Aspergillus nidulans catabolizes Leu to acetyl-CoA and acetoacetate through a pathway homologous to that used by humans. Fungal hlyA encodes a bifunctional polypeptide comprising the last two enzymes in this pathway, 3-methylglutaconyl-CoA hydratase and 3-hydroxy-3-methylglutaryl-CoA lyase. hlyA transcription is specifically induced by Leu. A ΔhlyA mutation removing the complete 3-methylglutaconyl-CoA hydratase C-terminal domain prevents growth on Leu but not on lactose or other amino acids and, in agreement with the predicted enzyme function, leads to Leu-dependent accumulation of 3-methylglutaconic acid in the culture supernatant. These data represent a formal demonstration in vivo of the specific involvement of 3-methylglutaconyl-CoA hydratase in Leu catabolism. Type I 3-methylglutaconic aciduria patients deficient in 3-methylglutaconyl-CoA hydratase show urinary excretion of 3-methylglutaconic acid and, in contrast to the other three types of methylglutaconic acidurias, 3-hydroxyisocaproic acid excretion. Gas chromatography-mass spectrometry analysis revealed an accumulation of both diagnostic compounds in ΔhlyA culture supernatants, illustrating that the metabolic consequences of equivalent inborn errors of metabolism are conserved from fungi to humans. Using our fungal type I 3-methylglutaconic aciduria model, we show that metabolites accumulating in the deficient strain are toxic, although less so than those accumulating in a ΔmccB strain deficient for the upstream enzyme 3-methylcrotonyl-CoA carboxylase. Diagnostic metabolite accumulation is Leu concentration-dependent, in agreement with the ability of Leu intake restriction to reduce the levels of offending metabolites. ΔmccB and ΔhlyA mutations show additive Leu toxicities. The double mutant accumulates 3-methylglutaconic acid, which can therefore be synthesized through 3-methylcrotonyl-CoA carboxylase-dependent and -independent reactions.

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3-Methylglutaconyl-CoA hydratase (EC 4.2.1.18) converting 3-methylglutaconyl-CoA to 3-hydroxy-3-methylglutaryl-CoA catalyzes the fifth step in the Leu degradation pathway (Fig. 1; Ref. 1). In humans, deficiency of this enzyme results in type I 3-methylglutaconic aciduria (MGA) (MIM 250950), a potentially pathogenic condition in which 3-methylglutaconic acid (3-MG), accumulating as a result of the metabolic block, is excreted in urine. However, urinary 3-MG excretion is a hallmark of three additional diseases: type II MGA (Barth syndrome (MIM 302060), resulting from a deficiency of a putative phospholipid acyltransferase), type III MGA or Costeff optic atrophy syndrome (MIM 258501, a deficiency of a predicted mitochondrial protein), and type IV MGA or “unspecified” MGA (MIM 250951, a heterogeneous class including patients for which all of the other three syndromes have been excluded). In contrast to type I MGA patients, type II, III, and IV MGA patients show abnormally elevated urinary excretion of 3-MG, but their 3-methylglutaconyl-CoA (3-MG-CoA) hydratase activity is normal. In addition to MGA patients, it has been shown that certain Smith-Lemli-Opitz patients also show abnormally increased plasma levels of 3-MG. This apparently complex scenario challenges our current understanding of the 3-MG-CoA metabolism. Remarkably, the characterization of the type II and III MGA genes gave no clues as to the biochemical mechanism underlying the abnormal accumulation of 3-MG, and it was not until the recent successful exploitation of a Saccharomyces cerevisiae model for type II MGA that direct evidence was obtained that tafazzin (the deficient protein in type II MGA) is involved in cardiolipin metabolism, illustrating the usefulness of microbial eukaryotic models.

Aspergillus nidulans is a filamentous fungus sharing with S. cerevisiae those features that make microbial eukaryotic models so valuable: a compact, haploid genome whose complete 31-Mb sequence is available, relatively easy gene inactivation procedures by homologous recombination/gene replacement, meiotic recombination, and small average intron size (less than 100 bp). A. nidulans grows in a chemically defined medium containing inorganic salts and sole nitrogen and carbon sources which can be changed as appropriate (see below). In contrast to budding yeast, however, A. nidulans has a much broader metabolic versatility. For example, A. nidulans is able to use most amino acids as sole carbon sources using metabolic pathways remarkably similar to those of human hepatocytes (5), a feature that we have exploited for designing fungal models for homogentisate dioxygenase (6), fumarylacetoacetate hydrolase (7), maleylacetoacetate isomerase, (8) and 3-methylcrotonyl-CoA carboxylase (9) deficiencies. These models have been instrumental for the identification of the human genes involved in alkaptonuria (6, 10, 11) and methylcrotonylglycinuria (12).

The use of A. nidulans models for Leu metabolism deficiencies is particularly advantageous (9). Leu is a ketogenic amino acid in humans/mammals which is catabolized to acetyl-CoA and acetoacetate (Fig. 1a). In contrast, fungi can convert acetyl-3-MG, 3-methylglutaconic acid; HL, 3-hydroxy-3-methylglutaryl-CoA lyase; GC/MS, gas chromatography-mass spectrometry; MCC, 3-methylcrotonyl-CoA carboxylase; 3-MG-CoA, 3-methylglutaconyl-CoA.
CoA into carbohydrates through the glyoxylate bypass of the Krebs cycle (13–15). Therefore, *A. nidulans* grows on Leu as the sole carbon source, enabling the use of simple growth tests to determine whether mutations impair this catabolic pathway. When this work was started, the gene encoding 3-MG-CoA hydratase had not been characterized in any organism. Here we use an *in silico* approach to identify the *A. nidulans* and human genes encoding this enzyme. As two recent papers reported the identification of the human gene and the characterization of loss-of-function mutations in type I MGA patients (16, 17), we focus this paper on the construction and physiological characterization of a fungal metabolic model for the disease. We provide here formal genetic evidence that 3-MG-CoA hydratase catalyzes in *vivo* one step in the Leu catabolic pathway shown in Fig. 1, and additionally, we demonstrate using epistasis tests that 3-MG can be synthesized without involvement of 3-methylcrotonyl-CoA carboxylase, a finding that may have implications in our understanding of the biochemical basis of those MGA types in which 3-MG-CoA hydratase is normal. Our data additionally suggest interplay between mitochondria and peroxisomes in the late steps of *A. nidulans* Leu catabolism.

**EXPERIMENTAL PROCEDURES**

**Fungal Strains, Media, and Growth Conditions**—*A. nidulans* strains carried markers in standard use (18). Complete and minimal medium were as in Ref. 19. Lactose was used at 0.05% (w/v) as the sole carbon source and amino acids at 30 μM, unless otherwise indicated. A *mccBΔhlyA* double mutant was constructed by crossing two parental strains of the complete genotype *biA1 argB2 methG1 (ΔhlyA::argB*) and *yA2 pabaA1 ppyG98 argB2 riboB2 (ΔmccB::pyr-4)*, respectively. argB+ progeny carrying the mutant hlyA allele were selected and analyzed by Southern for the presence of the *mccB* allele, using a probe specific for *Neurospora crassa* pyr-4. Six of thirteen analyzed strains were shown to be double mutants.

**RNA Isolation and Northern Blot Analysis and Transformation**—Fungal mycelia were pre-grown in glucose minimal medium and transferred to media with the indicated carbon sources. A cDNA library from mycelia grown under Leu catabolism-inducing conditions has been described. In Northern blots, a PCR cDNA fragment from positions 168 to 2053 of GenBank™ accession no. AY484417 was used as a hlyA-specific probe. MAD003 *biA1 argB2 methG1* was used as the recipient strain in transformation experiments (20). Southern blot analysis with argB- and hlyA-specific probes was used to confirm the expected hlyA truncation/deletion event.

**GC/MS Analysis of Culture Supernatants**—*Fungal* mycelia were pre-cultured as described for RNA isolation and transferred to minimal medium with one of the following carbon sources: 0.05% (w/v) lactose, 3 or 10 mM Leu. Secondary cultures were incubated for an additional 21 h. Culture supernatants were collected after removing mycelia by filtration. Organic acids were extracted, derivatized, and analyzed by GC/MS as described (9).

**RESULTS**

**In Silico Identification of an A. nidulans Gene Encoding 3-Methylglutaconyl-CoA Hydratase**—We predicted that 3-MG-CoA hydratase would belong to the enoyl-CoA hydratase/isomerase enzyme family, whose members share less than 20% amino acid sequence identity. Northern blot analysis failed to identify a putative Leu-regulated, candidate gene within or in the proximity of the *mccA-ivdA-mccB* leucine catabolic gene cluster (9), which led us to use two complementary strategies to identify the *A. nidulans* 3-MG-CoA hydratase gene. We first searched the genomes of metabolically versatile soil bacteria for Leu degradation operons, using the amino acid sequences of *A. nidulans* and *M. tuberculosis*, *P. aeruginosa* and *M. tuberculosis*, *P. aeruginosa* and *M. tuberculosis*. The genomes of *Pseudomonas aeruginosa* (21), *Agrobacterium tumefaciens* (22), *Streptomyces coelicolor* (23), and *Mycobacterium tuberculosis* (24) have, within 6 kb regions of their genomes, genes predictably encoding the above three proteins. We found that *M. tuberculosis*, *P. aeruginosa*, and *A. tumefaciens* (GenBank™ genome accession nos. NC_002755, NC_002516, and NC_003063, respectively) contain putative enoyl-CoA hydratases within these operons (see examples in Fig. 1b). Sequence alignments revealed that whereas the amino acid sequence identity between *P. aeruginosa* and *M. tuberculosis* *Leu* metabolism proteins ranged between 47% (in the case of isovaleryl-CoA dehydrogenase (IVD)) and 68% (in the case of MCCβ), enoyl-CoA hydratase candidates to 3-MG-CoA hydratase were only 33% identical, which illustrates the relatively low conservation of this family of proteins.

Secondly, we hypothesized that the 3-MG-CoA hydratase gene might be physically linked to the *A. nidulans* gene encoding 3-hydroxy-3-methylglutaryl-CoA lyase (HL), catalyzing the last step in the pathway (Fig. 1a). We used the amino acid sequence of human HL (GenBank™ accession no. P35914) to identify its *A. nidulans* orthologues using TBLASTN; two were
found. The one showing the highest score was mapped on chromosome V. Next, we used the deduced amino acid sequence of the putative \textit{P. aeruginosa} 3-MG-CoA hydratase (AAG05401) in a TBLASTN search of the \textit{A. nidulans} genome, which revealed a large number of putative genes for enoyl-CoA hydratases. However, the highest score (expect 2e^{-16} / H1100216) was obtained for a polypeptide encoded by a region adjacent to that encoding chromosome V HL. This open reading frame was a convincing candidate to encode \textit{A. nidulans} 3-MG-CoA hydratase.

Molecular Characterization of \textit{A. nidulans} hlyA—Inspection of the genomic sequence in this region of chromosome V showed that the HL coding sequence was located immediately upstream of that encoding the candidate 3-MG-CoA hydratase. Notably, no stop codons separating these open reading frames were found, strongly indicating that a single gene encodes a bifunctional polypeptide carrying an N-terminal HL domain and a C-terminal 3-MG-CoA hydratase domain (Fig. 2). PCR amplification using cDNA as the template (data not shown) demonstrated the existence of transcripts encoding both domains. Sequencing of a full-length cDNA clone confirmed the existence of a single open reading frame encoding a 599-residue polypeptide that included the two domains. The length of this cDNA clone (1841 bp without the poly(A) tail) agrees with the deduced size of the transcriptional unit as determined by Northern blot hybridization (Fig. 3), which additionally gave no indication of the existence of smaller transcripts. Comparison to the genomic DNA sequence revealed that the corresponding gene, which we denoted \textit{hlyA} (hydratase-lyase), is split by a short, 68-bp intron. We conclude that \textit{hlyA} encodes a bifunc-

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{alignment.png}
\caption{Alignment of HlyA with 3-MG-CoA hydratase and HL. Amino acid sequence alignment of \textit{A. nidulans} HlyA with human (GenBank\textsuperscript{TM} accession no. P35914) and \textit{Pseudomonas} (GenBank\textsuperscript{TM} accession no. AAG05399) HLs and with human (AUH-human, GenBank\textsuperscript{TM} accession no. Q13825) and \textit{Pseudomonas} (Hyd-Pseu, GenBank\textsuperscript{TM} accession no. AAG05401) 3-MG-CoA hydratase polypeptides. White lettering and black shadowing, identical residues in all three sequences; gray shadowing, similar (Arg/Lys/His; Glu/Gln/Asp/Asn; Ile/Leu/Met/Val; Ser/Thr; Phe/Tyr) residues.}
\end{figure}

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{northern.png}
\caption{Northern analysis of \textit{hlyA}. Mycelia were grown in minimal medium with 0.3\% (w/v) glucose as the sole carbon source for 16 h at 37 °C and transferred to media with the indicated carbon sources, where amino acids were used at 30 mM, glucose (Gluc) at 1\% (w/v), Tween at 0.1\% (v/v), and acetate (AcH) at 100 mM. After a 3-h additional incubation of these secondary cultures, mycelia were harvested and used to isolate RNA, which was analyzed by Northern blotting by using a hlyA-specific probe (upper panel). Lower panel, ethidium bromide staining of the gel to confirm approximately equal loading of RNA in the different tracks.}
\end{figure}
Involvement of hlyA in Leu Catabolism

To demonstrate the
linear DNA fragment in which 823 bp corresponding to A. nidulans argB
hlyA to the
333 (including an intron) and 1235 bp corresponding
codons 83
hlyA
that the inducer is a pathway metabolite located upstream of
expected for a gene playing a specific role in Leu, but not in Ile
had no inducing effect. The
bolic cluster (9). Acetate, another gluconeogenic carbon source,
mccA
hlyA
the transcriptional regulation of
any significant effect upon
stream enzymes IVD and MCC to the same extent as Leu, had
is induced by Leu and repressed by glucose. Of note, neither the
599 tripeptide matches the peroxisome C-terminal targeting
acid sequence identity). The C-terminal HlyA 597-Ala-Lys-Val-
3-MG-CoA hydratase domain including residues 320
ing residues 1 through 302 shows 48% identity to human HL
tional polypeptide catalyzing the two final steps in the Leu
degradation pathway.

Although the amino acid sequence of the HL domain including
residues 1 through 302 shows 48% identity to human HL (Fig. 2), the closest homologue of the predicted C-terminal
3-MG-CoA hydratase domain including residues 320–599 was the putative P. aeruginosa 3-MG-CoA hydratase (29% amino
acid sequence identity). The C-terminal HlyA 597-Ala-Lys-Val-599 tripeptide matches the peroxisome C-terminal targeting
signal consensus (Prosite Databank accession no. PS00342) (25). No mitochondrial signal peptide was evident in the
N-terminal region of the protein.

Northern blots (Fig. 3) demonstrated that hlyA transcription
is induced by Leu and repressed by glucose. Of note, neither the
branched chain amino acid Ile nor the aromatic amino acid
Phe, which induce transcription of genes encoding the upstream
enzymes IVD and MCC to the same extent as Leu, had
any significant effect upon hlyA transcription, indicating that the
transcriptional regulation of hlyA differs from that of the
ivdA, mccA, and mccB genes in the chromosome III Leu catalytic
cluster (9). Acetate, another gluconeogenic carbon source,
had no inducing effect. The hlyA transcriptional pattern is as
expected for a gene playing a specific role in Leu, but not in Ile
or Phe catabolism. Although we note that deletion of mccB
do not prevent induction, additional data precluded the conclusion
that the inducer is a pathway metabolite located upstream of
the MCC-catalyzed step (see below). The molecular basis of
hlyA induction has not been investigated further.

A Truncation/Deletion Mutation Demonstrates the Specific
Involvement of hlyA in Leu Catabolism—To demonstrate the
physiological involvement of hlyA in Leu catabolism, we
constructed a deletion/truncation allele by transformation with a
linear DNA fragment in which 823 bp corresponding to hlyA
codons 83–333 (including an intron) and 1235 bp corresponding
to the hlyA 3′-UTR were flanking a genomic DNA insert con-
taining the A. nidulans argB + gene (Fig. 4). Site-directed inte-
gration of this fragment mediated by a double cross-over event
results in a mutant hlyA in which the coding region is truncated
after residue 333. In this mutant, the sequence encoding the
C-terminal 3-MG-CoA hydratase domain (codons 334–599 of
HlyA) is replaced by the A. nidulans DNA insert containing
argB + (Fig. 4). An argB2 arginine-requiring strain was trans-
fomed with this fragment, and arginine-independent clones
were selected and purified. About 10% of the transformants
were unable to grow on Leu as the sole carbon source. Three
such transformants were analyzed by Southern hybridization
using argB- and hlyA-specific probes and were shown to carry
the truncation/deletion mutation shown in Fig. 4, denoted
ΔhlyA. All three transformants were found to be phenotypically
indistinguishable and unable to grow on Leu. Therefore, one
was chosen for further characterization. Northern blot analysis
of this strain confirmed the absence of full-length message
and the presence of a truncated message terminating within the
argB- containing DNA insert and including the region coding
for the N-terminal HL domain (Fig. 3, compare lanes 3 and 7; Fig. 4).

Although ΔhlyA prevents growth on Leu (Fig. 5), the mutation
has no effect upon the ability of the fungus to use other
branched chain amino acids (Ile, Val), Pro, Thr, or lactose as
the sole carbon source (data not shown). As shown below, this
ΔhlyA strain accumulates 3-MG when challenged with Leu,
strongly indicating that the truncation/deletion mutation results
in the specific impairment of 3-MG-CoA hydratase activity.
Taken together, these data formally demonstrate the physi-
ological, specific involvement of hlyA in Leu catabolism.

Biochemical Characterization of ΔhlyA: A Fungal Model for
3-Methylglutaconyl-CoA Hydratase Deficiency—To establish
that the ΔhlyA mutation blocks Leu catabolism at the level of
3-MG, we used GC/MS analysis of culture supernatants (9). Mycelia of the wild-type and ΔhlyA strains were pre-cultured
on glucose minimal medium and transferred to fresh minimal
medium containing either lactose (at 0.05% (w/v)) or Leu (at 3
or 10 mM) as sole carbon source. Organic acids in supernatants of
these secondary cultures were extracted and analyzed by
GC/MS. Fig. 6a and b show that the metabolic profiles of the
wild type and ΔhlyA strains are virtually indistinguishable
upon transfer to lactose minimal medium. In contrast, the
mutant specifically accumulated the E (trans) and Z (cis) iso-
mers of 3-MG upon transfer to 3 mM leucine minimal medium
(Fig. 6, c and d). 3-MG in the mutant culture supernatant
derives from 3-MG-CoA, the substrate of 3-MG-CoA hy-
ratase. The specific accumulation in the mutant culture supernatant of
3-MG was markedly more prominent upon transfer of 10 mM
Leu minimal medium (Fig. 6, e and f; see E and Z), demonstrating
that it is Leu-dependent. Both the wild-type and the muta-
ent supernatants showed an accumulation of 2-hydroxyisocaproic
acid resulting from Leu transamination. We have previously shown that branched chain ketoacid dehydrogenase
is limiting under these growth conditions (9).

In addition to 3-MG, we noted that the mutant also accumu-
lated 3-hydroxyisovaleric acid (3-HIVA) resulting from the re-
versibility of the pathway to 3-methylcrotonyl-CoA and hydration (see Fig. 1; Ref. 1). This is in marked similarity to human type I MGA patients, whose urine GC/MS analysis shows, in addition to the E and Z isomers of 3-MG, a significant amount of 3-HIVA. Indeed, the presence in urine of 3-HIVA in addition to 3-MG is a diagnostic feature of type I MGA patients which helps to differentiate them from the three other MGA types (see "Discussion"). Therefore, these data additionally illustrate the remarkably similar consequences of equivalent enzyme deficiencies in humans and fungi, giving credence to our metabolic model.

Identification of the Human Gene Homologue: Mutation Screen—The Human Genome databases were screened for candidate homologues using the HlyA amino acid sequence in TBLASTN searches. Relatively low sequence conservation among enoyl-CoA hydratases precluded the unambiguous identification of candidate genes but allowed our selection of two strong candidates, the 5-exon FLJ20909 gene and the 10-exon AUH gene. AUH encodes an RNA-binding protein with an enoyl-CoA hydratase domain. We screened these candidate genes in two type I MGA-affected brothers and detected a C589T (R197stop) truncating mutation in AUH. During our screen, Ijlst et al. (17) reported that AUH was the human 3-MG-CoA hydratase gene and the presence of the R197stop mutation in the same probands that we genotyped. This and a second report (16) (also including these siblings in the analysis) describe a total of five causative mutations in this gene, unambiguously demonstrating that type I MGA results from loss-of-function mutations in AUH.

Metabolites Accumulating in a Strain Deficient for 3-MG-CoA Hydratase Are Toxic, Although Less so than in a Strain Deficient in MCC—Only 11 type I MGA patients have been described in the literature (16) (which, in passing, explains why the two siblings described by Duran et al. in Ref. 26 have been
were pre-cultured as in Fig. 6, transferred to 3 mM Leu minimal, and incubated for a further 21 h before analysis with GC/MS organic acids. MCC is a heterodimeric enzyme composed of α and β subunits. ΔmccB is a null allele of the A. nidulans gene encoding MCCβ, phenotypically indistinguishable from a double mutant carrying null alleles in both genes encoding MCC subunits (9). A double ΔhlyA ΔmccB strain was constructed by meiotic recombination (see “Experimental Procedures”). Because the MCC-catalyzed step immediately precedes the 3-MG-CoA hydratase step in the Leu degradation pathway and the ΔmccB allele is a null allele, the finding that Leu inhibition was markedly more pronounced than that resulting from MCC deficiency (9), strongly indicating that the metabolites accumulated in the former are less toxic to the cells.

Unexpected Additivity of a Null mcc Mutation with ΔhlyA—MCC is a heterodimeric enzyme composed of α and β subunits. ΔmccB is a null allele of the A. nidulans gene encoding MCCβ, phenotypically indistinguishable from a double mutant carrying null alleles in both genes encoding MCC subunits (9). A double ΔhlyA ΔmccB strain was constructed by meiotic recombination (see “Experimental Procedures”). Because the MCC-catalyzed step immediately precedes the 3-MG-CoA hydratase step in the Leu degradation pathway and the ΔmccB allele is a null allele, the finding that Leu inhibition was markedly more pronounced than that resulting from MCC deficiency (9), strongly indicating that the metabolites accumulated in the former are less toxic to the cells.

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inefficiently. We cannot exclude the possibility that the enzyme(s) involved in this reaction might account for the partial bypass of the MCC step that we observe in vivo with the ΔmccB ΔhlyA double mutant. However, the fact that levels of this McCB-independent activity are almost negligible as compared with those attributable to 3-methylcrotonyl-CoA carboxylase strongly argues against a significant physiological role for this alternative enzyme(s) in 3-MG-CoA biosynthesis.

**DISCUSSION**

In contrast to other genetically amenable eukaryotic microbial models such as _S. cerevisiae_, _A. nidulans_ is able to use most amino acids as the sole carbon source (5), using pathways remarkably similar to those in human cells (6–10, 12). _A. nidulans_ is particularly useful for studying Leu (or other ketogenic amino acid) catabolism because acetyl-CoA resulting from its oxidation can be converted by the fungus (but not by human cells) into carbohydrates using the glyoxylate bypass of the Krebs cycle. Therefore, the fungus can grow on Leu as the sole carbon source. We have previously exploited this feature for the physiological characterization of the MCC-catalyzed step in the Leu catabolic pathway (9, 12).

We report here the physiological characterization of _A. nidulans_ 3-MG-CoA hydratase, the enzyme deficient in patients suffering from type I MGA. We used the amino acid sequence of the fungal enzyme to identify human homologues in sequence similarity searches. In two affected siblings, we detected a truncating mutation in a gene denoted _AUH_, encoding an enoyl-CoA hydratase. While this work was in progress, Jie et al. (17) and Nga Ly et al. (16) reported that ours and additional loss-of-function mutations in _AUH_ cause type I MGA, formally demonstrating that _AUH_ encodes 3-MG-CoA hydratase.

Evidence that this enzyme catalyzes a step of the Leu degradation pathway was based on the finding that excretion of 3-MG (and additional diagnostic metabolites, see below) by type I MGA patients increases 2- to 3-fold with a high protein diet, fasting, or a Leu challenge (26). We report here formal demonstration of the involvement of this enzyme in Leu catabolism: a mutant deleted for the complete 3-MG-CoA hydratase coding region is unable to grow on Leu (although its growth on Ile, Val, Phe, Pro, Thr, or carbohydrates is unaffected), and it shows dose-dependent 3-MG accumulation in the culture supernantant after a Leu challenge. This conclusion is relevant in view of the fact that 3-MG in the three other syndromes would appear not to proceed from Leu catabolism (Ref. 1, and see below). Our conclusion is further buttressed by the presence of _hlyA_ homologues within bacterial Leu degradation operons (Fig. 1b).

The amino acid sequence of _A. nidulans_ 3-MG-CoA hydratase and that of _HL_, the enzyme converting 3-hydroxy-3-methylglutaryl-CoA to acetyl-CoA and acetocacetate, are C- and N-terminal domains, respectively, of a bifunctional polypeptide encoded by the _hlyA_ gene. Transcription of _hlyA_ is specifically induced by Leu, which agrees with the physiological role or both enzyme activities in _Leu_ catabolism. _Leu_ degradation occurs in mitochondria (1). The predicted _hlyA_ bifunctional polypeptide would appear not to contain a potential mitochondrial targeting sequence but has a C-terminal peroxisomal targeting sequence. This result was unexpected. Although human _HL_ is located in both mitochondria and peroxisomes through N-terminal mitochondrial leader and C-terminal peroxisomal targeting peptides, respectively (27), the _AUH_ gene product (i.e. human 3-MG-CoA hydratase) has been reported to be mitochondrial (28); human MCC is mitochondrial (12), and _A. nidulans_ McCa and McCb have predicted mitochondrial leader peptide sequences in their N termini. The biochemical phenotype of _ΔhlyA_ strongly suggests that at least a proportion of _HlyA_ acts in mitochondria (see below). Mitochondrial protein import is post-translational, and certain mitochondrial proteins such as cytochrome c contain internal mitochondrial targeting sequences. Perhaps _HlyA_ is one such protein.

A distinctive hallmark of type I MGA is that urinary excretion of 3-MG is accompanied by a marked increase in levels of 3-HIVA which is not found in any of the other three MGA syndromes (1). We have previously emphasized the remarkable similarities in the metabolic consequences of equivalent amino acid degradation blocks between _A. nidulans_ and humans (6–9). Our GC/MS analyses of culture supernatants demonstrates that a _ΔhlyA_ strain also accumulates 3-HIVA, in addition to 3-methylglutaconic acid. This demonstrates that, from fungi to humans, these two metabolites are diagnostic markers of 3-MG-CoA hydratase deficiency, and that our metabolic model faithfully resembles the human biochemical phenotype.

We used our fungal type I MGA model to demonstrate that metabolites accumulating as a result of the enzyme deficiency are toxic (Fig. 5). However, this toxicity seems to be less pronounced than that resulting from MCC deficiency. We note that in _A. nidulans_, but not in our relatively isogenic fungal strains cultured under standardized conditions, other genetic or environmental factors almost certainly contribute to the clinical phenotype (for discussion, see Ref. 29). Such relatively low toxicity might be a possible explanation of the mild clinical phenotype found in certain type I patients (1). We speculate that the apparent low frequency of the disease reflects that individuals deficient for 3-MG-CoA hydratase do not always come to clinical attention.

An important finding of this paper which challenges our current understanding of Leu catabolism is that _ΔmccB_ and _ΔhlyA_ mutations affecting two sequential steps in the catabolic pathway showed phenotypic additivity in our _Leu_ toxicity test. Unexpectedly, GC/MS analysis unambiguously demonstrated that the double mutant accumulates 3-MG (resulting from 3-MG-CoA hydratase deficiency) in addition to 3-HIVA (resulting from MCC deficiency).

3-MG accumulation in type II and III MGA patients is modest (1, 2, 30). However, high 3-MG excretion has been reported for certain type IV patients (1). Although a deficient mitochondrial function underlies the biochemical phenotype of type II (4) and possibly of type III (3) patients, the primary enzymatic or molecular defect in type IV patients remains enigmatic. It has been suggested (30) that in type IV MGA patients increased levels of sterol/isoprenoid intermediates may be the source, through the mevalonate shunt proposed by Edmond and Popjak (31), of the abnormally elevated 3-MG levels. However, the “classical” mevalonate/isoprenoid shunt involves MCC (9) and, therefore, this cannot be the source of 3-MG found in our double mutant. It has also been suggested that abnormally elevated 3-MG plasma levels found in certain patients with Smith-Lemli-Opitz syndrome (a deficiency of cholesterol biosynthesis) result from increased flux through this shunt. However, this explanation does not explain why this increased flux would result in increased 3-MG levels without a detectable increase in 3-HIVA (which would reflect an overload of MCC). Our findings formally demonstrate that, at least in our metabolic model,
3-MG-CoA can be generated through an MCC-independent reaction(s), which might be responsible for increased 3-MG levels characteristic of type IV MGA patients.

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