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

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The catabolism of phenylalanine and tyrosine in humans is of both 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). Enzyme deficiencies in four other steps (those labeled as II, III, and VI in Fig. 1A) cause different hypertyrosinemias (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, 9), 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 I 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 deficiency (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 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

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AJ001836, AJ001837, AJ001838.

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The abbreviations used are: EST, expressed sequence tag; HT1, human type I hereditary tyrosinemia; GSH, reduced glutathione; PheAc, phenylacetic acid; FAAH, fumarylacetoacetate hydrolase; HGO, homogentisate dioxygenase; MAAI, maleylacetoacetate isomerase; TMS, trimethylsilyl; kb, kilobase(s); GC, gas chromatography; MS, mass spectrometry; ORF, open reading frame; UTR, untranslated region.

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should not result in the presence of succinylacetone in plasma and urine, as the latter compound is likely to be formed from succinylacetoacetate resulting from in vivo reduction of maleyl and fumarylacetoacetate (6, 11). Succinylacetoacetate is efficiently degraded by FAAH (1), and its hydrolysis would prevent succinylacetoacetone formation. Only one such succinylacetoacetone-negative patient showing type I tyrosinemia with non-detectable levels of MAI but normal levels of FAAH in liver has been described (14).

Mammalian MAI 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 MAI has not been cloned from any organism, and it is therefore the only structural gene of the Phe/Tyr degradation pathway that remains uncharacterized, precluding the analysis of the molecular basis of succinylacetoacetone-negative type I tyrosinemia. Here we successfully used our fungal model to identify cDNAs encoding human MAI. 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 MAI 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 MAI activity by an in vitro complementation assay using extracts from a recombinant fungal strain deficient for MAI. Notably, we detected no succinylacetone in culture supernatants of this strain.

EXPERIMENTAL PROCEDURES

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 bia1, methG1, ΔfahA strain has been described (2). A bia1, methG1 strain was used as the wild type in growth tests. Standard media for A. nidulans (20) were used for strain maintenance, growth conditions, and transcription. Culture conditions inducing high levels of expression of the fahA maiA hmgA genes, which were routinely used to grow mycelia for protein extraction, have been described (21).

Identification of the A. nidulans maiA Gene—Genomic maiA sequences were identified by Southern analysis of DNA from EMBL4 clones carrying the fahA and hmgA genes, using a subtracted cDNA probe representing genes induced by phenylacetate (“plus” probe) and a cDNA probe from glucose-grown mycelia (“minus” probe). A 2.5-kb EcoRI fragment contiguous to the fahA transcription unit (see Fig. 1B) showed no hybridization to a 35 μg probe used directly to inactivate LB liquid cultures cDNA library screening using the F2 fragment (see Fig. 1A) isolated in the addition, in addition to fahA cDNA clones, of a second class of cDNA clones representing maiA transcripts. The cDNA library enriched in transcripts induced by phenylacetate and the subtracted plus cDNA probe have been described (2, 3). The A. nidulans genomic library was a standard EMBL4 library constructed from the wild type strain. RNA isolation and Northern analysis followed (22). Equal loading of the different samples was confirmed using an actin probe.

Disruption of the maiA Gene—Transformation followed Tilburn et al. (23). For disruption of maiA we used a 4.2-kb linear DNA fragment in which the sequence between maiA codons 140–226 had been replaced by a 3.2-kb XbaI fragment carrying argB. A genomic fragment carrying the maiA sequence from an XbaI site at position −139 (relative to the initiation codon) to an XhoI site at position +655 (relative to the stop codon) was subcloned in pBS-SK+ (Stratagene). Substitution of an internal 0.26-kb SalI-EcoRI fragment by the above 3.2-kb A. nidulans genomic fragment (whose XbaI ends had been previously converted to EcoRI and XhoI) removed maiA sequences between codons 140 and 226 to yield pBS-ΔMAI. The transforming fragment was isolated from this plasmid after digestion with XbaI and wild type XbaI strains and the conditions for the homogenizable dioxygenase reaction have been described (21). For in vivo complementation assays, the initial homogentisate concentration was 100–125 μM. Maleylacetoacetate formation was monitored spectrophotometrically at 330 nm. When the reaction reached a plateau (with usually more than 80% of the substrate converted to maleylacetoacetate), 150 μM reduced glutathione was added to allow the MAI-dependent isomerization of maleylacetoacetate to fumarylacetoacetate (1, 16), which is then a substrate for FAAH. Complementation of ΔmaiA extracts was used to detect MAI activity in crude lysates of E. coli cells overexpressing fungal or human MAI, as described in the corresponding figure legends.

Overexpression of Proteins in E. coli—High levels of protein expression were achieved using the pD1 vector (a gift of E. Espeso). This is a modified pET11b (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 MGHHHHHHHHHHSSGGHIDDDDKHMGS at their amino termini. The MAI coding region was amplified using the following pair of primers (underlined sequences add or modify restriction sites): 5′-CCGG-GATCCTCCCGGACCCGTTCAAGATCTC-3′ (upper) and 5′-CGGAAT-TCGACCTAAATTCCGTTGTT-3′ (lower). The fusion protein contains the complete MAI sequence with four extra residues (PALE) between the above N-terminal tag and the MAI initiation methionine. The corresponding recombinant plasmid was denoted pD1::MaiA. The human MAI coding region was amplified using EST 263510 (5′) as template and primers 5′-CAGGGATCCAGCCCATC-CTTATTC-3′ (upper) and 5′-CAGGATTCCGGATGTCGCCCATC-3′ (lower). The recombinant gene fused the above N-terminal tag to residues 5–216 of the protein. The corresponding plasmid was denoted pD1::HISMAA. A pD1::HISGO plasmid (a gift from M. C. Estébanez), driving high level expression of human HGO, will be described elsewhere.

Recombinant plasmids were selected in E. coli DH1, purified, and transformed into E. coli BL21(DE3)pLyS S. Primary transformants were selected on LB plates containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) and used subsequently to inoculate LB liquid cultures that were grown at 37 °C until A600 reached 0.8–0.9 units. Expression of T7 RNA polymerase was induced after the addition of 0.4 mM isopropl-1-thio-β-p-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 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 ml 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 Filtrates—Fungal mycelia pregrown on 0.6% glucose (w/v) as the sole carbon source were transferred to appropriately supplemented minimal medium with 20 mM phenylacetae as the sole carbon source (see Ref. 21) and incubated for 20 h at 37 °C. Culture filtrates were extracted with ethyl acetate and derivatized with heptasilyl trifluoroacetamide as described (2). TMS derivatives were analyzed by GC-MS in a fused silica capillary column SBP-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.
A. nidulans fahA, HGO, MAAI, and FAAH. We have previously reported that the acetoacetate through the action of three enzyme activities, to homogentisate, which was then converted to fumarate and sole carbon source (Fig. 1).

organism. Southern blot hybridization of of these genes is expressed on glucose as the sole carbon source. Clustering of genes encoding FAAH and HGO, respectively, are closely linked and divergently transcribed (Fig. 1).

FIG. 1. The phenylalanine/phenylacetate degradation pathway. A, enzymes required for PhAc degradation to homogentisate are only present in A. nidulans. Enzymes for Phe catabolism to fumarate and acetoacetate (1) are present both in humans and A. nidulans. 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, alkaptonuria; V, (presumably) a variant of type 1 tyrosinemia; VI, type 1 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.

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) and 265310 (5) 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) and 265310 (5) were purchased from Genome Systems Inc., (St. Louis, MO).2

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 A. nidulans fahA and hmgA genes encoding FAAH and HGO, respectively, are closely linked and divergently transcribed from a 414-base pair intergenic region. fahA and hmgA gene transcription is strongly inducible by PhAc (or its structural relatives) or Phe and partially repressible by glucose. Neither of these genes is expressed on glucose as the sole carbon source. No gene encoding MAAI has yet been characterized from any organism. Southern blot hybridization of A. nidulans genomic DNA probes carrying the fahA and hmgA genes with a subtracted cDNA probe representing transcripts induced by PhAc revealed the presence of a third linked gene strongly hybridizing to this probe. As fahA and hmgA, this third gene, designated maiA, was not expressed on glucose. Clustering of genes encoding activities of the same catabolic pathway is not unusual in A. nidulans. Genomic and cDNA nucleotide sequencing of the region encoding this new transcript confirmed the presence of a third gene 3’ from fahA, transcribed in a tail-to-tail orientation (Fig. 1B). The transcribed region contains an intron-less ORF encoding a putative 230-residue polypeptide (Fig. 2) whose stop codon is 486 base pairs downstream from that of fahA. The nucleotide sequence of this three-gene cluster has been submitted to the DDBJ/EMBL/GenBankTM data bases under accession number AJ001836.

A. nidulans maiA Encodes Maleylacetoacetate Isomerase—Data base searches revealed that the predicted product of maiA shows strong amino acid sequence identity to glutathione S-transferases. For example, FASTA searches of the Swiss-Prot data base revealed that among the 30 protein sequences showing the highest alignment scores, 27 were glutathione S-transferases (data not shown). Similar results were obtained after searching the conceptual translation of EMBL + GenBankTM nucleotide sequence data bases with TBLASTN. The highest FASTA score corresponded to glutathione S-transferase 1 from Diantus caryophyllus, which showed 33.3% identity to MaiA in a 228-amino acid overlap including the complete sequence of both proteins (data not shown). The deduced molecular mass for MaiA (25,129 Da) is similar to the 25-kDa size of glutathione S-transferases.

Northern analysis showed that, as determined for fahA and hmgA, transcription of maiA was induced by either Phe or PhAc and was absent on glucose or gluconeogenic carbon sources (Fig. 3), strongly suggesting that maiA was a gene for Phe/PhAc catabolism and that its clustering with fahA and hmgA reflected its involvement in the same catabolic pathway. The only as yet unidentified gene encoding an enzyme essential for both Phe and PhAc catabolism is that encoding MAAI. The likely mechanism of this enzyme involves transfer of enzyme-bound GSH to C2 of maleylacetoacetate (26). Therefore, all the above data indicated that maiA might encode A. nidulans MAAI.

To confirm this, we replaced by transformation the wild type maiA gene by a mutant version in which the sequence encoding MaaI 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-

2 Details of EST libraries may be found in http://www-bio.llnl.gov/bbrp/image/humlib_info.html.
independent growth and purified by repeated streaking of conidiospores on minimal medium lacking arginine. Two independent transformants showing the expected \( \text{maiA} \) replacement were selected after Southern analysis. Both showed an identical phenotype, being unable to grow on either phenylacetate or phenylalanine as the sole carbon source. This confirmed that \( \text{maiA} \) is a gene of the common part of the \( \text{Phe/PhAc} \) pathways. MAAI assays were carried out with protein extracts from mycelia of the disrupted strains grown on glucose and transferred to \( \text{PhAc} \), 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 \textit{in vitro} by the homogenate dioxygenase activity present in mycelial extracts from the wild type strain or from either a mutant \( \text{D} \text{faA} \text{H} \) strain (lacking FAAH (2)) or a mutant \( \text{D} \text{maiA} \) 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 isomerase substrate (15, 16). On addition of GSH, wild type extracts catalyze the isomerization of maleylacetoacetate to fumarylacetoacetate 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 ultraviolet region, this coupled enzyme reaction can be monitored by the decrease of \( A_{330} \) (see Fig. 5; Refs. 15 and 16). Neither mutant extract alone would catalyze a decrease in \( A_{330} \), as maleylacetoacetate (which would accumulate with the \( \text{D} \text{maiA} \) extract; see below) and fumarylacetoacetate (which accumu-

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**Fig. 3.** Northern analysis of \( \text{maiA} \) transcript levels. Cells were grown on minimal medium with 0.3% (w/v) glucose as sole carbon source for 16 h at 37 °C and transferred to media with the indicated carbon sources. These secondary cultures were incubated for a further 1 h at 37 °C. Mycelia were then harvested and used to isolate RNA. The probe was a cDNA clone insert containing the complete \( \text{maiA} \) ORF. -C indicates no carbon source added.
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Fig. 4. Disruption of maiA. A, the wild type maiA gene was replaced by a mutant version by transformation with a linear XbaI-XhoI A. nidulans genomic DNA fragment in which maiA codons 140–226 have been replaced by a 3.2-kb fragment carrying the argB gene. Transformants carrying the integration event shown were identified by Southern analysis. Xb, XhoI; S, SalI; E, EcoRI; Xa, XbaI; (S), a destroyed SalI site. Dashed box, the maiA coding region; gray box, the 3.2-kb argB′ fragment. B, disruption of maiA results in Phe toxicity. Two independent clones (right and left colonies) carrying the above gene replacement were inoculated on minimal medium with lactose 0.05% (w/v) in the absence (left panel) or in the presence (right panel) of 25 mM Phe. A wild type (top) and a ΔfahA strain (bottom) were inoculated as controls. Plates were incubated at 37 °C for 96 h.

Fig. 5. Absence of MAAI activity in extracts of the A. nidulans strain deleted for the maiA gene. Large panel, equal amounts of protein extracts from the indicated mutant A. nidulans strains grown under inducing conditions were used either alone or in combination to synthesize maleylacetoacetate from homogentisate. When A. nidulans (O.D.330) did not increase further, GSH (an obligate cofactor of MAAI) was added at 0.1 mM. A decrease in A330 is indicative of maleylacetoacetate conversion to fumarate and acetoacetate through the coupled action of MAAI and FAAH. No decrease indicates that one of these enzyme activities is lacking. The smaller panel shows control reactions carried out with a wild type extract. GSH was added at the times indicated with an arrow.

Fig. 6. Absorption spectra of the compounds accumulated after in vitro incubation of maleylacetoacetate with mutant A. nidulans protein extracts. Maleylacetate (CONTROL spectra) was synthesized using bacterially expressed HGO and incubated in the presence of equivalent amounts of the indicated protein extracts. Reactions were carried out for 15 min at 23 °C as described under “Experimental Procedures.” A–D, acid pH; E–H, neutral pH; O.D., absorbance.

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).
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Expression of A. nidulans MaiA in E. coli—To definitively establish that MaiA is A. nidulans MAI, 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 with no insert. This protein, whose electrophoretic mobility was roughly consistent with the r predicted for the MaiA polypeptide as a fusion protein (see “Experimental Procedures”). Fig. 7 shows that an extract from A. nidulans 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, maleylacetoacetate and fumarylacetoacetate, 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), 154601 (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 Maleylacetoacetate 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') (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 (M, 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 MAAl (but see below). The complete nucleotide sequence of this cDNA has been submitted to DDBJ/EMBL/GenBankTM data bases (accession number AJ001838) The 3'9-UTR of this transcript was remarkably long (400 nucleotides, i.e. 30% of the transcript size). ESTs 290219 (59) (multiple sclerosis lesions) and 683733 (59) (germinal B-cells) represented cDNAs incomplete at their 5' ends, starting at codons 18 and 49, respectively, of the human MAAl 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 MAAl gene.

To confirm that the ORF of these transcripts encodes a human MAAl, the protein product encoded by the EST 265310 (5') 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 MAAl activity was dependent on the presence of GSH (not shown). By contrast, control cells showed no MAAl 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 maiA, a gene encoding an enzyme of the common part of the A. nidulans...
phenylalanine/phenylacetate pathways and provide definitive biochemical and genetic evidence that this gene encodes a maleylacetoacetate 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 in response to phenylalanine or phenylacetate as sole carbon sources; (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 maleylacetoacetate but cannot catalyze this compound further; (vi) mixing ΔmaiA and ΔfahA extracts results in reciprocal complementation of the corresponding enzyme deficiencies required for maleylacetoacetate catabolism to fumarate and acetoacetate; (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 and homogentisate to fumarate and acetoacetate 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 MAAI. 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 maleylacetoacetate, as substrates (26), which might suggest a detoxification function (6).

Our characterization of human MAAI 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 hepatoischemic 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 that of the toxic compounds accumulated as a result of a FAAH deficiency. Jorquera and Tanguay (12) have reported that, in contrast to fumarylacetoacetate, maleylacetoacetate 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 MAAI 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 MAAI deficiency in humans.

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

REFERENCES

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FIG. 10. The product of the human EST 265310 (5') ORF has MAAI activity. Detection of MAAI activity in cleared lysates of E. coli cells that overexpressed the protein encoded by this ORF under the control of a T7 polymerase-dependent promoter (see “Experimental Procedures”). The assay for MAAI, based on complementation of an A. nidulans ΔmaiA extract, was as in Fig. 7. The time points at which reduced glutathione (150 μg) and protein extracts (~50 μg) were added are indicated. Filled circles, a reaction to which a bacterial extract overexpressing the product of the EST 265310 ORF was added. The levels of expression achieved for this protein (not shown) were similar to those for A. nidulans MaiA. Triangles, an extract from induced E. coli cells carrying the expression vector with no insert. Open circles, a second, negative control reaction in which an extract from a recombinant bacterial clone that showed no overexpression of the desired EST 265310 protein (as determined by SDS-polyacrylamide gel electrophoresis) was added. O.D.330, A350.


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