The unpaired peroxisome-to-mitochondrion mistargeting of alanine:glyoxylate aminotransferase (AGT) in the hereditary disease primary hyperoxaluria type 1 is caused by the combined presence of a common Pro11 → Leu polymorphism and a disease-specific Gly170 → Arg mutation. The Pro11 → Leu replacement generates a functionally weak N-terminal mitochondrial targeting sequence (MTS), the efficiency of which is increased by the additional presence of the Gly170 → Arg replacement. AGT dimerization is inhibited in the combined presence of both replacements but not when each is present separately. In this paper we have attempted to identify the structural determinants of AGT dimerization and mitochondrial mistargeting. Unlike most MTSs, the polymorphic MTS of AGT has little tendency to adopt an α-helical conformation in vitro. Nevertheless, it is able to target efficiently a monomeric green fluorescent protein (GFP) fusion protein, but not dimeric AGT, to mitochondria in transfected COS-1 cells. Increasing the propensity of this MTS to fold into an α-helix, by making a double Pro11 → Leu + Pro10 → Leu replacement, enabled it to target both GFP and AGT efficiently to mitochondria. The double Pro11 → Leu + Pro10 → Leu replacement retarded AGT dimerization in vitro as did the disease-causing double Pro11 → Leu + Gly170 → Arg replacement. These data suggest that N-terminal α-helix formation is more important for maintaining AGT in a conformation (i.e. monomeric) compatible with mitochondrial import than it is for the provision of mitochondrial targeting information. The parallel effects of the Pro10 → Leu and Gly170 → Arg replacements on the dimerization and intracellular targeting of polymorphic AGT (containing the Pro11 → Leu replacement) raise the possibility that they might achieve their effects by the same mechanism.

Primary hyperoxaluria type 1 (PH1, McKusick 259900) is an autosomal recessive disorder of glyoxylate metabolism caused by a deficiency of the liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase 1 (AGT, EC 2.6.1.44) (1). In the absence of AGT, glyoxylate is oxidized to oxaloate rather than being transaminated to glycine. Excessive oxalate synthesis leads to the chronic deposition of insoluble calcium oxalate mainly in the kidneys and urinary tract. Following renal failure, the problem of excessive oxalate synthesis is compounded by an inability to remove it from the body, and in such circumstances calcium oxalate can deposit almost anywhere in the body (2).

Although most PH1 patients have a complete, or nearly complete, loss of AGT catalytic activity, a subset of patients have significant levels of activity that can approach those found in asymptomatic obligate heterozygotes (3). Disease in these patients is due to an unpaired protein trafficking defect in which AGT is mistargeted to mitochondria where it is unable to fulfill its metabolic function of glyoxylate transamination/ detoxification efficiently (4). Mistargeting results from the combined presence of a common Pro11 → Leu polymorphism, which is present in normal North American and European populations with an allelic frequency of ~20%, and a PH1-specific Gly170 → Arg mutation, which has a frequency of ~0.05% (5). The Pro11 → Leu polymorphism was predicted to increase the likelihood of the N terminus of AGT folding into an amphiphilic α-helix typical of mitochondrial targeting sequences (MTSs). However, this polymorphic putative MTS appeared to be functionally weak and was not cleaved after import (5, 6). Its efficiency was enhanced, at least in vivo, by the additional presence of the Gly170 → Arg mutation (5, 7). Neither amino acid replacement has any direct effect of on the import of AGT into peroxisomes, to which normal AGT is targeted by a C-terminal non-consensus type 1 peroxisomal targeting sequence (PTS1) Lys-Lys-Leu (7).

The explanation of how the Gly170 → Arg mutation can enhance the functional efficiency of the polymorphic MTS highlights one of the intriguing differences between the structural requirements for protein import into peroxisomes and mitochondria. Whereas at least some proteins, including AGT, can be imported into peroxisomes as fully folded oligomers (8–11), mitochondrial proteins can only be imported as unfolded, or at least loosely folded, monomers (12–15). AGT is a homodimeric pyridoxal phosphate-dependent enzyme (16) that dimerizes rapidly and irreversibly after synthesis into a conformation incompatible with mitochondrial import but perfectly capable of being imported into peroxisomes (10). Neither Pro11 → Leu nor Gly170 → Arg on their own interfere with AGT dimerization to any significant extent, but when present together dimerization is retarded. It has been suggested that polymorphic AGT (i.e. that containing the Pro11 → Leu-generated MTS but not the Gly170 → Arg mutation) cannot be imported into mitochondria efficiently because it dimerizes faster than its commitment to mitochondrial import (10). Therefore, it continues to be targeted to peroxisomes as efficiently as nor-
mial AGT$^2$ (7). When dimerization is blocked by the double Pro$^{11}$→Leu + Gly$^{170}$→Arg replacement, mitochondrial import is unimpaired and occurs at the expense of peroxisomal import, possibly because commitment to mitochondrial import precedes commitment to peroxisomal import (17). How AGT dimerization is retarded, apparently synergistically, by the double replacement and why the polymorphic MTS on its own is unable to retard dimerization are unknown.

In the present study, we have attempted to address these issues. We have examined more closely the structural basis for the functional weakness of the polymorphic MTS of AGT and how the Gly$^{170}$→Arg mutation might be able to retard the dimerization of polymorphic but not normal AGT. Our investigations lead us to conclude that the polymorphic MTS of AGT is not intrinsically weak and that it contains all the necessary topogenic information required to target efficiently a monocropic protein to mitochondria in vivo. Its functional weakness seems to be due entirely to its inability to prevent AGT acquiring a conformation (i.e. dimeric) incompatible with mitochondrial import. This inadequacy is likely to stem from the inefficiency with which the N terminus of polymorphic AGT acquires an α-helical conformation typical of other MTSs. Our data lead us to speculate that the PH1-specific Gly$^{170}$→Arg mutation might increase the functional efficiency of the polymorphic MTS of AGT by providing a molecular environment within which its N terminus can fold more efficiently into an α-helix and thereby retard folding and/or dimerization.

**MATERIALS AND METHODS**

*Circular Dichroism of Synthetic Polypeptides—Three 21-mer polypeptides based on the N-terminal 20 amino acids of naturally occurring and artificial AGT (see Table I), plus a C-terminal cysteine included for other purposes, were kindly made by P. Purkiss (now deceased) using an Applied Biosystems Inc. model 431A Peptide Synthesizer. They were purified by high pressure liquid chromatography and characterized by amino acid analysis and mass spectroscopy. The circular dichroism (CD) spectra of the polypeptides were measured at a concentration of 0.3–0.5 mg/ml at 20 °C, under nitrogen, in a 0.5-mm path length cuvette, using a Jasco J600 spectropolarimeter. Spectra are presented in terms of the differential molar extinction coefficient $\Delta\varepsilon = \varepsilon_{\alpha} - \varepsilon_{\beta}$ (mol$^{-1}$ cm$^{-1}$), based on a mean residue molecular weight. Secondary structure analyses were performed using the PLSIQ routines in the Galactic Industries GRAMS/32 computer program (61).*

*Expression Plasmids—General molecular biology procedures were carried out using standard methods (18). The primers used in the constructions of the expression plasmids, and the expression plasmids themselves, are described in Tables II and III, respectively. In vitro (pBluescriptKS$^+$) and in vivo (pHYK) expression clones pAGT, pAGTL($^{11}$), and pAGTL($^{11}$,R$^{70}$) were prepared as described previously (7) (previously described as pAGT-pgi, pAGT-lgi, and pAGT-iri, respectively).*
The Stepwise Replacement of Prolines by Leucines Enhances the Ability of the N-terminal 20 Amino Acids of AGT to Fold into an \( \alpha \)-Helix in Vitro—The N terminus of normal AGT (encoded by the more common major AGTX allele) contains two neighboring helix-breaking prolines at residues 10 and 11, suggesting that this region would be unlikely to fold into an \( \alpha \)-helix (5). However, the replacement of Pro\(^{10}\) with a helix-forming leucine, as occurs in the less common polymorphic variant of AGT (encoded by the minor AGTX allele), was predicted to increase the likelihood of the N terminus adopting an \( \alpha \)-helical conformation characteristic of an MTS (5). Although MTSs often contain a helix-breaking hinge region containing a single proline, two adjacent prolines are much less commonly found (19–22). We have attempted to test the above prediction by analyzing the CD spectra of various synthetic 21-mer peptides based on the N terminus of AGT, in which the Pro\(^{10}\) and Pro\(^{11}\) are sequentially replaced by leucines (see “Materials and Methods” and Table I). Although the interpretation of CD spectra of peptides smaller than 40 residues must be treated with caution (23), it is still possible to obtain an indication of the likelihood with which such peptides will adopt a particular conformation when part of a larger protein.

All three peptides containing either Pro\(^{10}\) + Pro\(^{11}\), or Pro\(^{10}\) + Leu\(^{11}\), or Leu\(^{10}\) + Leu\(^{11}\) (PP, PL, or LL, see Table I) when dissolved in 10 mM potassium phosphate buffer, pH 7.0, gave CD spectra characteristic of very low conformational order. The PP and PL peptides gave U-type spectra, whereas the LL peptide showed some evidence of order (Fig. 1) (24). Because trifluoroethanol (TFE) is a well known inducer of \( \alpha \)-helices, CD measurements were also carried out in a 50% TFE/water mixture to assess the relative abilities of the peptides to form \( \alpha \)-helices. Measurements in a 20 mM aqueous solution of micellar SDS at pH 7.0 gave effectively the same results (data not shown).

Even in 50% TFE, the PP peptide showed minimal tendency to form an \( \alpha \)-helix (Fig. 1 and Table I). Although this minor conformational change is not attributed to mitochondria in vivo or in vitro (6, 7) but is instead imported into peroxisomes due to the presence of a C-terminal non-consensus PTS1 Lys-Lys-Leu (7). Unexpectedly, the PL peptide containing the single Pro\(^{11}\) → Leu substitution showed only a marginally increased tendency to fold into an \( \alpha \)-helix (Fig. 1 and Table I). Although this minor conformational change is associated with the ability of the polymorphic N-terminal 20 amino acids to direct the import of AGT and the reporter protein DHFR to isolated rat liver mitochondria, the efficiency of the import was very low (6). In addition, the Pro\(^{11}\) → Leu
substitution was completely unable to target AGT to mitochondria in transfected tissue culture cells and to target only weakly AGT to mitochondria in human liver in situ (5, 7).

Although the single proline-to-leucine replacement had little effect on the structure of the peptide, when both Pro11 and its neighboring Pro10 were replaced by leucines, a change that does not occur naturally, there was a marked increase in the tendency of the peptide to fold into an α-helix (Fig. 1 and Table I).

The N-terminal 20 Amino Acids of Polymorphic AGT Containing the Single Pro11 → Leu Replacement Can Target Monomeric GFP, but Not Dimeric AGT, Efficiently to Mitochondria—The intrinsic ability of the various normal, polymorphic, and artificial N termini of AGT to behave as MTSs independently of any effect they might have on AGT folding and dimerization was investigated by transfecting COS-1 cells with various AGT-GFP constructs containing the first 20 amino acids of AGT attached to the N terminus of GFP. Unlike AGT which is a dimer that cannot readily exchange subunits (10), GFP is a monomeric protein, at least under conditions likely to prevail in the cell (26–28), although it can dimerize at high protein concentrations and in high salt (29–31). It is not known to contain any topogenic information itself, but it can be directed to a variety of intracellular compartments if fused to the appropriate targeting sequences. For example, N-terminal fusions with bona fide MTSs result in mitochondrial import (26, 32–35), and C-terminal fusions with bona fide consensus PTS1s result in peroxisomal import (36, 37).

As expected, the N-terminal 20 amino acids of normal AGT were unable to direct the mitochondrial import of a GFP fusion protein (AGT20-GFP), the distribution of which remained entirely diffuse (i.e. cytosolic) (see Fig. 3, I and J) (5, 7). However, when the equivalent 20 amino acids of polymorphic AGT (containing the Pro11 → Leu replacement) was fused to GFP (AGT20(L11)-GFP), most co-localized with the mitochondrial marker MitoTracker (Fig. 2, C and D), indicating efficient mitochondrial targeting. The AGT-GFP fusion protein containing both Pro10 → Leu and Pro11 → Leu replacements (AGT20(L10,L11)-GFP) was also targeted efficiently to mitochondria (Fig. 2, E and F).

These data show that the single polymorphic Pro11 → Leu replacement enables the N-terminal 20 amino acids of AGT to fold into a conformation that contains sufficient topogenic information to direct mitochondrial targeting, even though its tendency to fold into an α-helix, at least as a purified polypeptide in vitro, is low. Increasing the tendency to fold into an α-helix by the additional Pro10 → Leu replacement had little or no effect on mitochondrial targeting over and above that of the single Pro11 → Leu replacement. The effect of the single Pro11 → Leu replacement on the mitochondrial import of the AGT-GFP fusion stands in marked contrast to its lack of effect in AGT itself (see Fig. 3, I and J) (5, 7).
Previously we have shown that AGT(L11) is entirely peroxisomal. This distribution, being both mitochondrial and peroxisomal, whereas AGT(L10,L11) was distributed into compartments labeled with both MitoTracker (mitochondria) and catalase (peroxisomes) (Fig. 3, A–D) in a pattern that was indistinguishable from that of AGT(L11,R170) (Fig. 3, E–H) but very different from that of AGT(L11) (Fig. 3, I and J) which was entirely peroxisomal.

These results clearly show that, unlike polymorphic AGT containing the single Pro10 → Leu replacement, AGT containing the double Pro10 → Leu + Pro11 → Leu replacement could be efficiently targeted to mitochondria. Thus, the effect of the additional Pro10 → Leu replacement was similar to that of the additional presence of the PH1-specific Gly170 → Arg mutation (5, 7) (i.e. it markedly increased the efficiency of mitochondrial targeting). Peroxisomal targeting also occurs in these constructs due to the natural C-terminal PTS1 (7).

The Combined Pro10 → Leu and Pro11 → Leu Replacement, but Not the Single Pro11 → Leu Replacement, Blocks AGT Dimerization—Normal AGT dimerizes rapidly and irreversibly in vitro (10). We have previously demonstrated that chemical cross-linking genuinely reflects AGT dimerization in vitro and that an intermolecular BS3 cross-linking efficiency of 5–10% represents almost complete dimerization (10). The appearance of a cross-linked band at ~100 kDa following SDS-polyacrylamide gel electrophoresis indicates the presence of dimeric AGT. AGT cross-linked intramolecularly (as opposed to inter-molecularly) produces a widening and increased fuzziness of the normal non-cross-linked band at ~43 kDa (see Fig. 4A) (10).

We have shown previously that the synergistic effect of the Pro11 → Leu polymorphism and the PH1-specific Gly170 → Arg mutation on mitochondrial mistargeting was associated with an equally synergistic effect on AGT dimerization (10), so that whereas AGT, AGT(L11), and AGT(R170) dimerized rapidly, AGT(L11,R170) did not. We have replicated this result (as a control) in the present study (Fig. 4A), except that a slight effect was noticed with the dimerization efficiency of AGT(L11) which was not observed before. In addition, the present BS3 cross-linking experiments show that AGT containing the combined Pro10 → Leu and Pro11 → Leu replacement (AGT(L10,L11)) does not dimerize in vitro (Fig. 4A). Parallel studies, using an alternative cross-linker (i.e. sulfo-MBS), gave qualitatively similar results (Fig. 4B).

The Ability of the N Terminus of AGT to Act as an MTS Can Be Separated from Its Ability to Inhibit Dimerization—The vast majority of matrix MTSs are located at the N terminus, from which they can be easily cleaved following import. Therefore, it is not surprising that the addition of an extra 11 residues, by way of a His epitope tag (see Table III), to the N terminus of AGT containing the Pro11 → Leu and Gly170 → Arg replacements abolishes its import into mitochondria (Fig. 5, C and D).
instead targeted to the peroxisomes, as is His-tagged normal AGT (Fig. 5A). However, the presence of an N-terminal His tag does not seem to affect the ability of the natural N terminus of AGT to influence AGT dimerization. His-AGT still dimerizes, whereas His-AGT(L11,R170) does not (Fig. 4B). Therefore, although retardation of dimerization appears to be a prerequisite for the mitochondrial import of AGT, it does not necessarily lead to mitochondrial import without the correct presentation of the topogenic information (i.e. at the N terminus).

The Relative Abilities of the N-terminal Variants of AGT to Act as MTSs Is Unrelated to the Presence or Absence of Consensus or Non-consensus PTS1s—It has been suggested previously that the competition for the import of PH1 mutant AGT (AGT(L11,R170)) into peroxisomes and mitochondria is really a competition between the commitment to mitochondrial import and AGT dimerization (10). Nevertheless, interpretation of these data is complicated by the fact that the PTS1 of AGT does not fit, neither structurally nor functionally, the properties of the archetypal consensus PTS1 Ser-Lys-Leu (38). For example, the C-terminal Lys-Lys-Leu of AGT, although being necessary for its peroxisomal import, is unable to direct the peroxisomal import of reporter proteins such as firefly luciferase, bacterial chloramphenicol acetyltransferase (7), or GFP (data not shown). Whether there is any competition directly between MTSs and PTS1s is unclear. With AGTs found in some other mammals, which are normally targeted to both organelles and which contain bona fide cleavable MTSs very different to the polymorphic MTS found in human AGT, commitment to mitochondrial import appears to take precedence over commitment to peroxisomal import (17). The only way such proteins can ever be imported to any significant extent into peroxisomes is to remove the MTS from the open reading frame by the use of an alternative translation initiation site. Clearly this cannot be the case with the mutant AGT mistargeted from peroxisomes to mitochondria in PH1 patients, because the same N-terminal region is present in AGT targeted to both organelles.

The data presented in this paper, as well as our previous studies (see Introduction), show that the naturally occurring polymorphic N terminus of human AGT can act as a strong MTS when attached to GFP, which possesses no PTS1, and as a weak MTS when attached to AGT, which possesses a non-consensus PTS1. In order to determine whether it is still able to act as a functional MTS in the presence of a consensus PTS1, we have studied the effect on mitochondrial targeting of attaching Ser-Lys-Leu to the C terminus of the AGT-GFP fusions and replacing the C-terminal Lys-Lys-Leu by Ser-Lys-Leu in the PH1 mutant form of AGT.

When expressed in COS-1 cells, AGT20-GFP-SKL (Fig. 6A and B) was peroxisomal, whereas AGT20(L11)-GFP-SKL (Fig.
and AGT20(L10,L11)-GFP-SKL (Fig. 6, G–J) were both mitochondrial and peroxisomal. Therefore, although clearly some of the constructs (all in the case of AGT20-GFP-SKL) were diverted away from their original destinations by the attachment of a consensus PTS1 (compare with Fig. 2), its presence made little difference to the ability of the polymorphic and artificial N termini of AGT target GFP to mitochondria. Similarly, the distribution of AGT(L11,R170)-SKL (Fig. 7) was similar to that of AGT(L11,R170) (see Fig. 3, E–H), except that peroxisomal labeling appeared to be increased relative to that in the mitochondria.

These results show that, although the presence of Ser-Lys-Leu diverts some of the constructs away from the mitochondria, the efficiency of the N terminus of AGT to act as a MTS is largely unaffected. As we have previously shown (10) that manipulation of the C terminus of AGT has no effect on dimerization, it is likely that there is at least some direct competition between the mitochondrial and peroxisomal import pathways for the monomeric constructs, if not for the dimeric ones.

The N Terminus of AGT Is Essential for Dimerization and Mitochondrial Import but Not Peroxisomal Import—The data described above could be interpreted as showing that the N termini of normal and polymorphic AGT are deficient in the ability to stop AGT dimerizing because they have no or only a low tendency to adopt an α-helical conformation. When the N-terminal region of AGT can fold into an α-helix, due to the combined presence of the Pro10 → Leu + Pro11 → Leu amino acid replacements, dimerization is retarded. However, it is possible that the reverse is the case. That is, the N terminus of normal AGT contains information that is required for dimerization, this information being lost following α-helix formation. Some, but by no means conclusive, evidence for this proposition comes from studies in which the N terminus of AGT has been removed. When the first 37 residues are deleted, otherwise normal AGT appears to be stable in transfected COS-1 cells and is efficiently targeted to peroxisomes (Fig. 8, A and B). However, when such a construct is translated in vitro, it fails to dimerize (Fig. 4B).

Interference with Cofactor (Pyridoxal Phosphate) Schiff Base
Formation Does Not Interfere with AGT Dimerization or Peroxisomal Targeting—Like all aminotransferases, AGT is a pyridoxal phosphate-dependent enzyme. Previous studies on the homologous protein in the rat have shown the cofactor binding site to be Lys209 (39), each monomeric subunit having the potential to bind one pyridoxal phosphate molecule (40). Cofactor binding in general normally requires the protein to adopt its mature conformation before binding. Therefore, it might be expected that pyridoxal phosphate binding to peroxisomal and mitochondrial AGT would occur at different stages of their translocation pathways. It is likely that pyridoxal phosphate binds to peroxisomal AGT in the cytosol before import but to mitochondrial AGT in the mitochondrial matrix after import. In some systems, such as the bacterial secretion of redox proteins into the periplasm, cofactor binding is necessary for proper folding and membrane translocation (41). Whether cofactor binding is essential for the folding, oligomerization, and import of any peroxisomal proteins is not known.

As a preliminary attempt to determine whether pyridoxal phosphate binding is involved in AGT dimerization and intracellular targeting, Lys209 was mutated to Arg in order to interfere with Schiff base formation. Interestingly, the Lys209 → Arg replacement in normal AGT had no effect on AGT dimerization (Fig. 4A) or peroxisomal import (Fig. 8, C and D), even though as expected it completely abolished the catalytic activity of a His-tagged AGT construct expressed in Escherichia coli (data not shown).

**DISCUSSION**

The data presented in this paper, which are summarized in Table IV, show that the previously reported effects of the PH1-specific Gly770 → Arg mutation on the dimerization (10) and peroxisome-to-mitochondrion mistargeting (4, 5, 7) of polymorphic AGT (i.e. AGT containing the Pro11 → Leu replacement) can be duplicated by the artificial Pro10 → Leu replacement. In addition, the data show that, although the N terminus of polymorphic AGT can fold only very weakly into an α-helix in vitro, it still contains all the necessary topogenetic information to target GFP to mitochondria in vivo. However, it is unable to target AGT efficiently to mitochondria because, unlike GFP, AGT adopts a conformation (i.e. dimeric) incompatible with mitochondrial import. The effect of the artificial Pro10 → Leu replacement on the dimerization and mitochondrial mistargeting of polymorphic AGT is paralleled by a marked increase in the propensity of the N-terminal 20 amino acids to fold into an α-helix in vitro. This not only raises the possibility that N-terminal α-helix formation, retardation of dimerization, and mitochondrial mistargeting are causally related but also that the PH1-specific Gly770 → Arg mutation might achieve its essentially identical effect the same way as the Pro10 → Leu replacement (i.e. by increasing the tendency of the N terminus of polymorphic AGT to fold into an α-helix). Unfortunately, direct confirmation (or refutation) of this suggestion will have to wait for x-ray crystallographic structural analysis of the various normal and mutant forms of AGT. If indeed the Gly770 → Arg mutation does increase the propensity with which the N terminus of polymorphic AGT folds into an α-helix, then it provides a plausible molecular explanation for the synergistic effects of the Pro11 → Leu and Gly770 → Arg replacements found in PH1 (see Introduction).

There is accumulating evidence that MTSSs have multiple

---

**Table IV**

**Summary of the intracellular targeting and dimerization data presented in this paper**

A semi-quantitative estimate of the distribution of each construct in transfected COS-1 cells is shown, together with the extent to which each dimerizes in vitro. The data for AGT, AGT(L11), and AGT(L11, R170) are taken partly from our previous studies (10). For the dimerization data, Yes indicates that cross-linking was similar to that found with normal AGT; No indicates that there was no, or greatly reduced, cross-linking in vitro. The lack of dimerization of the GFP constructs is assumed as GFP is a monomer in dilute solution and cannot be cross-linked with BS3 using conditions that will cross-link AGT (data not shown).

| Construct | Distribution | Dimerization |
|-----------|--------------|--------------|
| AGT       | + ++ ++      | Yes          |
| His-AGT   | + + + +      | Yes          |
| AGT(L1)   | + + + +      | Yes          |
| AGT(L1, R170) | + + + + +   | No           |
| AGT(L11, R170)-SKL | + + + + +   | No           |
| His-AGT(L11, R170) | + + + + +   | No           |
| AGT(L11)  | + + + +      | No           |
| AGT(L11)-SKL | + + + + +   | No           |
| AGT20     | + + + +      | No           |
| AGT20-GFP | + + + +      | No           |
| AGT20(GFP-SKL) | + + + + +   | No           |
| AGT20(L11)-GFP | + + + + +   | No           |
| AGT20(L11, L13)-GFP | + + + + +   | No           |
| AGT20(L11, L13)-GFP-SKL | + + + + +   | No           |
functions (42). They have been shown to interact with a variety of cytosolic proteins, including mitochondrial import stimulat-
ing factor (43–45), presequence binding factor (46), and hsp70 (47), in addition to the outer mitochondrial membrane import receptors. These interactions mediate the dual roles of MTSs, namely targeting (i.e. the provision of topogenic information) and maintenance of import competent conformations (i.e. unfolded or loosely folded monomers).

However, at least some mitochondrial precursor proteins contain determinants of folding other than the MTS. For ex-
ample, a study of the mitochondrial and cytosolic isoforms of aspartate:2-oxoglutarate aminotransferase suggested that ill-
defined sequences at the N terminus of the mature mitochon-
drial protein play a more important role than does the leader sequence (48, 49). Nevertheless, interaction with hsp70, which is associated with the retardation of folding, is dependent on the leader sequence (50, 51). In aspartate:2-oxoglutarate aminotransferase, and probably many other mitochondrial precur-

sors, it is fortuitous that the N terminus of polymorphic

AGT, which has an allelic frequency of 20%, does not readily get into the mitochondrial import of mature mitochondrial alde-
hyde dehydrogenase because the latter interferes with the ability of the former to fold into an α-helix (53). Stabilization of the N-terminal α-helix, and hence efficiency of mitochondrial import, can be dependent on interaction with other parts of the protein (53, 57, 58).

Our studies on the dimerization and mitochondrial mistarget-
ing of human AGT are compatible with the putative dual (targeting and folding) roles of MTSs. In the case of human AGT, α-helix formation might be less important for targeting than it is for folding (or at least oligomerization). In many respects, it is fortuitous that the N terminus of polymorphic AGT, which has an allelic frequency of ~20%, does not readily adopt an α-helical conformation without the added presence of the PH1-specific mutation, otherwise ~4% of the population of Europe and North America would suffer from this debilitating disease.

Clearly, the polymorphic MTSs of human AGT are untypical of MTSs. Not only is it unable to target AGT to mitochondria without the presence of an additional, disease-causing, mutation, but also because it is not cleaved following import. Interestingly, a number of other animals, such as the marmoset, cat, and rat, normally target AGT to mitochondria (59). However, in these species targeting is directed by more typical cleavable MTSs encoded by a region of the gene upstream of that encoding the polymorphic MTS in human AGT (17). The roles of these more typical bona fide MTSs in the maintenance of import-competent conformations is currently unknown.
55. Thornton, K., Wang, Y., Weiner, H., and Gorenstein, D. G. (1993) J. Biol. Chem. 268, 19906–19914
56. Hammes, P. K., Waltner, M., Hahnemann, B., Heard, T. S., and Weiner, H. (1996) J. Biol. Chem. 271, 21041–21048
57. Karslake, C., Pirotto, M. E., Pak, Y. K., Weiner, H., and Gorenstein, D. G. (1990) Biochemistry 29, 9872–9878
58. Wang, Y., and Weiner, H. (1993) J. Biol. Chem. 268, 4759–4765
59. Danpure, C. J., Fryer, P., Jennings, P. R., Allsop, J., Griffiths, S., and Cunningham, A. (1994) Eur. J. Cell Biol. 64, 295–313
60. Danpure, C. J., Jennings, P. R., Fryer, P., Purdue, P. E., and Allsop, J. (1994) J. Inherited Metab. Dis. 17, 487–499
61. Malik, K. M. (1997) Ph.D. thesis, University of London, London