h. Under these conditions, the distribution of AGT[L₁₁,M₃₄₀] was peroxisomal and nuclear, no mitochondrial labelling being evident (Figure 1m). This suggests that peroxisome-to-mitochondrion mistargeting of AGT[L₁₁,M₃₄₀] and AGT[L₁₁] and cytosol-to-mitochondrion mistargeting of AGT[L₁₁,M₃₄₀,Δ_{390–392}] require a high temperature during synthesis and folding, etc., and is not dependent on the presence of accumulated heat-shock proteins. The reason for the presence of AGT[L₁₁,M₃₄₀] in the nucleus (in addition to that in the peroxisomes) 48 h after heat-shock is currently obscure.

Temperature dependence of the distribution of PH1 mutant AGT is unrelated to the presence of a non-consensus PTS1

At its C-terminus, AGT possesses an atypical non-consensus PTS1 that has a number of surprising properties [19,30,31]. To check whether the temperature dependence of AGT[L₁₁,R₁₇₀,M₃₄₀] targeting was a result of the unusual nature of its PTS1, a construct was made in which its C-terminal KKL was replaced by the canonical PTS1 SKL. When this construct (AGT[L₁₁,R₁₇₀,M₃₄₀,S₃₉₀]) was expressed in COS cells at different temperatures (Figures 5d and 5e), the distribution was indistinguishable from that of AGT[L₁₁,R₁₇₀,M₃₄₀] (see Figure 3), indicating that the specific nature of the PTS1 is unrelated to the temperature effects observed.

Normalization of the targeting of PH1 mutant AGT at 30 °C is stable

The normalization of AGT[L₁₁,R₁₇₀,M₃₄₀] targeting to peroxisomes at 30 °C is stable and does not appear to lead to any untoward effects on the cell. For example, when CHO cells stably expressing AGT[L₁₁,R₁₇₀,M₃₄₀] were cultured for 4 days at 30 °C, the distribution that was mainly mitochondrial was transformed into one that was mainly peroxisomal (results not shown). In addition, AGT[L₁₁,R₁₇₀,M₃₄₀] redirected to peroxisomes in COS cells at 30 °C remained there for at least 24 h if the temperature was brought back to 37 °C in the presence of cycloheximide (results not shown).

Glycerol normalizes the targeting of PH1 mutant AGT even at 37 °C

Decreasing the temperature is only one way to increase protein stability. To test whether chemical stabilization [21] could also normalize the targeting of PH1 mutant AGT, AGT[L₁₁,R₁₇₀,M₃₄₀] was expressed in COS cells for 24 h at 37 °C in the presence of the non-specific 'chemical chaperone' glycerol (5%). Although AGT[L₁₁,R₁₇₀,M₃₄₀] is mainly mitochondrial at 37 °C in the absence of glycerol (see above), in the presence of glycerol it is peroxisomal (Figures 6a and 6b). This effect is specific to AGT[L₁₁,R₁₇₀,M₃₄₀], as glycerol had no effect on the distribution

Figure 6 Effect of glycerol on the trafficking of PH1 mutant AGT and two other dual-targeted AGT constructs at 37 °C

AGT[L₁₁,R₁₇₀,M₃₄₀] (a, b), pre-mitoAGT (c) and AGT[L₁₁]_{1–20}-GFP-SKL (d) were expressed in COS cells for 24 h at 37 °C in the presence of 5% glycerol. Cells were then dual-labelled for either AGT (green) (a–c) or GFP autofluorescence (green) (d) and either MitoTracker (red) (a, c, d) or catalase (b). In the presence of glycerol, AGT[L₁₁,R₁₇₀,M₃₄₀] is targeted to the peroxisomes, whereas pre-mitoAGT and AGT[L₁₁]_{1–20}-GFP-SKL are targeted mainly to the mitochondria. Scale bar, 10 μm.

of two other dual-targeted proteins, namely pre-mitoAGT and AGT[L₁₁]_{1–20}-GFP-SKL (Figures 6c and 6d).

Cofactor and inhibitor binding do not influence the distribution of PH1 mutant AGT

The results above suggest that the targeting and distribution of polymorphic and PH1 mutant AGT depend to a great extent on their stabilities. In other instances, binding to small molecules, such as cofactors or inhibitors, can influence the stability of proteins [21]. To test whether this is the case for AGT[L₁₁,R₁₇₀,M₃₄₀], the ability of the cofactor pyridoxal phosphate and the competitive inhibitor amino-oxyacetic acid to influence the targeting of PH1 mutant AGT at different temperatures was studied. Under none of the conditions tested (0–10 mM pyridoxine, 0–1 mM pyridoxal phosphate or 0–5 mM amino-oxyacetic acid) was the distribution of AGT[L₁₁,R₁₇₀,M₃₄₀] affected (results not shown), suggesting that these chemicals did not affect the stability of the protein significantly under the present experimental conditions.

DISCUSSION

AGT in the normal population exists as a number of polymorphic variants, which vary in their amino acid composition. AGT

encoded by the major *AGXT* allele (often referred to as normal AGT in the present study), which has an allelic frequency of approx. 80% in European and North American populations, contains a proline residue at the 11th position and an isoleucine residue at the 340th position. AGT encoded by the minor *AGXT* allele (often referred to as polymorphic AGT) has an allelic frequency of approx. 20% and contains a leucine residue at the 11th position and a methionine residue at the 340th position [13]. In hepatocytes from individuals homozygous for the major allele, AGT is targeted exclusively to peroxisomes [32]. By contrast, in hepatocytes from individuals homozygous for the minor allele, although most AGT (approx. 95%) is still targeted to peroxisomes, a small proportion (approx. 5%) is also targeted to mitochondria [16].

The dual-distribution pattern of polymorphic AGT in hepatocytes *in situ* cannot be duplicated in transfected tissue culture cells *in vitro* at normal body temperature, so that AGT[L₁₁, M₃₄₀] appears to be exclusively peroxisomal in transfected COS cells or human fibroblasts [1,17,19]. This is not simply due to dominance of the peroxisomal over the mitochondrial import machinery, because blocking the former by removing the C-terminal PTS1 or by expressing in $\Delta pex5$ cell lines results in AGT[L₁₁, M₃₄₀] remaining cytosolic rather than being redirected to mitochondria [19]. So, it was surprising to find, in the present study, that increase in temperature was capable of redirecting a significant proportion of the construct to mitochondria. An explanation for the lack of mitochondrial targeting in the previous studies was that, despite possessing an MTS that was perfectly capable of targeting GFP to mitochondria [17], AGT[L₁₁, M₃₄₀] adopted a conformation (i.e. tightly folded and dimeric) that was incompatible with mitochondrial import.

High temperatures have many effects on cells and undoubtedly affect the functioning of protein trafficking pathways, but evidence presented in this study suggests that the temperature effects on the targeting of various AGT constructs are probably due to changes in the properties of AGT itself and not due to any changes in the cell. However, we cannot rule out the possibility that a cellular heat-shock response and the synthesis of heat-shock proteins delay the folding specifically of the polymorphic AGT constructs and consequently increases their targeting to mitochondria. Nevertheless, pre-heat-shock did not affect targeting AGT[L₁₁, M₃₄₀], which might have been expected if heat-induced molecular chaperones were involved in temperature-induced mistargeting. High temperatures can have considerable effects on protein structure, tending to decrease stability, encouraging open or loose conformations that are highly labile, thereby decreasing the extent of productive compact folding. Monomeric structures that are more loosely folded are quite probably much more compatible with the mitochondrial import machinery than tightly folded dimeric ones (see the Introduction section).

The necessary decrease in stability to allow mitochondrial import of polymorphic AGT can also be achieved by introducing an additional amino acid substitution. Normal and polymorphic AGT possess a glycine residue at the 170th position. However, in a large proportion (approx. 30%) of patients suffering from PH1, this residue is mutated to an arginine residue [13]. This G170R mutation is found only on polymorphic AGT (i.e. AGT encoded by the minor *AGXT* allele). Thus these PH1 patients express AGT (referred to as PH1 mutant AGT in the present study), which contains P11L, G170R and I340M substitutions. AGT[L₁₁, R₁₇₀, M₃₄₀] is mainly mitochondrial whether expressed *in situ* in the hepatocytes of PH1 patients [14] or when expressed in tissue culture cell lines *in vitro* [1,19]. The present study suggests that peroxisome-to-mitochondrion AGT mistargeting in PH1 results from polymorphism and mutation-induced changes in the stability

Table 2 Summary of subcellular distribution results

P/p, peroxisomes; M, mitochondria; C, cytosol; –, not determined (case represents the relative amounts).

Construct	Subcellular distribution		
	30 °C	37 °C	42 °C
AGT	P	P	P
AGT[L ₁₁]	P	P	P + M
AGT[L ₁₁ , M ₃₄₀]	P	P	P + M
AGT[L ₁₁ , M ₃₄₀ , $\Delta_{390-392}$]	–	C	C + M
AGT[L ₁₁ , R ₁₇₀ , M ₃₄₀]	P	p + M	M
AGT[L ₁₁ , R ₁₇₀ , M ₃₄₀] + glycerol	–	P	–
AGT[L ₁₁ , R ₁₇₀ , M ₃₄₀ , S ₃₉₀]	P	p + M	M
AGT[L ₁₁ , R ₁₇₀ , M ₃₄₀ , $\Delta_{390-392}$]	M*	M	M
AGT[L ₁₁] ₁₋₂₀ -GFP-SKL	p + M	p + M	p + M
AGT[L ₁₁] ₁₋₂₀ -GFP-SKL + glycerol	–	p + M	–
Pre-mitoAGT	p + M	p + M	p + M
Pre-mitoAGT + glycerol	–	p + M	–

* At 30 °C, mitochondrial import of AGT[L₁₁, R₁₇₀, M₃₄₀, $\Delta_{390-392}$] was observed after 48 h.

and conformation of the protein, because it can be prevented either by decreasing the temperature or by the addition of glycerol, both of which would be expected to stabilize the protein, enabling it to adopt a more compact dimeric conformation incompatible with the mitochondrial import machinery.

Although these studies strongly suggest the role of conformation of AGT in its intracellular targeting, it is still not clear whether this is entirely due to the fact that mitochondria are unable to import completely folded compact dimeric structures, whereas peroxisomes can. Another possibility is that the polymorphic MTS generated by the P11L substitution is hidden normally in the fully folded dimeric molecule, whereas the PTS is exposed. If this were the case, then destabilization caused by additional mutations or high temperatures could result in conformational rearrangement, which exposes the MTS so that it becomes available for interaction with the mitochondrial import machinery.

Although polymorphism-, mutation-, temperature- and glycerol-induced changes in AGT targeting (summarized in Table 2) are the most obvious manifestations of changes in its conformation and/or stability, there are other factors. For example, the presence of the P11L polymorphism markedly decreases specific catalytic activity and sensitizes AGT to the effects of at least four PH1-specific mutations [33]. The conformational instability of PH1 mutant AGT is demonstrated by its almost complete aggregation into inclusion bodies when expressed in *Escherichia coli* [33]. Normal and polymorphic AGT are fairly stable and soluble under such conditions. In addition, based on chemical cross-linking, the P11L polymorphism appears to decrease the efficiency with which AGT translated *in vitro* dimerizes, especially at higher temperatures (i.e. 37 °C compared with 30 °C) [1,17,33]. Under such conditions, PH1 mutant AGT does not seem to dimerize at all. The exact effects of amino acid substitution, temperature or glycerol on AGT conformation are not known currently. However, normal and polymorphic AGT have now been crystallized [34] and the structural co-ordinates have been deposited in the PDB databank (1H0C), so it will only be a matter of time before the exact relationship between AGT conformation and targeting will be determined. Whatever the outcome of these investigations, it is clear that the changes in AGT targeting induced by polymorphisms, mutations, temperature and glycerol highlight the widely different structural requirements of the mitochondrial and peroxisomal protein-translocation pathways (see the Introduction section). As far as polymorphic AGT is concerned, decrease

in stability favours mitochondrial import, whereas increase in stability favours peroxisomal import.

There is increasing evidence that, in many genetic diseases, the primary defect of mutations is to decrease protein stability. This can lead to protein misfolding, aggregation, accelerated degradation or aberrant trafficking [20]. This is well-characterized especially in proteins that travel through the endoplasmic reticulum en route to the lysosomes, plasma membrane or cell exterior, such as α -galactosidase in Fabry disease [35], the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis [36], α_1 -antitrypsin in α_1 -antitrypsin deficiency (emphysema) [25] and aquaporin-2 in nephrogenic diabetes insipidus [27]. In these cases, misfolding leads to aggregation in the endoplasmic reticulum, a block on further trafficking to the lysosomes, plasma membrane or cell exterior, and accelerated proteolytic degradation. However, unlike the aberrant trafficking of AGT, in all of these other cases the trafficking defect is manifested by a failure of the misfolded protein to get past the cell's quality control machinery [37,38]. Instead, the misfolded protein is rerouted down the default pathway of proteolysis. On the other hand, misfolded AGT not only sidesteps the quality control machinery [39], but it is also targeted specifically to an alternative destination where in some species it is found anyway [40].

Just as in the present study, previous workers have investigated the possibility of normalizing the consequences of mutations that cause protein misfolding by increasing protein stability. For example, lowering the temperature can at least partially normalize the trafficking of mutant CFTR to the plasma membrane [22]. The addition of non-specific 'chemical chaperones', such as glycerol, can normalize the trafficking of CFTR [23,24] and α_1 -antitrypsin [25].

Normalization of PH1 mutant AGT trafficking by lowering the temperature or addition of glycerol, as shown in the present study, opens up the possibility that pharmacological enhancement of AGT stability might be of use in the treatment of a significant subset of PH1 patients. AGT[L₁₁,R₁₇₀,M₃₄₀] is catalytically active when mistargeted to mitochondria, and there is no reason to believe that this activity would be lost if it was targeted to peroxisomes instead. Our recently acquired knowledge of the X-ray crystal structure of AGT [34,42] should enable the rational design of clinically useful pharmacological agents, which might increase AGT stability, normalize intracellular trafficking and cure an otherwise intractable hereditary disease.

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