

Cloning of Dimethylglycine Dehydrogenase and a New Human Inborn Error of Metabolism, Dimethylglycine Dehydrogenase Deficiency

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Dimethylglycine dehydrogenase (DMGDH) (E.C. number 1.5.99.2) is a mitochondrial matrix enzyme involved in the metabolism of choline, converting dimethylglycine to sarcosine. Sarcosine is then transformed to glycine by sarcosine dehydrogenase (E.C. number 1.5.99.1). Both enzymes use flavin adenine dinucleotide and folate in their reaction mechanisms. We have identified a 38-year-old man who has a lifelong condition of fishlike body odor and chronic muscle fatigue, accompanied by elevated levels of the muscle form of creatine kinase in serum. Biochemical analysis of the patient's serum and urine, using ¹H-nuclear magnetic resonance NMR spectroscopy, revealed that his levels of dimethylglycine were much higher than control values. The cDNA and the genomic DNA for human *DMGDH* (*hDMGDH*) were then cloned, and a homozygous A→G substitution (326 A→G) was identified in both the cDNA and genomic DNA of the patient. This mutation changes a His to an Arg (H109R). Expression analysis of the mutant cDNA indicates that this mutation inactivates the enzyme. We therefore confirm that the patient described here represents the first reported case of a new inborn error of metabolism, DMGDH deficiency.

Introduction

The catabolism of choline in mitochondria is a cyclical process, involving the formation of betaine, dimethylglycine, sarcosine, glycine, and serine, with the ultimate regeneration of choline (fig. 1). The interconversion of these species involves the transfer of "1-carbon" units at the level of active formaldehyde (Mackenzie and Abeles 1956; Mackenzie and Frisell 1958; Wittwer and Wagner 1981*a*). One methyl group is removed from dimethylglycine by dimethylglycine dehydrogenase (DMGDH) to form the modified amino acid sarcosine. A second methyl group is removed from sarcosine by sarcosine dehydrogenase (SDH) to form glycine (Frisell and Mackenzie 1962; Lang et al. 1994; Wittwer and Wagner 1981*a*, 1981*b*). Both enzymes are associated with a folate derivative as a coenzyme (Wittwer and Wagner 1980, 1981*a*, 1981*b*; Steenkamp and Husain 1982; Wagner et al. 1984). In each reaction, one methyl group is donated to one tetrahydrofolate (H₄PteGlu) molecule, forming 5,10-

methylenetetrahydrofolate (5,10-CH₂-H₄PteGlu), which is termed "active formaldehyde." This activated form of formaldehyde, in turn, is able to donate a methyl group in the formation of serine from glycine via serine hydroxymethyltransferase. In addition, 5,10-CH₂-H₄PteGlu can be used in the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate; it can be reduced to 5-methyltetrahydrofolate (5-CH₃-H₄PteGlu) for synthesis of methionine from homocysteine via homocysteine methyltransferase (methionine synthase); and it may be oxidized to 10-formyltetrahydrofolate (10-CHO-H₄PteGlu) for purine synthesis. In the case of DMGDH and SDH, the preferred folate derivative in the reaction is H₄PteGlu₅, although other derivatives may be used (Strong et al. 1989; Wittwer and Wagner 1981*b*). The DMGDH and SDH reactions may proceed in the absence of folate if folate becomes dissociated from the enzymes or if the person has a folate deficiency. However, the end products of the reactions when the folate cofactor is missing are formaldehyde and formate (Steenkamp and Husain 1982; Wittwer and Wagner 1981*a*). Both SDH and DMGDH have been shown to be major folate-binding proteins (Wittwer and Wagner 1980, 1981*a*, and 1981*b*).

In addition to their folate cofactor, both DMGDH and SDH have a covalently bound flavin adenine dinucleotide (FAD) (Wittwer and Wagner 1981*a*; Cook et al. 1984). Flavin peptides with a FAD covalently at-

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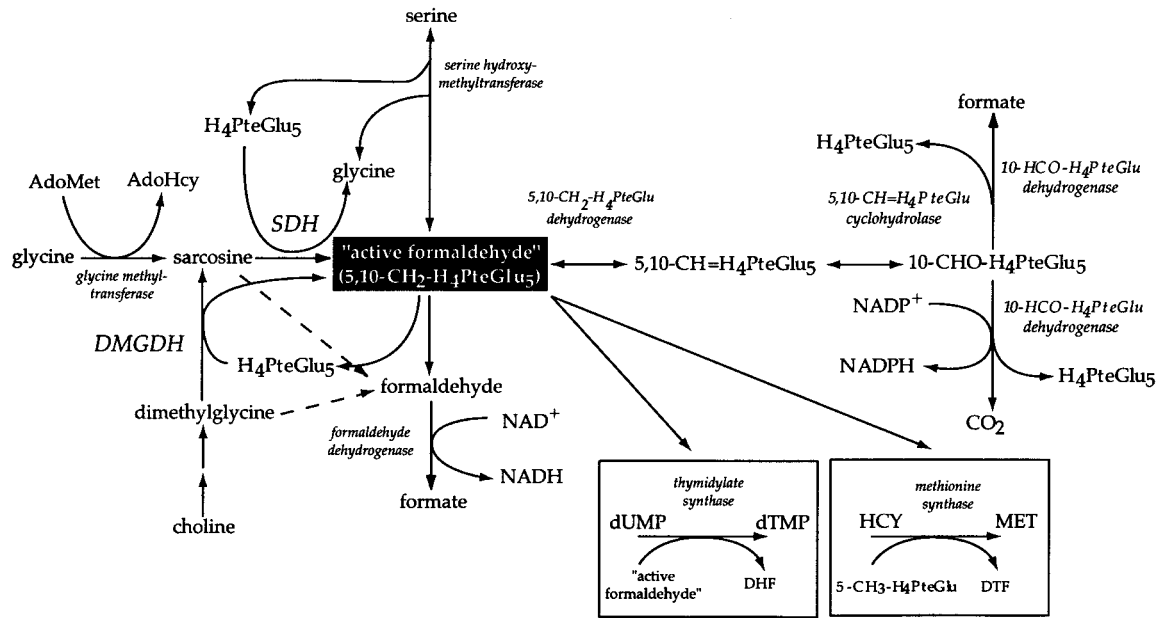


Figure 1 Metabolic pathways involving choline. The key relationships between glycine, serine, dimethylglycine, and sarcosine are indicated, as are the various fates of "active formaldehyde" (adapted from Wittwer and Wagner 1981a).

tached have been isolated from both rat DMGDH (rDMGDH) and rat SDH (rSDH) (Cook et al. 1984 and 1985). The covalent attachment occurs via a His(N3)/flavin (8 α carbon) linkage at H84 in rDMGDH, and the recent cloning of rSDH cDNA shows that covalent FAD attachment occurs at H109 in rSDH (Cook et al. 1984; Lang et al. 1991; Bergeron et al. 1998). Although the exact reaction mechanism for these two enzymes is unknown, electrons are subsequently transferred from this FAD to electron-transferring flavoprotein (ETF), then to ETF dehydrogenase, and finally to the mitochondrial electron-transport system (Hoskins and MacKenzie 1961).

A deficiency of SDH was first identified in a 10-month-old male infant, who died soon after reaching the age of 1 year. He had elevated levels of sarcosine in his urine and serum (Gerritsen and Waisman 1966). A total of 20 patients who are thought to have SDH deficiency have been described in the literature (Scott 1995). Clinical symptoms have been difficult to correlate with sarcosinemia/sarcosinuria, and some researchers believe that the diagnosis of sarcosinemia/sarcosinuria is unrelated to any clinical symptoms (Gerritsen 1972; Scott 1995). All patients, however, have been characterized by elevated levels of sarcosine in urine and/or serum.

Dimethylglycine is normally found in urine and serum at very low levels, and elevated levels of dimethylglycine are sometimes found to be secondary to another disorder, such as folate or cobalamin deficiency (Porter et al. 1985; Allen et al. 1993; Dudman et al. 1993). We

recently identified a patient who had elevated levels of dimethylglycine in serum and urine. The patient was identified through ¹H-nuclear magnetic resonance (NMR) studies of serum and urine that revealed dimethylglycine levels much higher than normal values (reported in detail by Moolenaar et al. [1999]). rDMGDH cDNA has been used to clone the cDNA for human DMGDH (hDMGDH) from expressed-sequence tag (EST) databases. A homozygous mutation was identified in DMGDH cDNA and genomic DNA from this patient (GenBank accession number AF111858). Studies of recombinant mutant protein show that the mutation inactivates the enzyme.

Patient and Methods

hDMGDH cDNA Cloning

Databases were searched using the rDMGDH cDNA sequence (Genbank accession number X55995, E.C. 1.5.99.2). The Human Genome Sciences Database was searched using facilities at SmithKline Beecham. All sequencing was performed by the Molecular Biology Core Sequencing Facility at the Mayo Clinic, using an ABI Prism model 377 sequencer (Applied Biosystems). PCR reactions to amplify gaps between ESTs were performed using PCR Supermix (Gibco BRL). Cycling conditions were as follows: denaturing at 95°C for 3 min; 35 cycles of denaturing at 94°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1 min; extension at 72°C

for 7 min; and soaking at 4°C. Human fibroblast cDNA served as a template. Primers used in the reaction were: 5'-AAGAGATGTTCCCTTTACTC-3' (forward) and 5'-TTTAATCTGTTTCAGTGCTTG-3' (reverse). A PAC clone containing hDMGDH sequence was obtained from Genome Systems. PAC DNA was induced to high copy number and was prepared according to the supplier's protocol. For direct sequencing of the PAC clone, DNA was prepared using kb-100s DNA purification columns (Genome Systems). The rDMGDH primer designed to the sequence of the flavin binding site and used for indirect PAC clone sequencing was 5'-GTGCCA-GGTAGAGCCAGCCGTGAGTTCCGA-3'. A second genomic clone (P1) was also obtained from Genome Systems, since the PAC clone was found to be missing at least one exon at the 5' end of the gene.

Patient-cDNA/Genomic-DNA Analysis

cDNA was prepared from patient and control fibroblast cultures, as described elsewhere (Mohsen et al. 1998). After the H109R mutation was identified, control alleles were sequenced around the mutated region. PCR reactions to amplify the region from cDNA were performed with the following primers: 5'-CAACTTACTTTCATCCTGGA-3' (forward) and 5'-GAGTAAAGGGAACATCTCTT-3' (reverse). PCR was performed using the genomic DNA as a template to amplify a region around the H109R mutation with the following primers: 5'-TGGCCTCCCCCATAGTTTTA-3' (forward) and 5'-GCCTATGGATACTCTTTTGC-3' (reverse).

Expression of hDMGDH in Mammalian Cells

The precursor sequence for hDMGDH was cloned into the *KpnI* site of the pcDNA6/V5-His plasmid (Invitrogen). Two hundred ninety-three cells (American Type Culture Collection) were grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Cells grown in 6-cm dishes were transfected with 3 µg of either the wild-type or mutant vectors, along with a pcDNA3.1 His/LacZ control plasmid (Invitrogen), using the Calcium Phosphate Transfection kit from the same company. Forty hours after transfection, the cells were harvested for western blot analysis or RNA extraction. Control plates were stained with the β-Gal staining kit (Invitrogen). For western blots, the isolated cell pellets were resuspended in PBS, pH 7.4, and were sonicated briefly; 30 µg of supernatant was loaded onto a 12% SDS polyacrylamide gel. Western blot analysis was performed, as described elsewhere, using a 1:1000 dilution of rabbit antiserum, which was raised against native DMGDH that had been purified from pig liver, as the primary antibody (Mohsen et al. 1998). To quantify the level of expression of the wild-type and mutant vectors, mRNA was extracted from transfected cells with

the QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech). Then 0.5 µg of RNA was used, with the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech), to generate first-strand cDNA. One microliter of the reverse-transcription product was subjected to PCR with primers specific to DMGDH (5'-ATGGATTGAAGAAGAAGCAGTC-3' and 5'-GATGTCGTGTTGCCAACCAC-3') or β-ETF (5'-CACCC-TGTAAGTGGCTGC-3' and 5'-ATGTTGAGAGTCA-GTTTTATTGCC-3'), with cycling conditions as described above. Amplification cycle number was varied to identify conditions that avoided saturation of the PCR reaction. PCR products were examined on 1% agarose gels.

DMGDH Expression Studies.—Mature wild-type rat and human DMGDH sequences (with amino termini beginning at Ser22 and Val29, respectively) were subcloned into the pKK223-3 expression vector (Amersham Pharmacia Biotech) in the *EcoRI/HindIII* sites. The H109R mutant, which represents residue 81 in the mature protein, was created using PCR-based mutagenesis and was inserted into compatible restriction sites in the wild-type vectors. Cultures (100 ml) of XL1-Blue cells containing wild-type, mutant DMGDH, and pKK223-3 with no insert were grown in 2xYT media plus ampicillin at 37°C, were induced in log-phase growth with 0.05 mM isopropyl-β-D-thiogalactoside, and were allowed to grow overnight at 30°C. Cell extracts were prepared as described elsewhere (Binzak et al. 1998). Extracts were tested for DMGDH activity using the ETF reduction assay, as described elsewhere, with 0.5 M dimethylglycine as substrate (Binzak et al. 1998) (*N,N*-dimethylglycine [hydrochloride] was purchased from Sigma). Protein assays were performed using the Bio-Rad DC Protein Assay (Bio-Rad). Western blot analysis was performed as described above.

¹H NMR Spectroscopy.—¹H NMR spectroscopy of serum and urine specimens was performed essentially as described elsewhere (Wevers et al. 1994).

Clinical History

The patient is a 38-year-old man of African ancestry who presented with a fishlike body odor that was first noticed when he was 5 years old. The intensity of the odor increases with physiological stress, such as illness, as well as during times of increased physical activity. The odor has led to severe psychological problems and professional difficulties. The patient had normal growth and development and is of normal intelligence with no unusual CNS symptoms, but he has experienced chronic muscular fatigue since adolescence. He has had no known hematologic problems or liver dysfunction. Routine clinical chemistry determinations yielded unremarkable results, with normal levels of cobalamin, folate, and

homocysteine in serum. Creatine kinase levels were consistently found to be four- to fivefold higher than normal levels. Metabolic investigations—including testing of amino acids, organic acids, carnitine ester profile, and serum levels of very-long-chain fatty acids—were normal. Trimethylaminuria, the only known specific defect leading to “fish-odor syndrome” was excluded using NMR spectroscopy of urine (Moolenaar et al. 1999). The patient does not use any dietary supplements containing either carnitine or dimethylglycine. There is no known consanguinity in the pedigree, but the patient’s parents were unavailable for interview. The brothers and sisters (a precise pedigree was unavailable) and the two sons of the patient are asymptomatic.

Results

^1H NMR

^1H NMR spectroscopic analysis of blood samples from the patient showed a dimethylglycine concentration of 449 mM creatinine (~20-fold higher than normal values) in urine and a concentration of 221 μM /liter (~100-fold higher than normal values) in serum. No other unusual metabolites were present. Figure 2 shows part of the ^1H NMR spectrum of serum from the patient. A singlet at 2.93 parts per million (ppm) is caused by dimethylglycine. Dimethylglycine was observed in serum spectra from healthy individuals only in low concentration (3.80-ppm singlet; data not shown). The identity of the dimethylglycine was confirmed using ^{13}C -NMR spectroscopy and gas chromatography–mass spectroscopy. Similar findings had been observed in the patient’s urine (complete details of biochemical analyses of the urine have been reported elsewhere [Moolenaar et al. 1999]).

cDNA Cloning

Because of the biochemical findings in this patient, we suspected a deficiency of DMGDH. To confirm this, we initiated the cloning of hDMGDH cDNA. The cDNA sequence for rDMGDH (Lang et al. 1991) was used to screen several databases to identify a human homologue. An EST was identified from the Human Genome Sciences database that aligns to the 3' end of the rat cDNA and is 92% identical over ~400 nucleotides. This clone also contains 1.1 kb of 3' UTR, including the poly A tail. A second human EST that aligns near the 5' end of the rat cDNA sequence was identified from Genbank. This EST is 81% identical to the rat sequence over ~170 nucleotides. PCR was then used to amplify the region between these two short sequences from human fibroblast cDNA made from control fibroblast mRNA. Various techniques to clone the extreme 5' end of the cDNA were unsuccessful; therefore, a human PAC clone was isolated using ~1.8 kb of the hDMGDH cDNA as a

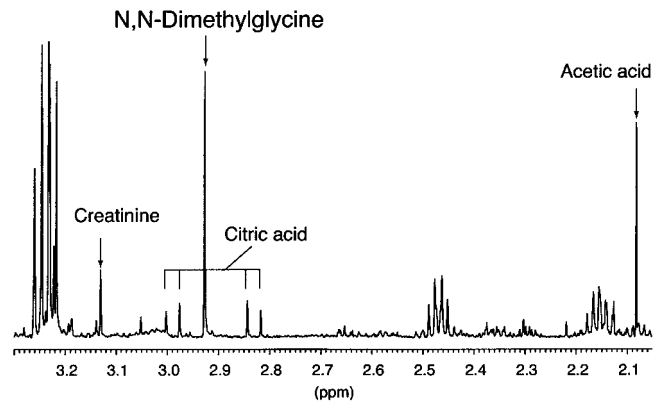


Figure 2 ^1H NMR spectrum of serum from the DMGDH-deficient patient. Peaks of relevant metabolites are labeled. The singlet peak at 2.93 ppm is caused by an accumulation of dimethylglycine in the patient’s serum.

probe. Both Southern blot analysis and direct sequencing of this PAC clone confirm that it contains the hDMGDH genomic sequence (Binzak et al. 2000). Since the extreme 5' end of the gene was still missing, a second genomic clone (P1) was obtained from Genome Systems, and it proved to contain the final 5'-most exon of coding sequence of the gene.

The hDMGDH cDNA is 2,598 nucleotides long, which suggests the presence of a protein with 866 amino acids and a 3' UTR of ~1,100 nucleotides (fig. 3). Overall, the precursor cDNA sequences for hDMGDH and rDMGDH share 85.6% nucleotide identity, as calculated using the GAP program from the University of Wisconsin GCG sequence-analysis software, although the mature proteins show 91.4% amino acid identity (93.9% amino acid similarity). The flavin peptide of rDMGDH (Wittwer and Wagner 1980) is 100% conserved, at the amino acid level, with the same region in hDMGDH; however, the area just upstream shows reduced homology. In fact, the cDNA sequence of hDMGDH suggests that seven additional amino acids are present in the mitochondrial targeting peptide, compared with rDMGDH. Val29 is predicted, on the basis of the known pattern of processing of mitochondrial leader peptides, to be the amino terminus of the mature human protein.

Analysis of Patient DNA

Analysis of hDMGDH cDNA and genomic DNA from the patient identified a homozygous A→G point mutation at nucleotide 326 of the precursor coding region (fig. 4). This suggests that the alteration of a His to an Arg at amino acid 109 (H109R or residue 81 of the predicted mature protein) occurs 18 amino acids downstream of the site of the FAD attachment that was

ATG CTC CGT CCC GGC GCG CAG CTG CTG CGG GGC CTC CTG CTG CGG 45
 M L R P G A Q L L R G L L L R
 AGC TGC CCG CTG CAG GGC TCC CCC GGG CGC CCG CGC TCT GTC TGC 90
 S C P L Q G S P G R P R S △ C
 GGC CGG GAA GGA GAG GAA AAA CCA CCC TTA TCT GCA GAA ACA CAA 135
 G R E G E E K P P L S A E T Q
 TGG AAA GAC AGA GCA GAA ACA GTG ATA ATT GGA GGT GGC TGT GTT 180
 W K D R A E T V I I G G G C V
 GGT GTG AGT CTG GCT TAT CAC CTG GCC AAA GCA GGG ATG AAA GAT 225
 G V S L A Y H L A K A G M K D
 GTG GTC CTG CTG GAG AAA TCA GAG CTC ACG GCT GGA TCT ACC TGG 270
 V V L L E K K S E L T A G S T W

CAC	GCA	GCA	GGT	TTA	ACA	ACT	TAC	TTT	CAT	CCT	GGA	ATA	AAC	TTG	315
H	A	A	G	L	T	T	Y	F	H	P	G	I	N	L	

 AAG AAA ATA CAT TAT GAT AGC ATC AAA CTT TAT GAG AAA CTG GAA 360
 K K I H Y D S I K L Y E K L E
 GAA GAA ACT GGT CAG GTG GTG GGA TTC CAT CAG CCA GGT AGT ATC 405
 E E T G Q V V G F H Q P G S I
 AGA CTT GCT ACC ACC CCT GTA AGG GTA GAT GAA TTT AAA TAT CAA 450
 R L A T T P V R A A G A R K Y Q
 ATG ACT CGG ACT GGC TGG CAT GCA ACA GAA CAG TAT CTC ATT GAA 495
 M T R T G W H A T E Q Y L I E
 CCT GAA AAA ATT CAA GAG ATG TTC CCT TTA CTC AAC ATG AAT AAG 540
 P E K I T M A L V R A A G A R K C G
GTT TTA GCT GGA TTG TAT AAT CCT GGA GAT GGT CAC ATT GAT CCT 580
 V L A G L Y N P G D G H I D P
TAT TCT CTA ACT ATG GCA CTG GCT GCT GGG GCT AGG AAA TGT GGT 630
 Y S L T M A L V R A A G A R K C G
GCC CTT TTA AAA TAT CCT GCA CCA GTA ACT TCT CTG AAA GCC AGG 675
 A L L K Y P A P V T S L K A R
TCA GAT GGA ACA TGG GAC GTT GAA ACA CCA CAG GGG TCT ATG AGA 720
 S D G T W D V E T P Q G S M R

 AAG TGT TCC ATG GGG TTC CAT GCT GGC TGG GAG CAG CCG CAC TGG 1485
 K C S M G F H A G W E Q P H W
 TTC TAC AAA CCA GGC CAG GAC ACT CAG TAC AGG CCA AGT TTT CGC 1530
 F Y K P G Q D T Q Y R P S F R
 CGC ACA AAC TGG TTT GAG CCT GTG GGC TCG GAG TAT AAA CAG GTT 1575
 R E N W F E L L I T G S E Y K Q V
 ATG CAA AGA GTA GGG GTA ACT GAC CTA TCA CCA TTT GGC AAG TTT 1620
 M Q R V G V T D L S P F G K P
 AAC ATC AAA GGC CAA GAT TCC ATT AGA CTA CTG GAC CAT CTC TTT 1665
 N I K G Q D S I R L D H L F
 GCA AAT GTC ATT CCA AAG GTG GGT TTT ACA AAT ATA AGT CAC ATG 1710
 A N V I P K V G F T N I S H M
 TTA ACA CCC AAG GGT CGA GTG TAT GCT GAG CTG ACT GTT TCT CAC 1755
 L T P K G R V Y A E L T V S H
 CAA TCT CCT GGG GAG TTT CTT TTA ATT ACT GGC TCT GGA TCA GAA 1800
 Q S P G E F L L I T G S G S E
 CTT CAT GAT CTT AGA TGG ATT GAA GAA GCA GTC AAA GGT GGA 1845
 L H D L R W I E E E A V K G G
 TAT GAT GTT GAA ATT AAA AAC ATA ACT GAT GAG CTT GGA GTT CTT 1890
 Y D V E I K N I T D E L G V L
 GGA GTT GCT GGG CCA CAG GCA AGA AAG GTC CTT CAG AAA CTG ACC 1935
 G V A G P Q A R K V L Q K L T
 CCT GAA GAT CTT AGT GAT GAT GTT TTC AAG TTT CTT CAA ACC AAG 1980
 P E D L K S D G V F X F L Q T K
 TCC TTA AAG GTT TCC AAC ATT CCT GTC ACT GCT ATT AGG ATA TCT 2025
 S L K V S N I P V T A I R I S

GCA AAT AGA ATT GTG AAT GCT GCA GGA TTT TGG GCT CGT GAA GTA 765
 A N R I V N A A G F W A R E V
GGT AAA ATG ATT GGA CTA GAA CAT CCT CTC ATT CCG GTT CAA CAT 810
 G K M I G L E H P L I P V Q H
CAA TAT GTT GTT ACA TCG ACT ATA TCT GAA GTG AAA GCT TTG AAA 855
 Q Y V V T S T I S E V K A L K
CGA GAA CTG CCT GTG CTC CGT GAC CTG GAA GGA TCA TAT TAT CTC 900
 R E L P V L R D L E G S Y Y L
CGA CAG GAA AGG GAT GGG CTT TTG TTT GGT CCA TAT GAA AGT CAA 945
 R Q E R D G L L F G P Y E S Q
GAG AAA ATG AAA GTT CAG GAC TCC TGG GTC ACC AAT GGA GTT CCT 990
 E K M K V Q D S W V T N G V P
 CCA GGT TTT GGA AAG GAA CTC TTT GAG TCT GAT CTA GAT CGA ATC 1035
 P G F G K E L F E S D L R I
 ATG GCA CAC ATC AAA GCT GCC ATG GAA ATG GTT CCT GTC TTG AAA 1080
 M E H I K A A M E M V P V L K
 AAG GCT GAC ATC ATC AAT GTT GTC AAT GGT CCT ATC ACG TAT TCT 1125
 K A D I I N V V N G P I T Y S
 CCT GAC ATT CTG CCT ATG GTG GGG CCC CAT CAG GGG GTC AGA AAN 1170
 P D I L P M V G P H Q G V R N
 TAC TGG GTG GCT ATA GGC TTT GGA TAT GGC ATA ATC CAC GCT GGT 1215
 Y W V A I G F G Y G I I H A G
 GGG GTA GGG AAA TAT CTC AGT GAC TGG ATC CTG CAT GGA GAA CCT 1260
 G V G K Y L S D W I L H G E P
 CCT TTT GAT CTG ATA GAA TTG GAT CCT AAT CGC TAT GGC AAA TGG 1305
 P F D L I E L D P N R Y G K W
 ACA ACA ACC CAG TAC ACT GAG GCC AAA GCA AGA GAA TCA TAT GGA 1350
 T T T Q Y T E A K A R E S Y G
 TTC AAC AAT ATT GTT GGT TAT CCT AAA GAA GAA CGG TTT GCT GGG 1395
 F N N I V G Y P K E E R F A G
 AGG CCG ACT CAA CGA GTC AGT GGG CTC TAT CAA AGG CTG GAG TCT 1440
 R P T Q R V S G L Y Q R L E S

 TAT ACT GGT GAG CTG GGT TGG GAG CTG TAT CAC AGA AGA GAA GAT 2070
 Y T G E L G W E L Y H R R E D
 TCT GTG GCG CTG TAT GAC GCT ATC ATG AAT GCA GGC CAG GAG GAG 2115
 S V A L Y D A I M N A G Q E E
 GGA ATC GAC AAT TTT GGA ACC TAT GCC ATG AAT GCC TTA CGC CTL 2160
 G I D N F G T Y A M N A R L G
 GAG AAA GCC TTC AGA GCC TGG GGG TTA GAG ATG AAC TGT GAT ACA 2205
 E K A F R A W G L E M N C D T
 AAT CCT TTG GAA GCT GGA CTG GAA TAT TTT GTG AAG TTA AAT AAG 2250
 N P L E A G L E Y F V K L N K
 CCA GCA GAC TTC ATA GGA AAG CAA GCA CTG AAA CAG ATT AAA GCC 2295
 P A D F I G K Q A L K Q I K A
 AAG GGG CTG AAA CGA AGA CTG GTC TGC CTC ACC TTG GCA ACG GAT 2340
 K G L K R R L V C L T L A T D
 GAT GTT GAT CCA GAG GGA AAT GAA AGC ATC TGG TAC AAT GGC AAG 2385
 D V D P E L N E S I W Y N G K
 GTG GTT GGC AAC ACG ACA TCT GGA AGC TAT AGC TAC AGC ATC CAG 2430
 V V G N T T S G S Y S Y S I Q
 AAG AGT CTG GCT TTC GCA TAT GTC CCT GTA CAA CTA AGT GAA GTG 2475
 K S L A F A Y V P V Q L S E V
 GGA CAG CAA GTG GAA GTT GAA CTA TTA GGC AAA AAT TAC CCA GCA 2520
 G Q Q V E V E L L G K N Y P A
 GTC ATC ATA CAA GAA CCT TTG GTA TTG ACC GAA CCA ACT AGA AAC 2565
 V I I Q E P L V L T E P T R N
 CGG CTT CAG AAA AAA GGT GGA AAG GAC AAA ACT TGA 2601
 R L Q K K G G K D K T *

Figure 3 The *hDMGDH* cDNA sequence. Only the sense strand is shown. Amino acids are represented by their one-letter codes. The italicized amino acid sequence with a single underline is the putative flavin-binding peptide. The Val residue at position 29 (precursor numbering), outlined by the triangle, was taken as the mature 5'-end of the cDNA. The boxed His residue is the putative flavin-binding site (corresponding to His84 in rDMGDH). The circled His residue is mutated in the patient. The underlined nucleotide sequence denotes the probe used to isolate the *hDMGDH* PAC clone.

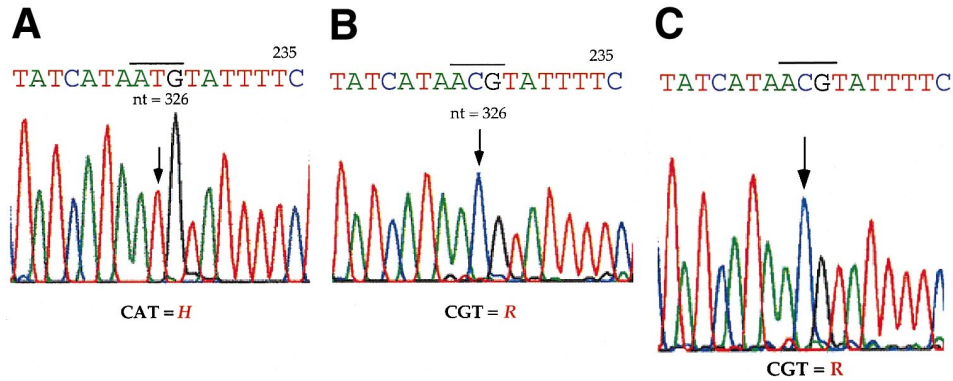


Figure 4 Patient mutation in the *hDMGDH* gene. *A*, Chromatograms of *hDMGDH* cDNA sequence from a control fibroblast sample and, *B*, from a patient fibroblast sample; *C*, genomic DNA sequence from the patient. mRNA, cDNA, and genomic DNA were prepared as described in the Patient and Methods section. Sequence shown is read in the 5′→3′ direction. The sequencing primer reads the sequence on the antisense strand. The arrow indicates the nucleotide that is mutated in the patient: T→C (A→G on sense strand), changing the residue from CAT (His) to CGT (Arg).

identified for *rDMGDH* (fig. 3) (Wittwer and Wagner 1981a; Cook et al. 1984). Southern blot analysis of genomic DNA from patient and control fibroblast DNA gave an identical pattern, suggesting that no genomic DNA deletions were present (data not shown). This region is 100% conserved between rats and humans, and the alteration was not present in 50 *DMGDH* alleles from control human fibroblast samples. The remainder of the cDNA sequence from the patient matched the control sequence. No information could be obtained about nucleotides 1–12, which were contained within the 5′-most PCR primer; however, this is within the mitochondrial targeting peptide and thus is not represented in the mature enzyme. Enzymatic assay and western blot analysis with *DMGDH*-specific antiserum were not sensitive enough to detect *DMGDH*, even in control fibroblasts. This is in keeping with previous observations with this enzyme (Moolenaar et al. 1999). To evaluate the effect of the H109R substitution on *DMGDH* enzyme activity, therefore, the nucleotide 326 A→G mutation was introduced into *hDMGDH* cDNA, and both the normal and mutant sequences were expressed in *Escherichia coli*, *Saccharomyces cerevisiae*, and a variety of mammalian tissue culture samples. No active enzyme could be detected in *E. coli* after expression of the normal *hDMGDH*, although immunoreactive material was present (data not shown). Similarly, expression studies in yeast yielded no *hDMGDH* antigen. After expression of the wild-type cDNA in 293 cells, *DMGDH* antigen could be detected, although the level of expression was not high enough to allow measurement of enzyme activity (fig. 5A). When a cDNA containing the patient mutation was similarly expressed, *hDMGDH* antigen was barely detectable (fig. 5A), although the transfection efficiency was similar for both vectors, as determined by

β -galactosidase staining of colonies on the transfection plates (data not shown). The levels of *DMGDH*-specific mRNA after transfection with the wild-type and mutant plasmids, as determined by a semiquantitative PCR technique (fig. 5B), were much lower in the mock-transfected cells. In contrast, β -ETF-specific primers used as a control showed a similar level of this message in all samples (fig. 5C).

Because the region containing the patient mutation is identical in rat and human genes, the mutation was introduced into *rDMGDH* in a prokaryotic expression vector previously made in our laboratory, and the wild-type and mutant proteins produced in *E. coli* were characterized. After overnight induction of a culture containing the wild-type vector, mean \pm SD *DMGDH* activity in cellular extracts was 0.14 ± 0.03 mU/mg protein ($n = 9$) (as measured with the anaerobic electron transferring flavoprotein assay), although no activity was detectable in extracts from cells containing a vector with the mutant *rDMGDH* cDNA or a vector with no insert (<10% control activity). In contrast, western blot analysis of the cellular extracts shows that levels of wild-type and mutant protein were similar after induction, indicating that the mutant enzyme was stable but inactive (fig. 6).

Discussion

Our findings unequivocally identify a deficiency of *DMGDH* as the cause of accumulation of dimethylglycine in the urine and serum of this patient. The accumulation appears to be the result of a homozygous point mutation in the *DMGDH* gene, although the presence of a large deletion on one allele at this locus, leading to hemizygosity for the point mutation cannot be excluded.

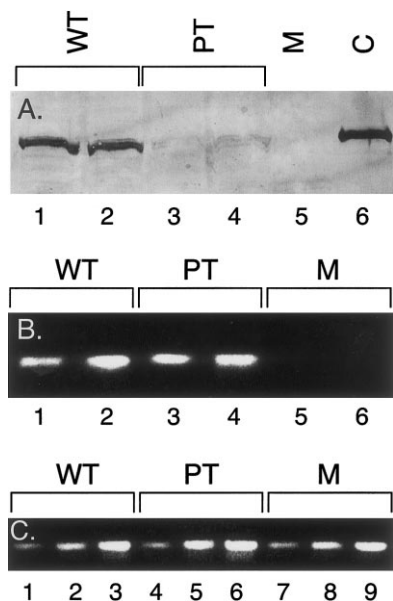


Figure 5 Expression of wild-type and mutant hDMGDH in 293 cells. *A*, Western blot analysis of cellular extracts after SDS-PAGE. Lanes 1 and 2, extracts from cells transfected in separate experiments with wild-type hDMGDH vector; lanes 3 and 4, extracts from cells transfected in separate experiments with an hDMGDH vector containing the patient mutation; lane 5, mock transfection; lane 6, partially purified pig liver DMGDH. *B*, RT-PCR results in transfected cells. Lanes 1 and 2, cells transfected with wild-type DMGDH vector; lanes 3 and 4, cells transfected with an hDMGDH vector containing the patient mutation; lanes 5 and 6, mock transfection. Lanes 1, 3, and 5 are products after 16 cycles of amplification. Lanes 2, 4, and 6 are products after 18 cycles of amplification. *C*, β -ETF message in samples prepared concurrently with those shown in panel B. Lanes 1–3, cells transfected with wild-type DMGDH vector; lanes 4–6, cells transfected with an hDMGDH vector containing the patient mutation; lanes 7–9 mock transfection. Lanes 1, 4, and 7 are products after 20 cycles of amplification. Lanes 2, 5, and 8 are products after 22 cycles of amplification. Lanes 3, 6, and 9 are products after 24 cycles of amplification.

Although the level of expression of the wild-type and mutant cDNAs in a eukaryotic expression system was too low to allow direct measurement of enzyme activity, the level of mutant enzyme antigen was clearly lower than that of the wild-type enzyme antigen, most likely indicating reduced stability of the mutant protein. This is consistent with the *E. coli* expression data for the mutant enzyme, because it was unstable in this system. Additional prokaryotic expression studies confirm that the point mutation inactivates enzymes when introduced into the rat coding sequence, which is 100% conserved in this region of the human enzyme. Thus, even if a mutant human enzyme were stably formed in the cell, it would not be active.

In view of the potential consequence of a disruption of metabolism of methyl groups related to DMGDH action, which are necessary for several other important

cellular reactions, this patient's clinical symptoms are surprisingly mild. Clearly, other sources of methyl groups for these reactions must be available when DMGDH is deficient. Because sarcosine in DMGDH deficiency can still be produced directly from glycine via glycine methyltransferase, which uses S-adenosylmethionine as a methyl donor, the remainder of the downstream reactions of choline catabolism can likely still proceed to some extent; however, chronic elevation of serum creatine kinase levels in this patient are indicative of ongoing muscle breakdown, suggesting that DMGDH deficiency does have physiologic repercussions.

The His \rightarrow Arg mutation demonstrated in the patient's DMGDH enzyme is in close proximity to the predicted FAD attachment site and could lead to a disruption of flavin binding. Alternatively, the mutation may disrupt interaction with folate. It has not been definitively determined where folate associates with the enzyme, but a compelling case has been made for association near the amino terminus of the protein based on structural information available for another folate-dependent enzyme, thymidylate synthase. The region starting 10 amino acids toward the C-terminus from the His91 (precursor numbering) flavin-binding site in DMGDH contains 3 Lys residues conserved in both the human and rat enzyme (residues 106, 107, and 114 in the precursor) and is homologous to a highly conserved motif present in several thymidylate synthase enzymes from various sources (Maley et al. 1982; Hardy et al. 1987; Lang et al. 1991). A thymidylate synthase has been crystallized from *Lactobacillus casei*, and the Lys residues in this region are important in binding its folate coen-

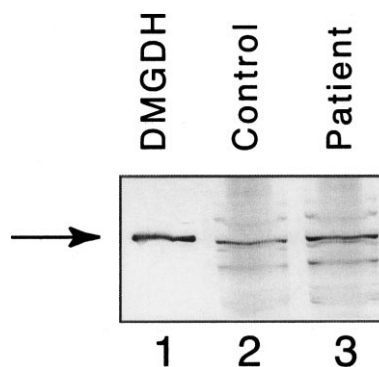


Figure 6 Western blot of wild-type and mutant rDMGDH produced in *E. coli*. DMGDH purified from pig liver for reference (lane 1). Extracts from induced cells containing the wild-type rDMGDH vector (lane 2) and rDMGDH containing the patient mutation (lane 3) were separated by SDS-PAGE, were transferred to a polyvinylidene fluoride membrane, and were examined using anti-DMGDH antibodies. No DMGDH-specific band was present in extracts from cells containing no plasmid or plasmid without insert (data not shown).

zyme, H₄PteGlu₇ (Hardy et al. 1987). It is important to note that, in the patient described here, the H109R mutation lies between these Lys residues and may lead to disruption of folate binding in the mutant DMGDH enzyme. However, these Lys residues are not conserved in the evolutionarily related SDH, suggesting that DMGDH and SDH may have a different mechanism of association with their folate coenzyme (Bergeron et al. 1998). Alternatively, some other binding motif may be responsible for folate association. The presence of a Lys/Arg-rich region between Lys740 and Arg764 in rDMGDH has also been implicated in folate binding (Otto et al. 1996). This region is similarly Lys/Arg rich in hSDH and hDMGDH (Bergeron et al. 1998).

Here we present the cloning of the cDNA for hDMGDH and the identification of a homozygous H109R mutation in a patient presenting with a fish odor and having high dimethylglycine levels in his serum and urine. In conjunction with our previous report (Moolenaar et al. 1999), this confirms that this patient represents the first identified case of DMGDH deficiency, a new inborn error of metabolism.

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