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Mutation in the 4a-Carbinolamine Dehydratase Gene Leads to Mild Hyperphenylalaninemia with Defective Cofactor Metabolism

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Summary

Hyperphenylalaninemias represent a major class of inherited metabolic disorders. They are most often caused by mutations in the phenylalanine hydroxylase gene and, less frequently but with usually more serious consequences, in genes necessary for the synthesis and regeneration of the cofactor, tetrahydrobiopterin. This cofactor is absolutely required for all aromatic amino acid hydroxylations, and, recently, nitric oxide production from L-arginine has also been found to be dependent on tetrahydrobiopterin. Phenylalanine hydroxylase catalyzes a coupled reaction in which phenylalanine is converted to tyrosine and in which tetrahydrobiopterin is converted to the unstable carbinolamine, 4a-hydroxytetrahydrobiopterin. The enzyme, carbinolamine dehydratase, catalyzes the dehydration of the carbinolamine to quinonoid dihydropterin. A decreased rate of dehydration of this compound has been hypothesized to be responsible for the production of 7-biopterin found in certain mildly hyperphenylalaninemic individuals. We have now identified nonsense and missense mutations in the 4a-carbinolamine dehydratase gene in a hyperphenylalaninemic child who excretes large amounts of 7-biopterin. This finding is consistent with the role of the carbinolamine dehydratase in the phenylalanine hydroxylation reaction. Together with previously identified inherited disorders in phenylalanine hydroxylase and dihydropteridine reductase, there are now identified mutations in the three enzymes involved in the phenylalanine hydroxylation system. In addition, the genetics of this system may have broader implications, since the product of the dehydratase gene has previously been shown to play an additional role (as dimerization cofactor for hepatocyte nuclear factor-1α) in the regulation of transcription, through interaction with hepatocyte nuclear factor-1α.

Introduction

The hydroxylation of phenylalanine to tyrosine is the first and rate-limiting step in phenylalanine catabolism in mammals (Kaufman and Fisher 1974; Milstien and Kaufman 1975). The reaction is catalyzed by phenylalanine hydroxylase, an enzyme with an absolute requirement for an unconjugated tetrahydropterin cofactor, tetrahydrobiopterin (Kaufman 1958*a*, 1963). During the hydroxylation reaction, tetrahydrobiopterin is converted to 4a-hydroxytetrahydrobiopterin, which is formed in equal amounts with the other product, tyrosine. Two enzymes are involved in the conversion of 4a-hydroxytetrahydrobiopterin back to tetrahydrobiopterin: 4a-carbinolamine dehydratase and the NADH-dependent dihydropteridine reductase (fig. 1) (Kaufman 1970, 1976*a*; Huang and Kaufman 1973; Huang et al. 1973; Lazarus et al. 1983). Thus, a three-enzyme system has evolved in mammals, for the efficient hydroxylation of phenylalanine. The first enzyme in conjunction with a reduced pterin cofactor performs

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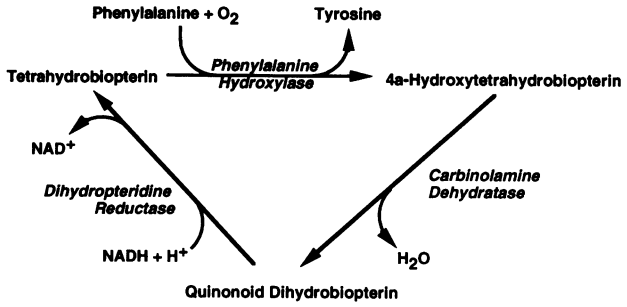

Figure 1 Phenylalanine-hydroxylating system. Tetrahydrobiopterin is converted to 4a-carbinolamine (4a-hydroxytetrahydrobiopterin) during the hydroxylation reaction. This compound is converted to quinonoid dihydrobiopterin by the dehydratase or, more slowly, by a nonenzymatic dehydration reaction. The quinonoid dihydropterin is reduced to tetrahydrobiopterin by dihydropteridine reductase (Kaufman 1958*a*, 1964). As demonstrated in vitro by Davis et al. (1991), the carbinolamine can rearrange to the 7-isomer in the absence of carbinolamine dehydratase.

the actual hydroxylation, whereas the other two enzymes catalyze the regeneration of the cofactor.

The hyperphenylalaninemias are inherited diseases that either directly or indirectly lead to defective hydroxylation of phenylalanine (Jervis 1947; Kaufman 1958*b*; Scriver et al. 1988). The frequency is approximately 1/10,000 live births. The most common cause of hyperphenylalaninemia, also called "phenylketonuria," is an inborn error in the gene coding for phenylalanine hydroxylase. When untreated, the afflicted individual becomes severely mentally retarded. Far less commonly, defects arise either in the biosynthetic pathway for tetrahydrobiopterin or in its regeneration system. In both cases, the resulting loss of the cofactor activity may cause not only severe mental retardation, because of the absence of phenylalanine hydroxylation, but also serious neurological disorders, because of the additional requirement of tetrahydrobiopterin in the biosynthesis of the catecholamines and serotonin (Kaufman 1976*b*).

To date, although mutations have been identified in the genes coding for phenylalanine hydroxylase (DiLella et al. 1986, 1987; Avigad et al. 1987) and dihydropteridine reductase (Howells et al. 1990), no patients have been diagnosed as having defects in 4a-carbinolamine dehydratase. Patients with GTP cyclohydrolase deficiencies would display lower overall levels of pterins; 6-pyruvoyltetrahydropterin synthase mutations would be characterized by high neopterin: biopterin ratios; and individuals with dihydropteridine reductase deficiency would have markedly decreased levels of tetrahydrobiopterin. Recently, we and others have predicted that children who have a form of mild hyperphenylalaninemia characterized by the excretion of 7-biopterin have a defective dehydratase (Curtius et al. 1988, 1990; Davis et al. 1991; Adler et al. 1992). This prediction was based on in vitro studies that demonstrated that the dehydratase could prevent the nonenzymatic conversion of 4a-hydroxytetrahydrobiopterin to 7-biopterin (Davis et al. 1991).

Previously, we cloned the cDNA for carbinolamine dehydratase (Citron et al. 1992). With this sequence information we examined the two alleles of the dehydratase from a mildly hyperphenylalaninemic patient who excreted 7-biopterin. The approach involved the amplification and sequence analysis of all of the coding regions of the carbinolamine dehydratase gene. We report here that both alleles in the patient are defective; one is a premature termination mutation, and the other is a substitution. These results are consistent with our in vitro studies and demonstrate that mildly hyperphenylalaninemic patients who excrete 7-biopterin have a defect in the gene coding for the dehydratase.

Material and Methods

Genomic DNA Isolation

Erythrocytes were removed from 0.1–1.0-ml samples of whole blood (stored with heparin) by centrifugation, at 14,000 *g* for 2 min, through a silica-based anion exchange column (Qiagen, Chatsworth, CA), followed by three washes with PBS, pH 7.4. The leukocytes were lysed on the column with detergent according to Qiagen's recommendation, and the released DNA was allowed to adsorb for 30 min at 23°C, was washed twice, was eluted with two 350-μl applications of 50 mM 3-[N-morpholino]propanesulfonic acid buffer, pH 8.3, 1.2 M KCl, and 15% ethanol (each time, centrifugation was at 7,000 *g* for 30 s), and was precipitated with 0.8 volume of 2-propanol. Yields were 10–30 μg of DNA/ml whole blood. This procedure has been reported to provide slightly sheared nucleic acids, which denature more readily during the PCR, and the template obtained in this manner did produce higher amplification yields than did the same DNAs that we have isolated by conventional lysis, proteolysis, and organic extraction techniques (Kawasaki 1990).

DNA Amplification

The PCR was performed in 100-μl volumes under paraffin oil or wax in an MJ Research (Watertown) thermal cycler using reagents supplied by Perkin-Elmer

(Norwalk). Reactions contained 200–800 pg of genomic template DNA, 0.5 μM each primer, 2.25 mM $MgCl_2$ (optimal value after testing was 1.5–5 mM), 200 μM each deoxynucleotide triphosphate, and 2.5 units of *Taq* polymerase (Amplitaq). The profile was as follows: 10 min at 98°C for initial denaturation without polymerase, cooling to 90°C for the addition of the enzyme, and then generally 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 0.25–2.5 min. Finally, the reaction was extended for 5 additional min and cooled to 4°C for 1–12 h. Subsequent amplification was performed similarly with 0.25%–1.5% of the previous amplification product and a total of 20 additional cycles. Products were evaluated by agarose gel electrophoresis of 10%–30% of the reaction.

Subcloning DNA Amplification Products

DNA fragments generated by the PCR, which commonly harbor single A overhangs, were ligated to the linearized and modified plasmid pCRII (Invitrogen, San Diego), which contains single T overhangs in 10 μl containing 50 ng of vector DNA and 0.2–2 μl of amplified DNA, at 12°C for 16–20 h, and then were used to transform *Escherichia coli* strain DH5α. The transformants were plated on LB media containing ampicillin, isopropyl-β-D-thiogalactoside, and X-gal, to identify cells harboring clones with inserts interrupting a β-galactosidase α-peptide, and the cells and plasmids were handled by standard procedures (Miller 1972; Ausubel et al. 1987; Sambrook et al. 1989). Of the transformants with putative recombinant clones (white colonies), 25%–100% in various experiments contained carbinolamine dehydratase gene inserts. The C-terminal region primers seemed less efficient in the production of useful inserts; however, nested amplifications always yielded desired carbinolamine dehydratase sequence in 100% of the plasmids examined.

DNA Sequence Analysis

The clones containing inserts were grown in small cultures, and the plasmids were purified. DNA sequences for both strands were determined (Sanger et al. 1977) from the double-stranded plasmids by modified T7 DNA polymerase (Sequenase, United States Biochemicals, Cleveland), according to the protocol recommended by EG&G Berthold (Natick, MA) for application to an EG&G Acugen automated DNA sequencer. The DNA sequences obtained were examined with the aid of software developed by the University of Wisconsin Genetics Computing Group (Devereux et al. 1984).

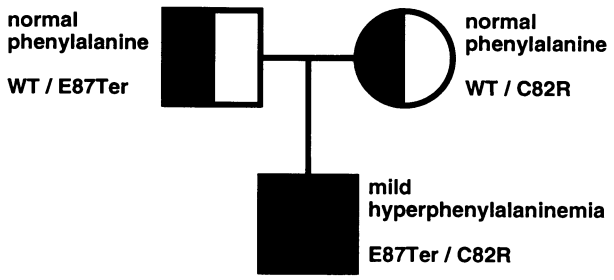

Figure 2 Pedigree of family with mild hyperphenylalaninemic child. Carbinolamine dehydratase alleles are indicated.

At least six clones were sequenced from at least two independent amplification experiments for each individual.

Results

A male 1-year-old Caucasian patient was identified as mildly hyperphenylalaninemic at newborn screening. He had a positive Guthrie test, and his blood phenylalanine level was 700 μM, compared with a normal range of 50–60 μM. Classical phenylketonuric patients would have phenylalanine levels in excess of 1 mM. The levels of a variety of pterin intermediates and metabolites, such as neopterin:biopterin ratios, and overall pterin and reduced pterin levels indicated that this patient harbored an unusual cofactor disorder. This patient had normal urinary neopterin and biopterin levels, with the exception of the presence of an abnormally high abundance of 7-biopterin in his urine. Therefore, the biochemical profile of this patient was consistent with loss of carbinolamine dehydratase activity. The patient exhibited no other clinical abnormalities. Sera from both parents were examined and found to have normal phenylalanine levels as well as normal pterin levels, and the parents displayed no other clinical signs (see fig. 2).

DNA was isolated from blood samples from the patient, his parents, and an unrelated normal individual, for use as templates in multiple DNA amplification experiments with a variety of primers (see fig. 3). The amplified fragments were subcloned into plasmid vectors and were sequenced independently. Examination of the coding sequence by this procedure confirmed the wild-type gene in a normal subject. The patient was found to carry two mutant alleles of the carbinolamine dehydratase gene: one, a G→T transversion at base 259, which replaced a glutamic acid codon with a premature termination codon at position 87 in the 104-

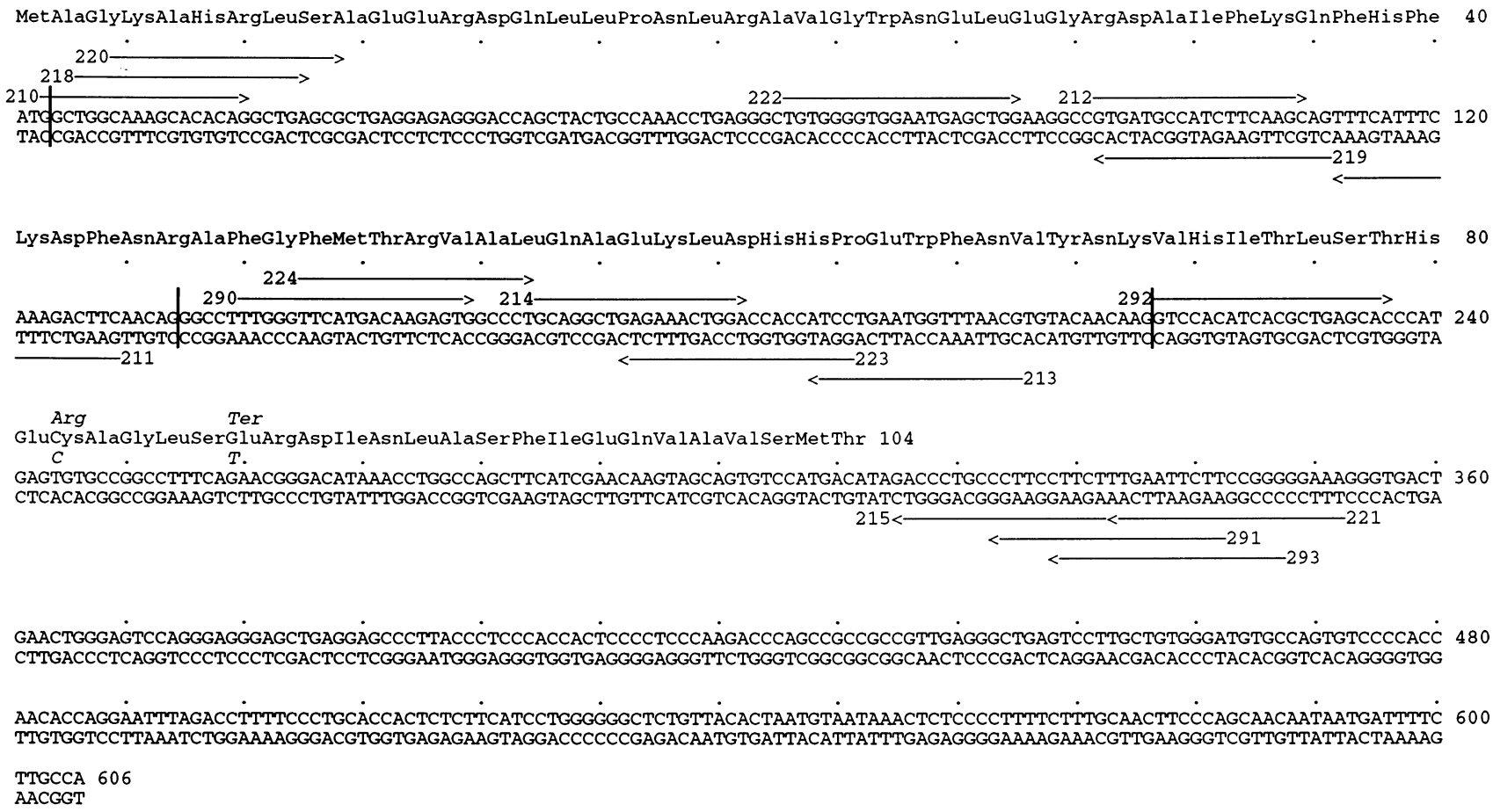

Figure 3 Coding sequence of the human carbinolamine dehydratase gene. Oligonucleotide primers used to generate genomic clones are indicated, by arrows, in a 5′→3′ orientation. Exon boundaries are indicated by vertical bars. Partial intron sequences have been included in the GenBank submission (accession numbers L15410, L15427, and L15428). The mutant alleles, denoted by italics, are at positions 244 (cysteine 82) and 259 (glutamic acid 87).

amino-acid coding sequence (fig. 3), and a T→C transition at position 244, resulting in a substitution of an arginine for a cysteine residue. The father harbored the wild-type and termination alleles, and the mother carried the wild-type and the substitution mutation. Figure 2 depicts the hyperphenylalaninemia and allele pattern in this family.

Discussion

The electron donor, tetrahydrobiopterin, is necessary for both the metabolism of phenylalanine and the synthesis of dopamine, norepinephrine, epinephrine, and serotonin, since it serves as a cofactor for the aromatic amino acid hydroxylases (Kaufman and Fisher 1974). Tetrahydrobiopterin is present in virtually every tissue, whereas the hydroxylases are present mainly in the liver, adrenal and pineal glands, and certain regions of the brain, including the striatum and brain stem. Recently, nitric oxide synthase has also been shown to have an absolute requirement for tetrahydrobiopterin, for the oxidation of arginine to nitric oxide. Nuclear functions for tetrahydrobiopterin, such as control of cell proliferation or transcription regulation, have also been implicated (Tanaka et al. 1989) or suggested (Mendel et al. 1991; Citron et al. 1992; Hansen and Crabtree, in press).

In the absence of carbinolamine dehydratase in the liver, the rate of phenylalanine hydroxylation may be depressed because of decreased availability of the active tetrahydro form of the natural cofactor, combined with inhibition (Davis et al. 1992*b*) and partial uncoupling of the hydroxylation reaction in the presence of the 7-substituted pterin (Davis and Kaufman 1991). In contrast, dihydropteridine reductase mutations (which severely depress the rate of quinonoid dihydrobiopterin conversion to tetrahydrobiopterin) lead to greater elevations in phenylalanine levels and also to central nervous system disorders associated with significant decreases in dopamine and serotonin levels.

Carbinolamine dehydratase deficiencies seem to be rare, although such cases may often remain hidden because of the mild nature of the hyperphenylalaninemia. Clones of this gene initially studied as the homeodomain transregulator, DCoH (dimerization cofactor for hepatocyte nuclear factor-1α [HNF-1α]), have been characterized from several mammalian species (Mendel et al. 1991). The protein is also very small—104 residues—and is very highly conserved. The identification of these alleles will facilitate the analysis of at least one subset of cofactor disorders.

The allele analysis indicates that the carbinolamine dehydratase mutations exhibit autosomal recessive inheritance. The nonsense mutation would, at best, yield a protein with a 17% deletion at the C-terminal end. The arginine substitution introduces a basic side chain adjacent to a carboxyl group and removes the only cysteine residue. Either the loss of this sulfhydryl group or the altered electrostatic charge could have an adverse affect on the catalytic domain or on possible dimerization that might be necessary for catalysis of the dehydratase reaction.

Neutral variation in this gene may be minimal, since the enzyme is so highly conserved through evolution. For example, the rat and human proteins have identical amino acid sequences (Mendel et al. 1991).

The characterization of the mutant alleles in this family supports the postulate that carbinolamine dehydratase (Curtius et al. 1990; Davis et al. 1991; Adler et al. 1992; Blau et al. 1992), by accelerating the conversion of the 4a-carbinolamine to quinonoid dihydrobiopterin, minimizes its conversion to 7-biopterin during phenylalanine hydroxylation. The mild nature of this hyperphenylalaninemia is consistent with the ability of this step to occur nonenzymatically (Kaufman 1976*a*) at a slower rate that may be sufficient to maintain acceptable phenylalanine levels. Newborn screening should identify these patients as mildly hyperphenylalaninemic, and pterin analysis should help identify the basis as carbinolamine dehydratase deficiency.

Questions remain concerning additional biological roles for tetrahydrobiopterin. There are no phenotypic manifestations visible in this family, other than the presence of 7-biopterin and elevated blood levels of phenylalanine in the patient. Mammalian tissue distribution of carbinolamine dehydratase seems broad, with the highest levels present in the liver and kidney (Davis et al. 1992*a*). Standard clinical liver-function tests of our patient were all well within the normal range. It is possible that the carbinolamine dehydratase enzyme with the amino acid substitution is sufficiently active in its HNF-1α binding role yet inactive with respect to its dehydratase activity. Whether subtle effects could be detected or whether affected individuals might present any abnormalities either during development or other stages such as pregnancy or in combination with other medical disorders remains to be examined. We have also begun construction of carbinolamine dehydratase mutations in a prokaryotic expression system to include these as well as substitutions at other positions, with the goal of elucidating the structural features that

are important for the dual biological activities of this protein.

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