Characterization of a Fungal Maleylacetoacetate Isomerase Gene and Identification of Its Human Homologue

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We have previously used Aspergillus nidulans as a fungal model for human phenylalanine catabolism. This model was crucial for our characterization of the human gene involved in alcaptonuria. We use here an identical approach to characterize at the cDNA level the human gene for maleylacetoacetate isomerase (MAAI, EC 5.2.1.2), the only as yet unidentified structural gene of the phenylalanine catabolic pathway.

We report here the first characterization of a gene encoding a MAAI enzyme from any organism, the A. nidulans maiA gene. maiA disruption prevents growth on phenylalanine (Phe) and phenylacetate and results in the absence of MAAI activity in vitro and Phe toxicity. The MaiA protein shows strong amino acid sequence identity to glutathione S-transferases and has MAAI activity when expressed in Escherichia coli. maiA is clustered with fhaA and hmgA, the genes encoding the two other enzymes of the common part of the Phe/phenylacetate pathways.

Based on the high amino acid sequence conservation existing between other homologous A. nidulans and human enzymes of this pathway, we used the MaiA sequence in data base searches to identify human expressed sequence tags encoding its putative homologues. Four such cDNAs were sequenced and shown to be encoded by the same gene. They encode a protein with 45% sequence identity to MaiA, which showed MAAI activity when expressed in E. coli.

Human MAAI deficiency would presumably cause tyrosinemia that would be characterized by the absence of succinylacetone, the diagnostic compound resulting from fumarylacetoacetate hydrolase deficiency in humans and fungi. Culture supernatants of an A. nidulans strain disrupted for maiA are succinylacetone-negative but specifically contain cis and/or trans isomers of 2,4-dioxohept-2-enoic acid. We suggest that this compound(s) might be diagnostic for human MAAI deficiency.

The catabolism of phenylalanine and tyrosine in humans is both of intrinsic and clinical interest. The enzymatic steps of this pathway were definitively established in the '50s by the work of Knox and colleagues (see Fig. 1A; Ref. 1). However, two of its structural genes remained uncharacterized. We recently used a novel approach based on the development of a fungal model to characterize one of them (2–4). Here we report our successful application of this approach to the characterization of the other and address by reverse genetics the consequences of the corresponding enzyme deficiency in our model organism.

An enzyme deficiency in any of the steps of this pathway causes in humans a known metabolic disease. For example, a deficiency in phenylalanine hydroxylase causes phenylketonuria (reviewed by Scriver et al. (5)). Enzyme deficiencies in four other steps (those labeled as II, III, and VI in Fig. 1A) cause different hypertyrosinemas (reviewed by Mitchell et al. (6)), and absence of homogentisate dioxygenase (IV, see Fig. 1A) causes alkaptonuria (4, 7). Although the historical interest in the later is notable as it enabled Archibald Garrod to coin the term of “inborn error of metabolism” (8), the gene had not been characterized until recently (3, 4, 10). Crucial for the isolation and characterization of this gene was our establishment of a fungal model for human phenylalanine catabolism based on the filamentous ascomycete Aspergillus nidulans (2).

We cloned its homogentisate dioxygenase gene (the first gene encoding this enzyme identified for any organism) and used its derived amino acid sequence as a probe to identify in similarity searches of the human expressed sequence tag data base (EST)1 cDNAs encoding its human homologue (3).

Type 1 hereditary tyrosinemia (HT1, hepatorenal tyrosinemia, McKusick 276700) is the most severe disease in human Phe catabolism, affecting liver, kidney, and peripheral nerves. HT1 patients surviving infancy develop chronic liver disease with a high incidence of hepatocellular carcinoma (6). HT1 results from fumarylacetoacetate hydrolase (FAAH) deficiency (11). It is generally accepted that fumarylacetoacetate and its spontaneous reaction product, succinylacetone (the diagnostic compound of the disease), are toxic due to their considerable reactivity with key cellular molecules (6, 11), and fumarylacetoacetate has been shown to be mutagenic in Chinese hamster cells (12). In agreement with this, growth of an A. nidulans strain disrupted for the FAAH-encoding gene is prevented by phenylalanine even in the presence of an alternative carbon source (2). succinylacetone is accumulated in culture supernatants of this strain, illustrating the equivalent consequences of a FAAH deficiency in humans and A. nidulans (2).

The clinical consequences of a MAAI (MAAI, EC 5.2.1.2; step V in Fig. 1A) deficiency in humans are largely unknown. It is predicted that this deficiency should also lead to HT1, as maleylacetoacetate has similar reactivity to fumarylacetoacetate (for example, see Ref. 13). By contrast, it is thought that it...
should not result in the presence of succinylacetone in plasma and urine, as the latter compound is likely to be formed from succinylacetocetate resulting from \textit{in vivo} reduction of maleyl and fumarylacetocetate (6, 11). Succinylacetocetate is efficiently degraded by FAAH (1), and its hydrolysis would prevent succinylacetocetone formation. Only one such succinylacetone-negative patient showing type I tyrosinemia with non-detectable levels of MAAI but normal levels of FAAH in liver has been described (14).

Mammalian MAAI has been little studied since its original characterization (1, 15, 16), possibly due, among other possible reasons, to the instability of the substrate (6). The gene encoding MAAI has not been cloned from any organism, and it is therefore the only structural gene of the PheTyr degradation pathway that remains uncharacterized, precluding the analysis of the molecular basis of succinylacetone-negative type I tyrosinemia. Here we successfully use our fungal model to identify cDNAs encoding human MAAI. The liver enzyme requires glutathione (1, 15, 16) as does the equivalent bacterial enzyme that has been purified to homogeneity (17). Our characterization of fungal and human MAAI cDNAs revealed strong amino acid sequence identity of their derived protein sequences to glutathione S-transferases, in agreement with the proposed mechanism of the isomerization (18). We also extend the work of Edwards and Knox (16) and demonstrate MAAI activity by an \textit{in vitro} complementation assay using extracts from a recombinant fungal strain deficient for MAAI. Notably, we detected no succinylacetone in culture supernatants of this strain.

**EXPERIMENTAL PROCEDURES**

\textbf{Fungal Strains, Media, and Growth Conditions—}A. nidulans strains carried markers in standard use (19). A bia1 strain was used as a source of cDNA and wild type protein extracts. The \textit{bia1}, \textit{methG1}, \textit{\Delta fahA} strain has been described (2). A \textit{bia1}, \textit{methG1} strain was used as the wild type in growth tests. Standard media for \textit{A. nidulans} (20) were used for strain maintenance, growth strain, transformation, Culture conditions inducing high levels of expression of the \textit{fahA/maiA/hmgA} genes, which were routinely used to grow mycelia for protein extraction, have been described (21).

**Identification of the \textit{A. nidulans} \textit{maiA} Gene—**Genomic \textit{maiA} sequences were identified by Southern analysis of DNA from \textit{A. nidulans} clones carrying the \textit{fahA/maiA/hmgA} genes, which were routinely used to grow mycelia for protein extraction, have been described (21).

**Disruption of the \textit{A. nidulans} \textit{maiA} Gene—**Disruption followed Tilburn et al. (23). For disruption of \textit{maiA} we used a 4.2-kb linear DNA fragment in which the sequence between \textit{maiA} codons 140–226 had been replaced by a 3.2-kb \textit{xbol} fragment carrying \textit{argB}. A genomic fragment carrying \textit{fahA} from an \textit{xbol} site at position –133 (relative to the initiation codon) to a \textit{Xhol} site at position +655 (relative to the stop codon) was cloned into pBS-SK (Strategen). Substitution of an internal 0.26-kb \textit{SacI-EcoRI} fragment by the above 3.2-kb \textit{A. nidulans} genomic fragment (whose \textit{xbol} ends had been previously converted to \textit{EcoRI} and \textit{Xhol}) removed \textit{maiA} sequences between codons 140 and 226 to yield pBS-\textit{\Delta MAI}. The transforming fragment was isolated from this plasmid after digestion with \textit{BglII} and the resulting \textit{XhoI} and \textit{SacI} ligations were used for electroporation of the 10 g strain. DNA and protein sequences were determined (22).

**Enzyme Assays—**Maleylacetocetate, which is not commercially available, was synthesized enzymatically from homogentisate (1, 15) using homogentisate dioxygenase from \textit{A. nidulans} extracts or from \textit{Escherichia coli} cells expressing the human enzyme. The procedure used to obtain mycelial protein extracts from \textit{\Delta fahA}, \textit{\Delta maiA}, and wild type \textit{A. nidulans} strains and the conditions for the homogentisate dioxygenase reaction have been described (21). For \textit{in vitro} complementation assays, the initial homogentisate concentration was 100–125 μM. Maleylacetocetate formation was monitored spectrophotometrically at 330 nm. When the reaction reached a plateau (with usually more than 80% of the substrate converted to maleylacetocetate), 150 μM reduced glutathione was added to allow the MAAI-dependent inactivation of maleylacetocetate to fumarylacetocetate (1, 16), which is then a substrate for FAAH. Complementation of \textit{\Delta maiA} extracts was used to detect MAAI activity in crude lysates of \textit{E. coli} cells overexpressing fungal or human MAAI, as described in the corresponding figure legends.

**Overexpression of Proteins in \textit{E. coli}—**High levels of protein expression were achieved using the pD1 vector (a gift of E. Espeso). This is a modified pET19b (Novagen) derivative that was engineered to introduce a single BamHI site allowing in-frame fusion of the desired coding region to an N-terminal His tag. Details of this vector will be described elsewhere. Proteins overexpressed in this system carry the sequence MGGHHHHHHHHHHHHHSSGHHDDDDKHMGS at their amino termini. The MAIA coding region was amplified using the following pair of primers (underlined sequences add or modify restriction sites): 5′-CGGGATCCCACGACGGTAAATGCGTGTTGAC3′ (upper) and 5′-CGGAAATTCACACTAAATTTCTGGCTTGAC3′ (lower). The fusion protein contains the complete MAIA sequence with four extra further residues (PAPL) between the above N-terminal tag and the MAIA initiation methionine. The corresponding recombinant plasmid was denoted pD1::MAIA. The human MAIA coding region was amplified using EST 265310 (5′) as the template and primers 5′-CAGGATCTAAAAGGCTCTCCTATTACC-3′ (upper) and 5′-CGGAGTGCTGAGTTGAGGCCTCCT-3′ (lower). The recombinant gene fused the above N-terminal tag to residue 121 of the protein. This recombinant plasmid was denoted pD1::HISMAA A.pD1::HISMAA A (a gift from M. C. Estébanes), driving high level expression of human HGO, will be described elsewhere.

Recombinant plasmids were selected in \textit{E. coli} DH1, purified, and transformed into \textit{E. coli} BL21(DE3)plyS. Primary transformants were selected on LB plates containing ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml) and directly used to inoculate LB liquid cultures that were grown at 37 °C until \textit{Amp} reached 0.8–0.9 units. Expression of T7 RNA polymerase was induced after the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside and further incubation for 2.5 h. 0.5 ml samples were taken before and after induction, and bacteria were collected by centrifugation and resuspended in SDS-polyacrylamide gel electrophoresis loading buffer. Samples were boiled for 3 min before loading appropriate aliquots onto a 12% SDS-polyacrylamide gel alongside Bio-Rad wide-range protein markers. Proteins were detected by Coomassie staining.

For preparation of protein extracts, bacteria from a 50-ml culture were collected by centrifugation, washed in 100 mM potassium phosphate buffer, pH 7.0, resuspended in 4 ml of the same buffer, and lysed by sonication. Lysates were clarified by centrifugation at 10,000 rpm and 4 °C for 20 min in an SS34 rotor. Protein concentrations were estimated by the Bradford assay (24).

**GC-MS Analysis of Culture Fibrates—**Fungal mycelia pregrown on 0.6% glucose (w/v) as the sole carbon source were transferred to appropriately supplemented minimal medium with 20 mM phenylacetate as the sole carbon source (see Ref. 21) and incubated for 20 h at 37 °C. Culture filtrates were derivatized and analyzed by gas chromatography/mass spectrometry (GC/MS) as described (25). TMS derivatives were analyzed by GC-MS in a fused silica capillary column SPB-1 (30 m × 0.25 mm; 0.2-mm film thickness) with a temperature program from 80 to 280 °C (4 °C/min), and a Q-MASS (Perkin-Elmer) mass detector. Identification of peaks was carried out by comparison of sample spectra with reference spectra from the NIST/EPA/NIH mass spectral data base.

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\textbf{Fungal and Human Genes for Maleylacetocetate Isomerase}
**Fungal and Human Genes for Maleylacetoacetate Isomerase**

**RESULTS**

**A Cluster of Three Genes Encoding Enzymes of the Common Part of the A. nidulans PhAc/Phe Catabolic Pathway**—The ascomycete fungus *A. nidulans* can use either Phe or PhAc as sole carbon source (Fig. 1A). Both compounds were catabolized to homogentisate, which was then converted to fumarate and acetoacetate through the action of three enzyme activities, HGO, MAAI, and FAAH. We have previously reported that the enzymatic steps in the Phe pathway are denoted with roman numbers. The abbreviations for the enzymes used throughout this work are also shown. Single enzyme deficiencies cause the following diseases: I, phenylketonuria; II, type II (oculocutaneous) tyrosinemia; III, type III tyrosinemia; IV, alkakinopenia; V, (presumably) a variant of type I tyrosinemia; VI, type I tyrosinemia. B, the *A. nidulans* cluster for the three genes encoding the common part of the Phe/PhAc degradation pathway. Contiguous genomic DNA fragments (F1–F4) and their sizes are indicated. hmgA encodes HGO (3), fahA encodes FAAH (2), and maiA encodes MAAI (this work). Arrows indicate the direction of transcription. E, EcoRI.

**DNA Sequencing**—Genes and cDNAs were sequenced using a Dye Terminator Cycle sequencing kit (Perkin-Elmer) and *Taq* FS DNA polymerase with universal and custom primers. Sequencing reactions were resolved on an ABI Prism 377 automatic sequencer and analyzed with the ABI analysis software (Version 3.1). Genomic and cDNA versions of maiA and human EST cDNA clones 265310 (5'), 290219 (5'), 683733 (5'), and 52677 (5') encoding human MAAI were completely sequenced in both strands. cDNA clones of the IMAGE consortium (25) were purchased from Genome Systems Inc., (St. Louis, MO).

To confirm this, we replaced by transformation the wild type *maiA* gene by a mutant version in which the sequence encoding *maiA* residues 140–226 had been substituted by a genomic DNA fragment containing the *argB* + gene (Fig. 4A). Transformants were selected in an *argB2* background for arginine-
independent growth and purified by repeated streaking of con- 
idospores on minimal medium lacking arginine. Two 
independent transformants showing the expected 
maiA 
replacement were selected after Southern analysis. Both showed 
an identical phenotype, being unable to grow on either phen- 
ylacetate or phenylalanine as the sole carbon source. This 
confirmed that 
maiA 
is a gene of the common part of the 
Phe/PhAc pathways. MAAI assays were carried out with pro- 
tein extracts from mycelia of the disrupted strains grown on 
glucose and transferred to PhAc, which showed them to be 
deficient for MAAI activity (Fig. 5). By contrast, these extracts 
showed normal levels of either FAAH or HGO (data not shown).

Maleylacetoacetate can be synthesized 
in vitro 
by the homogen- 
tisate dioxygenase activity present in mycelial extracts from 
the wild type strain or from either a mutant 
DfahA 
strain 
(lacking FAAH (2)) or a mutant 
DmaiA 
strain (presumably 
lacking MAAI) and detected by its absorption at 330 nm (1, 15, 
16). In the absence of GSH, an obligate cofactor of MAAI, 
maleylacetoacetate is not isomerized to fumarylacetoacetate, 
thereby providing an enzymatic method to obtain the isomer- 
ase substrate (15, 16). On addition of GSH, wild type extracts 
catalyze the isomerization of maleylacetoacetate to fumarylac- 
etoacetate and the conversion of the latter to fumarate and 
acetoacetate. As neither of these two latter compounds shows 
the characteristic absorption of diketoacids in the near ultra- 
violet region, this coupled enzyme reaction can be monitored by 
the decrease of 
A330 (see Fig. 5; Refs. 15 and 16). Neither 
mutant extract alone would catalyze a decrease in 
A330, as 
maleylacetoacetate (which would accumulate with the 
DmaiA extract; see below) and fumarylacetoacetate (which accumu-
absorbing in the near ultraviolet region at either acidic or neutral pH (Fig. 6, D and H). By contrast, the characteristic absorption of maleylacetoacetate at neutral but not at acidic pH was still detectable upon incubation with the ΔmaiA extract (Fig. 6, B and F). Incubation of the substrate with the ΔfahA extract (which contains the isomerase but not the hydrolase) produced a compound (fumarylacetoacetate) showing strong absorbance in the above region both at acidic and neutral pH (Fig. 6, C and G).
Expression of A. nidulans MaiA in E. coli—To definitively establish that MaiA is A. nidulans MAIA, we expressed the polypeptide as a fusion protein (see “Experimental Procedures”) in E. coli under the control of a T7 RNA polymerase-dependent promoter. Promoter induction resulted in the synthesis of a markedly abundant 32-kDa protein that was absent from extracts of induced cells carrying the expression vector without insert. This protein, whose electrophoretic mobility was roughly consistent with the large arrows from control E. coli cells or from E. coli cells overexpressing MaiA were added as indicated. Degradation of maleylacetate to fumarate and acetacetate was monitored by the decrease of absorbance at 230 nm (OD230), which was used in three identical, separate reactions to synthesize maleylacetate using recombinant human homogentisate dioxygenase. It showed the diagnostic high absorbance in the near ultraviolet region under neutral but not under acidic pH conditions. This conversion did not take place when extracts overexpressing the protein were substituted by control extracts (data not shown). As a whole, these results provide formal evidence that A. nidulans maiA encodes MAIA.

Analysis of A. nidulans Culture Supernatants for Diagnostic Compounds of MAIA Deficiency—An A. nidulans strain disrupted for the gene encoding FAAH accumulates succinylacetone, the diagnostic compound for HT1 (caused by FAAH deficiency) in the urine of human patients. Growth of a ΔfahA strain on lactate (which is unaffected by the mutation) is strongly inhibited by Phe ((2); see Fig. 4B). Phe toxicity in this mutant background is due to fumarylacetate and/or its spontaneous reaction product, succinylacetone, which accumulate(s) as a result of the enzyme deficiency. Notably, growth of a strain disrupted for maiA was also inhibited by Phe (Fig. 4B), although clearly to a lesser extent than that of the ΔfahA strain, strongly suggesting that different catabolites with distinct toxicities accumulate in each disrupted strain (but see “Discussion”).

No specific diagnostic method is available for a deficiency in human MAIA. We therefore investigated by GC-MS the compounds accumulated in a culture supernatant of our ΔmaiA strain transferred to phenylacetate and detected four peaks almost certainly corresponding to cis and/or trans isomers of 4,6-dioxohept-2-enoic acid (Fig. 8), as shown by comparison of their mass spectra with those for the above compound(s) deposited in the data bases. These four peaks presumably represent different TMS derivatives of the above compound(s) (see legend to Fig. 8). Neither of the two possible cis-trans isomers of 4,6-dioxohept-2-enoic acid, maleylacetone and fumarylacetone, had been detected in culture supernatants of either the wild type or a ΔfahA strain (2), strongly suggesting that its (their) presence specifically results from the ΔmaiA mutation. Notably, no peaks corresponding to succinylacetone were detected.

Identification of Human EST Clones Encoding Homologues of A. nidulans MaiA—We next used the fungal MAIA amino acid sequence to screen the EST data bases for human and murine ESTs encoding putative MaiA homologues, in analogy with the protocol already established for the AKU gene (3, 4). BLAST searches identified a number of these human ESTs. The 10 highest scores were obtained with the derived amino acid sequences of the following human ESTs (with the source of RNA for each EST cDNA clone in parentheses): 683733 (germinal B cells), 290219 (multiple sclerosis), 290775 and 265310 (melanocytes), 52677 (infant brain), 156401 (breast), 309975 (senescent fibroblasts), 240726 and 246479 (fetal liver/spleen), and 66e04 (skeletal muscle). These (partial) amino acid sequences showed more than 40% identity to that of A. nidulans MaiA, strongly suggesting that they represent its human homologue(s). The fact that only minor differences in the sequence (presumably resulting from automated sequencing errors) were found between these derived human proteins strongly suggests that all these cDNAs correspond to a single gene. Tissue-specific expression of such a gene does not appear to be as restricted as that of HGO (3, 4), and only two of the 10 cDNA clones represented liver transcripts (see above). In addition to the above human protein sequences, data base searches detected MaiA homology to derived protein products of mouse and Arabidopsis thaliana EST clones (not shown).

Molecular Characterization of Human cDNAs Encoding Maleylacetate Isomerase—We fully sequenced four of the above ESTs. (Fig. 9A). Nucleotide sequencing showed that they were indeed encoded by the same gene, despite the fact that
they had been isolated from different tissues. EST 265310 (5'9) (melanocytes) is the longest of these cDNAs. It is 1155 base pairs long (excluding the poly(A) tail) and contains a 216-codon ORF whose derived protein product (Mr 24,083) shows 45% identity in amino acid sequence to A. nidulans MAAI (Fig. 9B). This represents nearly definitive evidence that this cDNA encodes a human MAAI (but see below). The complete nucleotide sequence of this cDNA has been submitted to DDBJ/EMBL/GenBank\textsuperscript{TM} data bases (accession number AJ001838) The 3'-UTR of this transcript was remarkably long (400 nucleotides, i.e. 30% of the transcript size). ESTs 290219 (5'9) (multiple sclerosis lesions) and 683733 (5'9) (germinal B-cells) represented cDNAs incomplete at their 5' ends, starting at codons 18 and 49, respectively, of the human MAAI ORF. The precise site of polyadenylation and the sequence of the 400-nucleotide 3'-UTR of EST 290219 were identical to those of the longest cDNA. Polyadenylation of the 683733 cDNA occurred two nucleotides upstream of the above site, but no other nucleotide sequence difference was observed either in the 3'-UTR or in the coding region. Finally, we detected no differences between the nucleotide sequences of EST 52677 (brain) and EST 265310 up to position 1045, where the former is prematurely polyadenylated as compared with the latter. This strongly suggests that the 5' ends of these two transcripts represent a transcription start site for the human MAAI gene.

To confirm that the ORF of these transcripts encodes a human MAAI, the protein product encoded by the EST 265310 (5'9) ORF was overexpressed in E. coli as above. High levels of a protein with the expected mobility for this polypeptide product were detected by SDS-polyacrylamide gel electrophoresis when recombinant E. coli cells in which its expression was driven by a T7-polymerase promoter (see "Experimental Procedures") were grown under inducing conditions (data not shown) and were absent from these cells grown under noninducing conditions. Extracts from E. coli cells overexpressing this protein showed strong maleylacetoacetate isomerase activity, as shown by its ability to complement an A. nidulans extract deficient for the enzyme (Fig. 10). This strong MAAI activity was dependent on the presence of GSH (not shown). By contrast, control cells showed no MAAI activity. These results establish that the product of the EST 265310 ORF (and, by extension, of the above four cDNAs) is a human maleylacetoacetate isomerase.

**DISCUSSION**

We describe here the molecular characterization of \textit{maia}, a gene encoding an enzyme of the common part of the \textit{A. nidulans}}
phenylalanine/phenylacetate pathways and provide definitive biochemical and genetic evidence that this gene encodes a maleylacetate isomerase. This paper represents the first characterization of a gene encoding MAAI from any organism. Compelling evidence for the above conclusion can be summarized as follows: (i) maiA is clustered with fahA and hmgA, the two other structural genes of this common part of the pathways; (ii) transcription of the gene is induced by phenylalanine; (iii) the deduced amino acid sequence of its encoded protein shows identity to glutathione S-transferases, as expected for a MAAI enzyme; (iv) disruption of the gene prevents growth on either Phe or phenylacetate; (v) protein extracts from this disrupted strain convert homogentisate to maleylacetate but cannot catalyze this compound further; (vi) mixing ΔmaiA and ΔfahA extracts results in reciprocal complementation of the corresponding enzyme deficiencies required for maleylacetate catabolism to fumarate and acetooacetate; (vi) expression of maiA in E. coli results in bacterial protein extracts showing MAAI activity. Two technical developments were crucial to obtaining some of the above evidence. First, we used either recombinant HGO or fungal extracts showing high HGO activity to efficiently synthesize maleylacetate. Second, we used a complementation assay for MAAI based on a protein extract from our A. nidulans strain deleted for maiA. This extract converted homogentisate to fumarate and acetooacetate only when supplied with GSH and a source of MAI.

We next used the MaiA-derived sequence to identify human, mouse, and plant ESTs encoding proteins showing high amino acid sequence identity to A. nidulans MAI. Four such human cDNAs were fully sequenced and shown to encode a protein with 45% identity to MaiA. Although they were isolated from different tissues, these four cDNAs (and the other ESTs detected in our searches) almost certainly represent transcripts of the same gene. The protein encoded by this transcript(s) has MAAI activity when expressed in E. coli. Our electronic screening of the human EST data base would suggest that expression of this gene would be more ubiquitous than that of HGO, whose expression is largely restricted to liver, kidney, colon, small intestine, and prostate. This apparently less restricted pattern of expression might be related to its ability to use other compounds, in addition to maleylacetate, as substrates (26), which might suggest a detoxification function (6).

Our characterization of human MAI cDNAs represents the identification of the only as yet undescribed gene of the human Phe catabolic pathway. These results further confirm the validity of our fungal metabolic model and open the possibility of analyzing at the molecular level the predicted disease (a possible variant of HT1) resulting from MAAI deficiency. The incidence of this inborn error of metabolism is presently unknown, perhaps due to the absence of clear biochemical and/or molecular diagnostic criteria. A single patient with a putative MAAI deficiency has been reported in an abstract (14). Notably, this patient suffered from severe hepatorenal and brain damage. Our results with the fungal model show that the metabolite(s) accumulated as a result of a MAAI deficiency is indeed toxic for Aspergillus, but their toxicity is detectably lower than the toxicity of those accumulated as a result of a FAAH deficiency. Jorquera and Tanguay (12) have reported that, in contrast to fumarylacetate, maleylacetate was not mutagenic in Chinese hamster cells. We have not yet addressed if either of the above deficiencies is mutagenic in Aspergillus.

We detected no succinylacetone in culture supernatants of the A. nidulans ΔmaiA strain. This would be expected from the presumed origin of succinylacetone from decarboxylation of succinylacetate, as normal levels of FAAH in this strain would degrade the latter (1). Therefore, this absence of succinylacetone might, at least in part, account for the lower Phe toxicity found in an A. nidulans strain deficient for MAI as compared with a strain deficient for FAAH. Analysis of culture filtrates of the A. nidulans ΔmaiA strain specifically detected the presence of 4,6-dioxohept-2-enoic acid. This chemical structure would be consistent with maleylacetone (cis isomer) and/or fumarylacetone (trans isomer). These isomers cannot be reliably distinguished by the methodology used here. Taking into account the remarkable similarities in the consequences of equivalent metabolic blocks in human and fungal Phe catabolism, we suggest cis and/or trans isomers of 4,6-dioxohept-2-enoic acid as possible diagnostic compound(s) for MAI deficiency in humans.

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Note Added in Proof—An unpublished sequence recently submitted to the GenBank™/EBI Data Bank with accession number U86529 and described as a cDNA encoding a human glutathione transferase Zeta 1 is the same as our cDNA sequence for human maleylacetate isomerase.

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