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Molecular basis of ornithine aminotransferase deficiency in B-6-responsive and -nonresponsive forms of gyrate atrophy

(pyridoxal phosphate/retinal degeneration/hyperornithinemia/missense mutation/transient expression)

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ABSTRACT **Gyrate atrophy (GA), a recessive eye disease involving progressive loss of vision due to chorioretinal degeneration, is associated with a deficiency of the mitochondrial enzyme ornithine aminotransferase (OATase; ornithine–oxo-acid aminotransferase; L-ornithine:2-oxo-acid aminotransferase, EC 2.6.1.13) with consequent hyperornithinemia. Genetic heterogeneity of GA has been suggested by the demonstration that administration of pyridoxine to increase the level of pyridoxal phosphate, a cofactor of OATase, reduces hyperornithinemia in a subset of patients. We have cloned and sequenced cDNAs for OATase from two GA patients, one responsive and one nonresponsive to pyridoxine treatment. The respective cDNAs contained different single missense mutations, which were sufficient to eliminate OATase activity when each cDNA was tested in a eukaryotic expression system. However, like the enzyme in fibroblasts from the pyridoxine-responsive patient, OATase encoded by the corresponding cDNA from this individual showed a significant increase in activity when assayed in the presence of an increased pyridoxal phosphate concentration. These data firmly establish that both pyridoxine responsive and nonresponsive forms of GA result from mutations in the OATase structural gene. Moreover, they provide a molecular characterization of the primary lesion in a pyridoxine-responsive genetic disorder.**

Gyrate atrophy of the choroid and retina (GA) is a severe inherited eye disease involving progressive loss of vision due to chorioretinal degeneration. The disorder is associated with a deficiency of the mitochondrial matrix enzyme ornithine aminotransferase (OATase; ornithine–oxo-acid aminotransferase; L-ornithine:2-oxo-acid aminotransferase, EC 2.6.1.13). The enzyme defect is expressed in many tissues, including skin fibroblasts (1, 2), lymphocytes (3), and liver (4), but the deleterious consequences are largely confined to the visual system. Pyridoxal phosphate, a cofactor of OATase, administered as pharmacological doses of pyridoxine or vitamin B-6, alleviates hyperornithinemia in some GA patients (2, 5, 6). Consequently, GA has been subdivided into two distinct clinical types: B-6 responsive and B-6 nonresponsive. Several other inherited metabolic disorders, including homocystinuria, also show B-6-responsive and B-6-nonresponsive variants (7, 8), although in no case has the defect been elucidated at the gene level.

We and others have reported (9, 10) the cloning and characterization of OATase cDNA from human liver and retinoblastoma, respectively. We have also shown that the structural gene for OATase maps to chromosome 10 and OATase-related sequences map to the X chromosome (11). Preliminary molecular analysis of GA with a human OATase probe (HOAT1) suggested that if the defect occurs in the OATase gene, it must frequently involve a subtle sequence alteration in the mRNA that does not affect its apparent size (9). To prove that GA is caused by mutation at the OATase locus, we have cloned and sequenced cDNAs for OATase from one B-6-responsive and one nonresponsive GA patient. In each case, the cDNA contained a different point mutation predicting a single amino acid change in the mature protein. Transient expression studies with normal and mutant OATase clones have confirmed that these mutations are associated with the loss of OATase activity. In the presence of 10-fold excess vitamin B-6, a partial restoration of OATase activity was observed only in the clone derived from the B-6-responsive patient. This report reveals the nature of the molecular defect at the DNA level in a B-6-responsive metabolic disorder.

MATERIALS AND METHODS

Isolation and Sequencing of cDNAs from GA Patients. Poly(A)$^+$ RNA was prepared from the fibroblast cell lines of a B-6-responsive GA patient (MGF1152) and a B-6-nonresponsive GA patient (MGF1148). The clinical phenotype of each patient has been described (as patients 5 and 1, respectively) (5). The synthesis of cDNA was carried out according to the method of Gubler and Hoffman (12). The double-stranded cDNA was cloned into λgt10 by the method of Huynh *et al.* (13). The libraries were screened by the procedure of Benton and Davis (14) and the inserts from the isolated clones were subcloned into the *Eco*RI site of pUC19. DNA sequencing was carried out directly on supercoiled plasmid DNA as described by Chen and Seeburg (15) and compared with the published sequence of HOAT1 (9).

Construction of Plasmids for Transient Expression of OATase Clones. To study the expression of normal and mutant OATase clones, the inserts were cloned into the eukaryotic expression vector CLH3A, which was derived from CL28X (16). CLH3A has a unique *Bgl* II site for inserting sequences to be expressed. The insert is flanked on the 5′ side by ≈1950 base pairs (bp) of the mouse metallothionein (MT-1) gene, including the promotor and glucocorticoid and heavy metal response elements, and on the 3′ side by the simian virus 40 early poly(A) signal. The remainder of the plasmid has MT-1 3′ sequences, along with a ColE1 origin and *amp* gene from pML. CL3HA was kindly provided by Nancy Hsiung (Integrated Genetics, Framingham, MA).

HOAT1 and OAT1148 were full-length clones containing the entire protein coding region and were inserted into

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Abbreviations: GA, gyrate atrophy; OATase, ornithine aminotransferase.

CL3HA as single *Bgl* II/*Sal* I fragments extending from 14 bp 5′ of the ATG initiation codon to the *Sal* I site within the pUC19 polylinker. The simian virus 40 early poly(A) addition signal was therefore deleted from the final constructs, which contained the endogenous poly(A) addition site from the OATase cDNAs. OAT1152 was 1821 bp long and lacked 150 bp at the 5′ end of the coding region and therefore required a more complicated construct. An internal *Pst* I site at 905 bp of the coding sequence was used in combination with a 3′ flanking site in the pUC19 polylinker to attach the 3′ portion of OAT1152, containing the putative mutation at 994 bp, to the 5′ end of the wild-type OATase cDNA. This chimeric cDNA was then inserted into the expression vector as described above. The construct was verified by sequencing across the *Pst* I site as well as the region containing the substituted base. The expression vector constructs were designated as CLHOAT1, CLHOAT1148, and CLHOAT-1152.

Like the CHOK1 cell line from which it was derived, the hamster cell line tsH1 is a proline auxotroph due to the failure to express OATase activity and pyrroline-5-carboxylate synthetase activity (which can provide proline via P5C from glutamate) (17). The absence of OATase mRNA in this cell line was confirmed by RNA blot analysis. tsH1 cells were transfected by the calcium phosphate precipitation method (18) with each recombinant clone (18 μg per 10-cm^2 plate) along with pRSVCAT (2 μg per 10-cm^2 plate), included as an internal control. Cells were harvested 48 hr after transfection and OATase activity was measured in the cell extracts by the sensitive radiometric method of Ohura *et al.* (19). Chloramphenicol acetyltransferase assays were performed on the same extracts by the method of Grove *et al.* (20). Total protein was monitored with a dye reagent (Bio-Rad) based on the method of Bradford (21).

RESULTS

Identification of Mutations in B-6-Responsive and -Nonresponsive Forms of GA. cDNA libraries were constructed in λgt10 with poly(A)$^+$ RNA from fibroblast cell lines of two independent GA patients, one B-6 responsive (MGF1152) and one nonresponsive (MGF1148). Each cell line displays <4% of normal OATase activity, although the size and level of OATase mRNA in both are comparable to normal controls. Approximately 100,000 plaques were screened from each library, with HOAT1 used as probe (9). Each library yielded three positive clones, and those with the largest inserts were chosen for sequence analysis. On complete sequencing, the clone derived from cell line 1152, designated OAT1152, was 1821 bp long and lacked 150 bp at the 5′ end of the coding region of 439 amino acids. The clone derived from the cell line 1148, designated OAT1148, was 2044 bp long and contained the entire coding region for OATase, including the mitochondrial leader peptide.

The nucleotide sequence in the coding region of OAT1152 was identical to the normal OATase (HOAT) cDNA sequence (9, 10) except for a transition mutation (guanine to adenine) at 994 bp that changed valine-332 to a methionine residue (Fig. 1). On the other hand, the nucleotide sequence of OAT1148 revealed a single transversion mutation (cytosine to adenine) at 162 bp, changing asparagine-54 to lysine (Fig. 2). Neither mutation alters a restriction endonuclease recognition site. It should be noted that both OAT1152 and OAT1148 also differ from the published sequence of HOAT1 at two positions in the 3′ untranslated region. Base 1855 in each of the new clones is a thymine, whereas HOAT1 has a cytosine. This is apparently a polymorphism without functional significance, since the normal OATase sequence published by Inana *et al.* (10) also has a thymine at this position. Resequencing of the HOAT1 clone revealed that the second

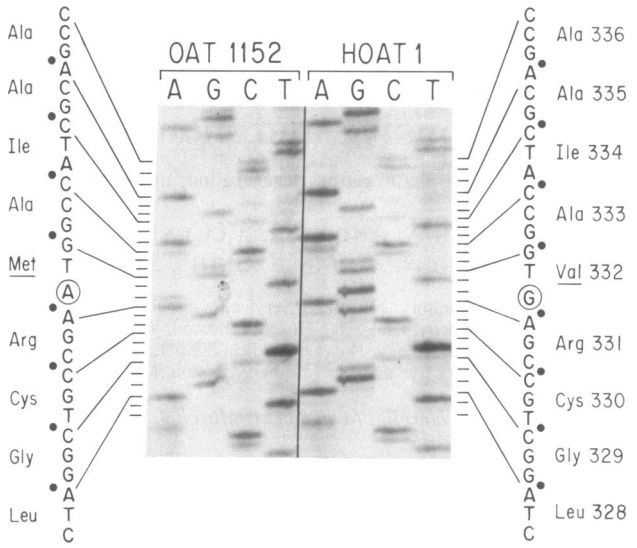

FIG. 1. Comparison of the nucleotide sequence of OATase cDNA from a pyridoxine-responsive GA patient to the normal OATase cDNA. HOAT1, normal OATase sequence; OAT1152, pyridoxine-responsive patient. Deduced amino acid sequence is shown next to the nucleotide sequence. Substituted bases are circled and altered amino acids are underlined.

difference, the presence of an extra guanine at position 1949 of the normal clone, represents a misreading in the previously published HOAT1 sequence (9).

Transient Expression of Normal and Mutant OATase Clones. To ensure that the sequence differences detected in OAT1152 and OAT1148 are indeed responsible for the loss of OATase activity, we examined transient expression of wild-type OATase cDNA (HOAT1) and the mutant OATase cDNAs by using the eukaryotic expression vector CLH3A for transfection into the hamster cell line tsH1, which is a proline auxotroph. Constructs for the wild-type and mutant OATase cDNAs are described in *Materials and Methods* and shown schematically in Fig. 3. OATase activity was readily

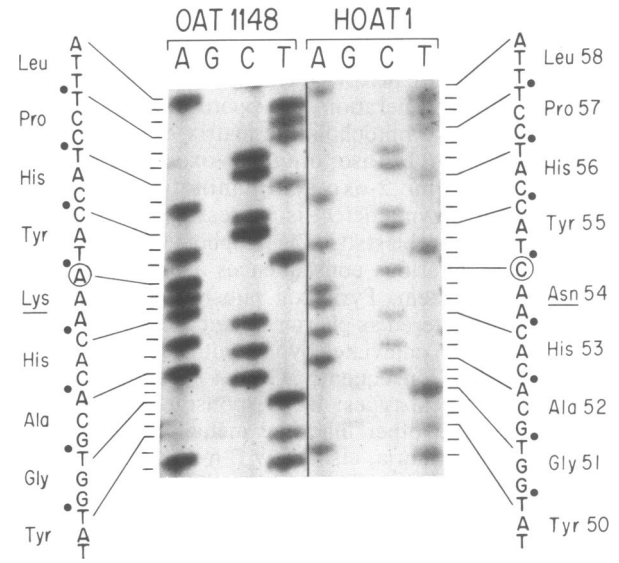

FIG. 2. Comparison of the nucleotide sequence of OATase cDNA from a pyridoxine-nonresponsive GA patient to the normal OATase cDNA. HOAT1, normal OATase sequence; OAT1148, from the pyridoxine-nonresponsive patient. Deduced amino acid sequence is shown next to the nucleotide sequence. Substituted bases are circled and altered amino acids are underlined. HOAT1, normal clone; OAT1148, pyridoxine-nonresponsive mutant clone. Substituted bases are circled and altered amino acids are underlined.

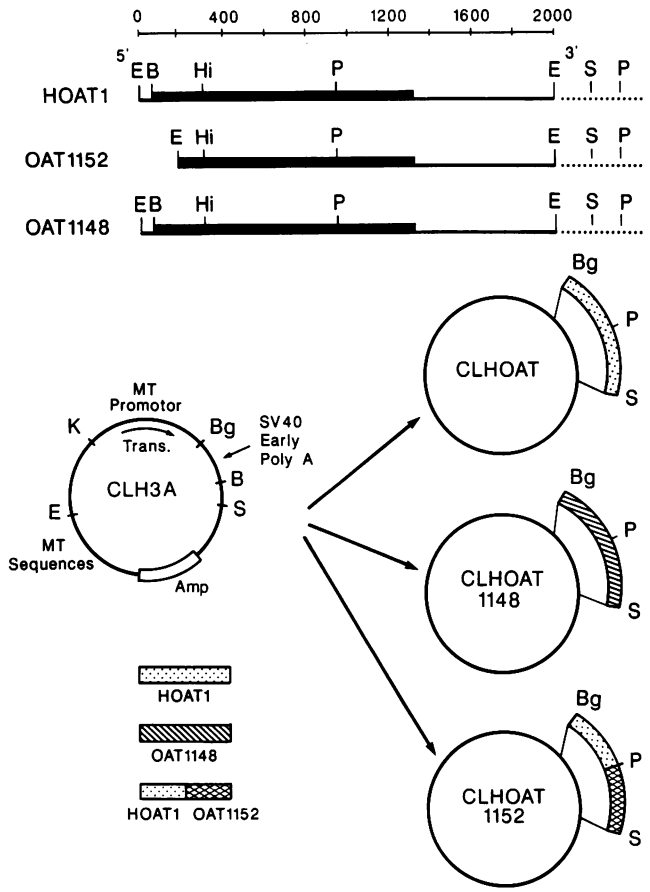

FIG. 3. Construction of plasmids for the expression of OATase clones. Inserts from wild-type (HOAT1) and mutant (OAT1148, OAT1152) OATase clones are shown at the top as linear fragments. Solid bar, coding region; dotted line, polylinker sequence of pUC19. A map of the expression vector CLH3A, which has a mouse metallothionein (MT-1) promoter, is shown on the left. SV40, simian virus 40; Amp, ampicillin. The HOAT1 and OAT1148 clones are inserted into the expression vector as single *Bgl* II/*Sal* I fragments extending from 14 bp 5′ of the ATG initiation codon to the *Sal* I site within the pUC19 polylinker sequence (CLHOAT and CLHOAT-1148, respectively). CLHOAT1152 was constructed by replacing an internal *Pst* I fragment of the HOAT1 cDNA with the same fragment from OAT1152 clone containing the putative mutation at base-pair 994. K, *Kpn* I; E, *Eco*RI; Bg, *Bgl* II; B, *Bam*HI; S, *Sal* I; P, *Pst* I; Hi, *Hin*cII.

observed in tsH1 cells transfected with CLHOAT, containing the wild-type cDNA, but was negligible when either CLHOAT1148 or CLHOAT1152 was used (Table 1). Similarly, extracts from untransfected cells or from cells transfected with either the CLH3A or pRSVCAT vector showed no measurable OATase activity. However, when the assay was performed in the presence of a 10-fold excess of the cofactor pyridoxal phosphate, a 20-fold increase in OATase activity was observed in cells transfected with CLHOAT1152 but not in cells transfected with CLHOAT1148. With CLHOAT, a decline in OATase activity was obtained (Table 1), in agreement with the previously discovered inhibitory effect of high pyridoxal phosphate concentrations on the catalytic activity (22). Thus, the relative level of OATase activity in the transient expression assay parallels that expected based on the clinical categorization of patients 1148 and 1152 and indicates that the sequence alterations observed are not neutral polymorphisms but missense mutations associated with loss of OATase activity in GA.

Table 1. OATase activity in extracts of tsH1 cells transfected with wild-type or mutant OATase cDNA clones cloned into expression vector CLH3A

DNA construct	OATase activity ± SD, nmol of product·mg^{-1}·hr^{-1}	
	Pyridoxal phosphate (20 μM)	Pyridoxal phosphate (200 μM)
Wild type (CLHOAT)	41.17 ± 1.73	32.51 ± 2.64
B-6-nonresponsive mutant (CLHOAT1148)	0.62 ± 0.09	0.25 ± 0.04
B-6-responsive mutant (CLHOAT1152)	0.11 ± 0.09	2.65 ± 0.82

OATase activities from triplicate experiments are shown. The tsH1 cell extracts or the cells transfected with the expression vector alone showed no detectable OATase activity. Chloramphenicol acetyltransferase activity was comparable in all of the transfections.

DISCUSSION

We have reported elsewhere the cloning and characterization of human OATase cDNA and shown that the majority of the GA patients do not show any gross abnormality in gene structure or expression of OATase mRNA (9). However, suggestive evidence that the primary defect in GA does indeed reside in the OATase structural gene has been provided by the analysis of restriction fragment length polymorphisms at the OATase locus (23). In this report, we provide direct proof that molecular defects in the OATase gene underlie both the B-6-responsive and B-6-nonresponsive forms of GA. Both cases examined represent missense mutations in the OATase gene that potentially alter the conformation of the mature protein.

The phenomenon of pyridoxine responsiveness is not confined to GA but is common to several inherited metabolic disorders. Pyridoxine-responsive and -nonresponsive variants have been described in other metabolic disorders (24–26). However, in each of these disorders, the mechanism of pyridoxine responsiveness has been addressed only at the level of enzyme activity and the exact nature of the underlying defect is not known. Our data provide the first characterization of a B-6-responsive mutation at the DNA level.

The B-6-responsive phenotype in patient 1152 is associated with a transition mutation in the OATase gene, replacing valine-332 with a methionine. When the OAT1152 cDNA was expressed in Chinese hamster ovary cells, it yielded negligible OATase activity assayed under standard conditions, whereas in the presence of a 10-fold higher concentration of pyridoxal phosphate, the significant OATase activity was readily detectable. This phenomenon is also observed when extracts from the MGF1152 fibroblast line are assayed under similar conditions (2).

Simmaco *et al.* (27) have provided protein sequence data from rat liver OATase indicating that lysine-292 is the critical residue that binds to pyridoxal phosphate. The coding region of rat and human OATase cDNAs share 90% similarity at the amino acid level with lysine-292 conserved in the human sequence, suggesting that it also represents the B-6-binding site in human OATase. The OAT1152 mutation at residue 332 is in the approximate vicinity of lysine-292, rather than immediately adjacent to the B-6-binding site. The replacement of a branched chain amino acid (valine) by a larger sulfur-containing amino acid (methionine) might interfere with the binding of pyridoxal phosphate to lysine-292, either directly or by altering the folding of the protein. The ability of the enzyme to bind its cofactor is not completely eliminated, however, since high levels of pyridoxal phosphate lead to partial restoration of activity. The degree of B-6 responsiveness varies both within and among different disorders, probably reflecting differences in the precise nature and position of individual mutations with respect to the lysine

residue that binds pyridoxal phosphate. Consequently, among cases of pyridoxine-responsive GA there may be more than one mutation that can affect the binding of pyridoxal phosphate. Comparison of several such mutants could provide a better understanding of the structural requirements for B-6 binding and enzyme function.

By contrast with patient 1152, total loss of OATase activity in patient 1148 was due to a transversion (cytosine to adenine) replacing the neutral amino acid asparagine-54 with a positively charged lysine. Expression of the OAT1148 cDNA yielded negligible OATase activity assayed in either high or low pyridoxal phosphate, reflecting the B-6-nonresponsive classification of the patient. This missense mutation could potentially alter mitochondrial processing and transport, enzyme activity, or enzyme stability, possibilities that must be distinguished by protein studies.

GA is rare, but patients are not confined to a single ethnic group. The two mutations reported here, combined with the recent suggestion of an initiation codon mutation in Lebanese GA patients (28), imply that the clinical and biochemical heterogeneity observed in GA will be reflected in diverse mutations at the OATase locus. Haplotyping of 19 independent GA patients with six OATase restriction fragment length polymorphisms has revealed that a single haplotype is present on the majority of GA chromosomes (including both chromosomes from patient 1152), but that at least six other haplotypes are also associated with OATase deficiency (23). Patient 1148 is heterozygous at the OATase locus, carrying both the common haplotype associated with GA along with a rarer haplotype. Thus, this patient probably possesses a different mutation on each chromosome. Unfortunately, the direct approach of comparing GA alleles at the DNA level, by either specific oligonucleotide hybridization or by "polymerase chain reaction" amplification, is complicated by the presence of highly homologous OATase-like sequences on the X chromosome. However, since the latter are not expressed as mRNA, it may be fruitful to apply these techniques at the RNA level, although some GA alleles may not be expressed. The identification and characterization of GA mutations associated with other OATase haplotypes will likely yield several potential mechanisms of OATase deficiency and reveal the true extent of genetic heterogeneity in this disorder.

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