Hereditary tyrosinemia type 1 (HT1) is an autosomal recessive disease caused by a deficiency of the enzyme involved in the last step of tyrosine degradation, fumarylacetoacetate hydrolase (FAH). Thus far, 34 mutations in the FAH gene have been reported in various HT1 patients. Site-directed mutagenesis of the FAH cDNA was used to investigate the effects of eight missense mutations found in HT1 patients on the structure and activity of FAH. Mutated FAH proteins were expressed in Escherichia coli and in mammalian CV-1 cells. Mutations N16I, F62C, A134D, C193R, D233V, and W234G lead to enzymatically inactive FAH proteins. Two mutations (R341W, associated with the pseudo-deficiency phenotype, and Q279R) produced proteins with a level of activity comparable to the wild-type enzyme. The N16I, F62C, C193R, and W234G variants were enriched in an insoluble cellular fraction, suggesting that these amino acid substitutions interfere with the proper folding of the enzyme. Based on the tertiary structure of FAH, on circular dichroism data, and on solubility measurements, we propose that the studied missense mutations cause three types of structural effects on the enzyme: 1) gross structural perturbations, 2) limited conformational changes in the active site, and 3) conformational modifications with no significant effect on enzymatic activity.

Type 1 hereditary tyrosinemia (HT1, OMIM 276700) is an autosomal recessive disease caused by a deficiency of fumarylacetoacetate hydrolase (FAH, EC 3.7.1.2), the last enzyme involved in the tyrosine catabolic pathway. FAH catalyzes the hydrolysis of fumarylacetoacetate into fumarate and acetoacetate (1). FAH is mainly expressed in mammalian liver. It is also expressed, in lesser amounts, in cells from a wide range of tissues such as kidneys, adrenal glands, lungs, heart, bladder, intestine, stomach, pancreas, lymphocytes (2), skeletal muscle, placenta, fibroblasts, chorionic villi (3), and some glial cells of the mammalian brain (4). A deficiency of FAH causes the accumulation of succinylacetone (1), maleylacetoacetate, and fumarylacetoacetate (FAA), the latter presenting a mutagenic potential (5–7). Both an acute and a chronic form of the disease have been described on the basis of the clinical severity and/or the age at diagnosis. The acute form occurs early in infancy and causes severe liver damage leading to liver failure and death. The chronic form manifests itself later in infancy or childhood with symptoms such as progressive liver cirrhosis, renal tubular dysfunction, and a high incidence of hepatocellular carcinoma (8). HT1 is the most severe of the diseases associated with the enzymes of the tyrosine catabolic pathway. Although its prevalence worldwide is low (1:120,000 births), it shows a high incidence in some populations such as that of the Saguenay-Lac-St-Jean region (Canada), where it affects 1:1,846 newborns indicating a carrier frequency of 1:20 inhabitants (9). The high incidence of HT1 in this region is presumably the result of a founder effect involving mostly the IVS12+5g→a splice mutation (10, 11).

The human FAH gene is localized to the q23-q25 region of chromosome 15 (12), contains 14 exons, and covers ~35 kilobases of DNA (13, 14). At this time, 34 mutations have been reported (8, 15, 16). These include 18 missense mutations, 10 splice mutations, 5 nonsense mutations, and 1 silent mutation. These mutations are evenly spread along the FAH gene but with a slightly higher frequency in some parts of exons 8 and 13.

The human FAH enzyme has been purified to homogeneity (2, 17), and the crystal structure of recombinant mouse FAH has recently been reported (18). FAH represents a new class of metalloenzymes that possess a unique α/β fold. The crystal structure of FAH and its active site should prove particularly helpful in understanding the effects of mutations on both the structure and the activity of the enzyme.

To determine the effects of missense mutations on the structure and activity of FAH, we used site-directed mutagenesis to generate mutant FAHs and examined the expression and enzymatic activity of mutant proteins in a bacterial expression system and by transient expression after transfection in mammalian cells. Circular dichroism spectra were measured for the wild-type FAH and four variants containing HT1-associated amino acid substitutions, and structural descriptions of HT1-associated amino acid substitutions were made based on crystal structure of murine FAH (18). Eight missense mutations (all previously reported in HT1 patients) were analyzed: N16I (19), F62C (14), A134D (13, 20, 21), C193R (22), D233V (20, 23), W234G (24), Q279R (25), and R341W, the so-called pseudo-deficiency mutation (26, 27). There is still no clear relation between the genotype and the phenotype in HT1, which varies from an acute to a more chronic form. Mutations analyzed in...
this study were chosen in a manner to include mutations affecting residues in different parts of the FAH structure and involving mostly residues conserved from Caenorhabditis elegans to Homo sapiens. Some of the mutations were studied for specific reasons. For example, the C193R mutation was analyzed to determine whether the cysteine residue at position 193 was essential to the structural integrity of the enzyme. Other mutations were examined because they were located in the enzyme’s active site (D233V and W234G). The molecular basis of the R341W mutation, described as a pseudo-deficiency mutation, was investigated because of the unusual phenotype observed in homozygote individuals who show no symptoms of the disease. Finally, the Q279R mutation was studied because it represented a new mutation for which the molecular basis had not yet been described. Many of the mutated proteins were found to be inactive, probably as a result of misfolding of the mutated FAH.

EXPERIMENTAL PROCEDURES
FAH Cloning in the pET30 Vector—A human FAH cDNA clone was obtained by amplification of cDNA reverse-transcribed from mRNA of a normal liver (patient 8888). The amplification reaction mixture (50 µl) contained 5 µl of cDNA, 5 µl of 10× PCR buffer (Expand Long Template PCR System, Roche Molecular Biochemicals), 500 µM of dNTPs, 200 ng of primers TANR130 and RT025, and 2.5 units of recombinant Taq polymerase (Roche Diagnostics). TANR130 primer (5'-GGG AAT TCT GTC ACT GAA-3') and RT025 (5'-GGG AAT TCT GTC ACT GAA-3') are located in the 3' coding end of FAH cDNA, a cytosine has been added in front of the start codon of the FAH gene. RT025 (5'-GGG AAT TCT GTC ACT GAA-3') is located in the 3'-noncoding end of the gene. The 5' extremity of RT025 is not complementary to the cDNA but contains an EcoRI restriction site used to clone the amplification product in a vector. The reaction mixture was covered with 50 µl of mineral oil and incubated at 95 °C for 5 min, 55 °C for 5 min, and 72 °C for 40 min to allow the synthesis of the second strand of the cDNA. The PCR performed on a DNA Thermal Cycler (model N801–0150, PerkinElmer Life Sciences) included 35 cycles of the following program: 40 s at 95 °C, 1 min at 53 °C, and 2 min at 72 °C. A final extension of 15 min at 72 °C was done to complete the elongation. The amplification product was digested with NcoI and Pfu (2,700 units/ml, Amersham Pharmacia Biotech) and was used to transform competent bacterial cells. Clones containing the site-directed mutations were verified by sequencing (results not shown) to ensure that no other mutations were present in the gene.

FAH Cloning in pCEP4 Mammalian Expression Vector—The wild- and mutant pCEP4FAH mammalian expression vectors were constructed by inserting the fragments of pET30FAH vectors digested with KpnI (10,000 units/ml, New England Biolabs) and HindIII (20,000 units/ml, New England Biolabs) and containing the wild-type or mutant FAH gene into KpnI-HindIII-cut pCEP4 vector (Invitrogen). The fah fragments were ligated to digested pCEP4 vector using T4 DNA ligase (40,000 units/ml, New England Biolabs).

Preparation of Samples for pET30FAH Expression in Bacterial Cells and Purification—The wild-type vector pET30FAH and mutant vectors were transformed in the GJ1158 strain of Escherichia coli. The cells were grown to an A900 of 0.4–0.5 at 37 °C in Luria-Bertani medium with NaCl omitted, containing 10 g of tryptone and 5 g of yeast extract per liter (pH 7.0), with 50 µg/ml kanamycin. The temperature was then lowered to 30 °C; 0.3 x NaCl was added to the medium, and cultures were further incubated for 5 h (28). Cells were harvested and pellets resuspended in 20 ml of 10 mM phosphate buffer (pH 7.3). Each sample was introduced into a French press cell and lysed at 800 p.s.i. of pressure. Afterward, samples were centrifuged at 10,000 × g for 30 min at 4 °C. Following this centrifugation, the soluble supernatant was recovered and protein concentration was determined using the Bio-Rad protein assay. For CD measurements, the recombinant proteins were purified by affinity chromatography through their His tag on nickel-nitrilotriacetic acid superflow columns (Qiagen).

CD Measurements—Samples for CD measurements were dialyzed overnight against a 1000-fold excess volume of 10 mM sodium phosphate (pH 7.0) at 4 °C. Wild-type FAH remains soluble in excess of 1 M NaCl under these conditions; however, precipitation was noted for several of the variants and was removed by centrifugation at 14,000 × g. The yield of soluble N16I, F62C, and W234G from the bacterial expression system was not sufficient for making CD measurements. Concentrations of supernatants of the dialyzed samples were determined by scanning UV absorbance spectra using an extinction coefficient of 1.3 mM-1 cm-1 at 280 nm. Less than 3% of the C193R sample remained soluble following dialysis, preventing further CD analysis of this sample. Samples were diluted to 0.12 mg/ml, and CD spectra were recorded as an average of 12 scans at room temperature using a Jasco J720 instrument and a 1-mm light path. Base-line spectra were recorded using individual dialysis solutions as a blank for each sample. Mean residue ellipticities were calculated using a mean residue weight of 110. Final protein concentrations were calculated using photomultiplier tube voltages and standard curves based on serial dilutions of wild-type FAH. Data smoothing has not been performed. Protein concentration-dependent differences between the CD spectra were assessed based on the proportionality between variant and the wild-type spectra in the 240–190 nm range, and by calculating the ratio of ellipticities measured at fixed wavelengths (data not shown) and measured at two minima apparent in the spectra (Fig. 2 and Table IV). Differences between the wild-type and Q279R spectra were judged to be insignificant, based on these criteria.

Expression in Mammalian Cells—CV-1 (kidney African Green monkey cells, American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Medicorp Inc.) at 37 °C with 5% CO2. The day prior to transfection, 2 × 104 CV-1 cells were seeded in a 75-cm² flask and grown at 37 °C. The cells were transfected with 2 µg of pCEP4FAH wild-type or mutant DNA using the FuGENE6 kit (Roche Molecular Biochemicals) according to the manufacturer’s recommendations. Six hours after transfection, cells were washed twice in PBS and fresh DMEM, 10% fetal bovine serum medium was added to the cells. Forty hours after transfection, cells were washed in PBS, trypsinized and

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Oligonucleotide sequence (5' → 3')</th>
<th>Location in FAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAH-N16I</td>
<td>CAC TCA CCA CCT GCC CTA CGG C</td>
<td>127</td>
</tr>
<tr>
<td>FAH-F62C</td>
<td>CAA ACA CCA GGA TGT CTA CCA TCA GCC TAC ACT C</td>
<td>185</td>
</tr>
<tr>
<td>FAH-A134D</td>
<td>CTC TCG GCA GCA TGA TAC CAA CGT CGG AAT C</td>
<td>401</td>
</tr>
<tr>
<td>FAH-C193R</td>
<td>GAA TAT GGT GCC CCG AAG CTC TTG GAC</td>
<td>577</td>
</tr>
<tr>
<td>FAH-W234G</td>
<td>GCT CTT GAA CGA CGG GAG TGC ACG AG</td>
<td>688</td>
</tr>
<tr>
<td>FAH-R341W</td>
<td>CTT GAA CCT GCG GGC GCG GGG GGG C</td>
<td>1021</td>
</tr>
</tbody>
</table>
TABLE II
Characteristics of missense mutations studied in HT1 patients

<table>
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<tr>
<th>Mutation</th>
<th>Characteristics</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>N16I</td>
<td>Normal mRNA, no activity</td>
<td>Heterozygous, N16I/ U*</td>
<td>Acute</td>
</tr>
<tr>
<td>F62C</td>
<td>No activity</td>
<td>Homozygous</td>
<td>ND b</td>
</tr>
<tr>
<td>A134D</td>
<td>Reduced mRNA, no activity</td>
<td>Heterozygous, A134D/ E364K</td>
<td>Chronic-intermediate</td>
</tr>
<tr>
<td>C193R</td>
<td>Normal mRNA</td>
<td>Heterozygous, C193R/ E364K</td>
<td>Subacute</td>
</tr>
<tr>
<td>D233V</td>
<td>Normal mRNA</td>
<td>Homozygous</td>
<td>Chronic-intermediate</td>
</tr>
<tr>
<td>W234G</td>
<td>Reduced mRNA, no activity</td>
<td>Heterozygous, W234G/ IVS12 + 5g → a</td>
<td>Chronic</td>
</tr>
<tr>
<td>Q279R</td>
<td>Uncharacterized</td>
<td>Heterozygous, Q279R/ IVS6 − 1g → t</td>
<td>Mild</td>
</tr>
<tr>
<td>R341W</td>
<td>Pseudo-deficiency, normal mRNA reduced activity</td>
<td>Homozygous</td>
<td>Normal</td>
</tr>
</tbody>
</table>

a U, undetermined.
b ND, not described.

resuspended in 10 mM phosphate buffer (pH 7.3) for homogenization using a Teflon pestle and a syringe. The homogenized extract was centrifuged 15 min at 10,000 × g, both the supernatant and the pellet were recovered, and protein concentration was determined using the Bio-Rad protein assay. These samples were used for kinetics assays as well as for Western blot analysis. Nontransfected CV-1 cells showed no FAH activity (data not shown).

FAH Activity Assay and Western Blot Analysis—FAH hydrolytic activity was determined as described previously using about 50 μM FAA as a substrate (2). Briefly, the decrease of absorbance at 330 nm, which corresponds to hydrolysis of the FAA substrate, was measured at room temperature using a spectrophotometer (Ultraspec III (Amersham Pharmacia Biotech) or Cary Varian 100) for the CV-1 cell extracts (using 40–150 μg of total protein/assay) and the bacterial extracts (using 5–10 μg of total protein/assay). To correct for differences in transfection efficiency and expression of the mutant FAH proteins, the hydrolytic activity of FAH against FAA was expressed as nanomoles of FAA hydrolyzed/min × mg of FAH, as determined by the following equation.

FAH activity (Eq. 1)

\[
\Delta \text{OD}(330 \text{ nm}/\text{min}) = \frac{1.35 \times \text{FAA molar extinction coefficient (ml/nmol)} \times \text{FAH (mg)}}{1 \text{ml (reaction volume (ml))}}
\]

As shown in Fig. 1 A, all mutated FAH proteins with the exception of N16I, F62C, and W234G were expressed in the soluble fraction of GJ1158 E. coli strain. The FAH harboring N16I, F62C, and W234G were expressed but were retained in the insoluble cell fraction (data not shown). Next, the hydrolytic activity of the mutated FAH proteins was measured using FAA as the substrate. As shown in Table III, all mutated FAHs with the exception of R341W and Q279R were inactive in this hydrolytic assay. R341W and Q279R showed activities equal to that of normal FAH.

Characterization of Mutant FAH Proteins by Expression in Bacterial Cells—To study the structure-function of FAH, various missense mutations found in the FAH gene of different HT1 patients (Table II) were introduced in a human FAH cDNA by site-directed mutagenesis. These patients were either homoallelic or heteroallelic and exhibited different phenotypes ranging from normal to an acute form. For example, the patient heteroallelic for the Q279R and the IVS6 − 1g → t mutations showed mild clinical symptoms until 36 years but then developed hepatoocellular carcinoma (25).

The mutated FAH constructs were introduced in E. coli and the corresponding proteins induced with NaCl (28). The soluble recombinant protein fractions were analyzed by Western blot and for enzymatic activity. As shown in Fig. 1A, all mutated
and W234G mutations were only present in the insoluble fraction. However, proteins containing the N16I, F62C, C193R, and all mutant proteins were expressed in CV-1 seen in Fig. 1B, top), which suggests that these proteins are subject to misfolding and aggregation in mammalian CV-1 cells. This would explain their absence from the soluble fraction of cellular extracts. To assess whether the expressed proteins were localized properly in the cell, immunofluorescent staining with anti-FAH was performed on CV-1 cells 48 h after transfection. The mutated proteins found in the insoluble fraction of the cellular extracts by Western blot analysis (N16I, F62C, C193R, and W234G) were shown to form aggregates mainly in the perinuclear-Golgi region of the cell (data not shown). This observation supports the hypothesis that the presence of these mutations interferes with the proper folding of the enzyme. The other FAH variants (A134D, D233V, Q279R, and R341W) were evenly distributed in the cytoplasm, much like the wild-type protein (data not shown).

Assay of the hydrolytic activity of the mutant FAHs in transfected mammalian cells gave results identical to those seen for proteins expressed in bacteria, i.e. only the R341W and Q279R proteins had hydrolytic activity (Table III), which was similar to that of the wild-type enzyme. The specific activity of FAH expressed in bacterial cells is notably much higher than that measured in transfected mammalian CV-1 cells. The activity in these cells is comparable to that measured in pork liver (780 nmol/min/mg FAH).

**Effects of Mutations on FAH Structure**—To assess structural perturbations caused by HT1-associated amino acid substitutions, circular dichroism (CD) spectra were measured for wild-type FAH and the A134D, D233V, Q279R, and R341W variants. Representative spectra are shown for wild-type FAH, FAH D233V, and FAH A134D in Fig. 2. The wild-type FAH CD spectrum is characterized by minima at 225.0 and 208.8 nm, a crossover point at 201.9 nm, and a maximum at 196.0 nm. This spectrum is consistent with an α/β structure in solution. The FAH crystal structure contains 27% α-strand and 18% α-helical secondary structure (18). Significant differences were observed with all the HT1 variants, except Q279R (Fig. 2 and Table IV). The largest differences involved the position of the minimum between 220 and 225 nm and the intensity of the CD bands across the spectrum. The spectrum for A134D, having a minimum at 220.0 nm, enhanced negative ellipticity between 220 and 200 nm, and diminished positive ellipticity below 200 nm, deviated most from the wild-type spectrum (Fig. 2). The R341W FAH spectrum shows enhanced negative ellipticity above 201 nm and diminished positive ellipticity below 201 nm, relative to wild-type. D233V FAH also showed enhanced negative ellipticity above 201 nm, but nearly identical ellipticity below 201 nm, relative to wild-type. As the far UV CD spectrum of a protein is primarily due to the environment of the peptide chromophore (30), differences in the CD spectra of the FAH variants relative to wild-type FAH are consistent with small changes in secondary structure. Spectra for N16I, F62C, C193R, and W234G could not be accurately measured due to problems with precipitation and solubility (see “Experimental Procedures”). Thus, all of the HT1-associated amino acid substitutions studied here, with the exception of Q279R, have effects on the FAH structure in vitro as indicated by altered CD spectra and/or reduced solubility. These structural perturbations may, in turn, account for the functional loss of FAH activity in individuals carrying these HT1-associated amino acid replacements. Structural representation of FAH and effects of these different mutations are shown in Fig. 3 (see “Discussion”).

**DISCUSSION**

Using site-directed mutagenesis, we generated a number of naturally occurring missense mutations detected in the FAH gene of HT1 patients to try to understand the structure-function relationship of FAH. Although some of these mutations had previously been tested in an in vitro translation system (13, 14, 19, 21, 23, 24, 27), no studies examining the properties of the mutated proteins in a bacterial and in a mammalian background have been reported. The HT1-associated amino acid substitutions studied here can be grouped into two classes based on the solvent inaccessibility of the altered residue. Nonconservative substitutions involving solvent-inaccessible residues are more likely to have adverse steric effects and are less likely to be tolerated within enzyme structures than solvent-accessible residues. Indeed, the FAH substitutions N16I, F62C, C193R, and W234G appear to cause gross structural misfolding and/or instability that results in aggregation and precipitation when expressed in either mammalian or bacterial cells. Phaneuf et al. (19) identified N16I as the first causal mutation in FAH responsible for HT1. After transfecting the mutant cDNA in CV-1 cells, they reported that these cells produced FAH mRNA but FAH was not detected by Western blot analysis nor was any FAH activity detected. They suggested that the mutated protein was unstable. The present data show that the N16I protein is produced in bacterial cells and in transfected mammalian CV-1 cells but that it is insoluble. In fact, the Asn-16 side chain forms hydrogen bonds with main chain atoms of both the N- and C-terminal domains (Fig. 3 B). The N16I substitution disrupts these electrostatic contacts between domains and the bulkier Ile side chain could cause steric problems between the domains, which would explain the gross structural impairment we observe for FAH N16I.

Awata et al. (14), who first documented the F62C mutation of
FAH, had demonstrated that this mutation was responsible for the decreased activity of the enzyme in a patient. The present study agrees with their data. Thus, the Phe-62 side chain stacks between the Phe-50 and Phe-70 side chains to form a hydrophobic core in the N-terminal domain (Fig. 3C). The F62C substitution introduces a cavity into this hydrophobic core. This effect of the mutation on the structure of the enzyme causes its retention as aggregates in the perinuclear-Golgi region of the cell.

The C193R mutation (22), also disrupts the structure of the protein since it forms insoluble aggregates mainly localized in the perinuclear-Golgi region of the cell. The Cys-193 side chain

**TABLE IV**

Major features in CD spectra

The positions of the major features present in the indicated CD spectra were identified using the Jasco Standard Analysis Program version 1.20. Min1:Min2 is the ratio of the minimal mean residue ellipticity values measured at the wavelengths indicated in columns two and three.

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Minima</th>
<th>Min1:Min2</th>
<th>Crossover</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>225.0</td>
<td>208.8</td>
<td>1.25</td>
<td>201.9</td>
</tr>
<tr>
<td>A134D</td>
<td>220.0</td>
<td>209.0</td>
<td>1.04</td>
<td>200.5</td>
</tr>
<tr>
<td>D233V</td>
<td>224.6*</td>
<td>208.8</td>
<td>1.18</td>
<td>201.3</td>
</tr>
<tr>
<td>Q279R</td>
<td>223.2</td>
<td>209.6*</td>
<td>1.23</td>
<td>201.5*</td>
</tr>
<tr>
<td>R341W</td>
<td>222.2</td>
<td>210.2</td>
<td>1.17</td>
<td>201.1</td>
</tr>
</tbody>
</table>

* Indicates values that do not differ significantly between the wild-type and variant samples.

**FIG. 3.** Structural representation of FAH (A) and mutations (B–E). A shows the dimeric structure of FAH. The effects of the mutations are shown in B–E (B, N16I; C, F62C; D, C193R; E, D233V and W234G). Carbon atoms are in yellow, nitrogen atoms in blue, oxygen atoms in red, sulfur atoms in green, Ca2+ in purple, and Mg2+ in gray; black lines represent hydrogen atoms.
is located in a C-terminal hydrophobic core (Fig. 3D) in van der Waals contact with the α-helix that contributes the Arg-237 and Gln-240 side chains to the FAH oxyanion hole. A conservative C193S substitution is found in the wild-type rat FAH. The C193R substitution would introduce unfavorable steric contacts in this region and might also alter the conformation of these two critical active site residues. This would explain the loss of activity that we observed.

The W234G mutation was reported in an American HT1 patient compound for this mutation and the IVS12+5g → a splice mutation (24). We observed that the mutated protein showed no FAH activity and was enriched in a cell-insoluble fraction. Trp-234 is located adjacent to the metal ion binding residue Asp-233 (described below). In addition to affecting the binding of two metal ions, the W234G substitution introduces a large cavity in a hydrophobic core (Fig. 3E). Thus, substitutions of the solvent-inaccessible residues described above (N16I, F62C, C193R, and W234G) result in gross structural defects associated with the disruption of native structural interactions or by the introduction of cavities and bad steric contacts.

Two exceptions to the gross structural perturbations described above are the A134D and D233V substitutions. Immunofluorescent staining of A134D FAH shows that the protein is evenly distributed throughout the cytoplasm, which suggests that this mutation does not severely impair its structure (data not shown). Ala-134 is located in a hydrophobic environment adjacent to the His-133 catalytic base and also near Tyr-128 at the entrance of the active site (18). The A134D substitution introduces a charged residue and bad steric contacts into this hydrophobic environment, presumably affecting substrate binding and catalysis by perturbing the conformation of the active site. Patients homozygous for the D233V mutation have a normal amount of liver FAH mRNA but a reduced amount of immunoreactive material and no FAH activity (23). In the present study, Western blot analysis as well as immunofluorescent staining show a comparable level of expression of D233V-substituted and wild-type FAH. The mutated enzyme showed no hydrolytic activity either when expressed in bacteria or in mammalian cells, a finding similar to measurements in liver extracts of patients reported by Rootweit et al. (23). The Asp-233 side chain is a ligand for the catalytic metal ion and binds a second metal ion that stabilizes the conformation of a loop between residue Lys-253 of the FAH oxyanion hole and Thr-257 (Fig. 2E). The D233V mutation introduces a hydrophobic group into a charged environment and presumably disrupts two metal ion binding sites and the conformation of important catalytic residues. It is surprising that significant amounts of soluble protein are produced in mammalian and bacterial systems for both of these variants. Loss of metal ion binding in the active site would be expected to cause charge repulsion of the acidic side chain ligands of the metal ions. The CD spectra for A134D differs most from the wild-type FAH spectrum (Fig. 2 and Table IV), indicating that substantial structural changes are associated with this substitution. Differences between the D233V and wild-type CD spectra were less pronounced, but still consistent with minor structural perturbations (Table IV). Although A134D and D233V involve residues that are inaccessible to bulk solvent in the surrounding solution, Ala-134 and Asp-233 are in close proximity to ordered water molecules bound in the FAH active site. The FAH active site shows considerable flexibility when different crystal structures are compared. Therefore, limited conformational changes associated with these substitutions appear to be tolerated within the flexible FAH active site.

The Q279R and R341W substitutions affecting solvent accessible side chains had no significant effect on enzymatic activity. The Q279R mutation was reported in a compound heterozygote patient (Q279R/IVS6–1g → t), who is unusual in the sense that she represents a rare case of HT1 to have lived over 30 years of age and showed few symptoms associated with the disease until she developed hepatocellular carcinoma (25). Site-directed mutagenesis and expression of Q279R in E. coli cells and transiently transfected CV-1 cells show that the catalytic activity of the mutated protein is similar to that of the wild-type protein and a normal localization pattern of expression. The Gln-279 side chain forms two hydrogen bonds with the Gln-328 side chain and a single hydrogen bond with the Tyr-323 main chain oxygen atom in a solvent-accessible loop. Although these interactions are likely to be disrupted by the Q279R substitution, solvent accessibility in this region is likely to tolerate this amino acid replacement by allowing the Arg side chain to rotate out into the surrounding solution, or by allowing slight alteration of the loop conformation to enable the Arg side chain to participate in hydrogen bonding similar to the Gln-279 side chain. In fact, no structural perturbation was indicated by the CD spectrum for Q279R relative to wild-type FAH. Therefore, the present study shows no direct effect of the Q279R mutation on the structure or activity of the enzyme. However, since the Q279R mutation is located in the 5’ donor splice region of exon 9, we believe that this mutation acts as a splice mutation in vivo, which would explain the HT1 phenotype of the patient.3

The last mutation analyzed is R341W described as a pseudo-deficiency mutation because patients homozygous for the mutation show no pathological symptoms of the disease (26). Northern blot analysis showed a normal amount of FAH mRNA in fibroblasts from homozygous patients but very little immunoreactive FAH (27). Measurement of enzymatic activity of the mutated FAH in an in vitro protein synthesis system showed a reduced activity on the order of 25–30% of that of normal FAH (27). This was higher than the activity measured in fibroblasts leading to the suggestion that the R341W protein was more rapidly degraded in vivo than in vitro.

In the present study, the R341W protein had a specific activity very similar to that of wild-type FAH when expressed either in bacteria or in CV-1 cells. Structural data indicate that the Arg-341 side chain protrudes into a water-filled space near the FAH dimer interface and makes van der Waals contacts with the Ser-164 side chain of the opposite subunit. The solvent accessibility of these residues suggests that this interaction is not very significant and similar contacts could be made by the Trp side chain in the R341W substituted protein. Despite having good enzymatic activity (Table III), it is interesting to note that the R341W spectrum was altered relative to wild-type FAH (Table IV). Reduced enzymatic activity of the pseudo-deficiency phenotype associated with R341W has been suggested to result from decreased stability or increased turnover in patient fibroblasts (27). Although the studies presented here do not address enzymatic half-life within mammalian cells, it is conceivable that the perturbation of the FAH structure caused by substituting the charged surface Arg side chain with an indole ring is sufficient to account for a decreased enzyme stability.

In summary, three kinds of mutations in the FAH gene were characterized: 1) mutations resulting in gross structural perturbations (N16I, F62C, C193R, and W234G), 2) mutations causing limited conformational changes tolerated within the

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flexible active site (A134D and D233V), and 3) mutations that display no significant effect on enzymatic activity (Q279R and R341W). The availability of the crystal structure of FAH can provide helpful hints in predicting and testing the effects of newly discovered mutations by their localization within the enzyme's tertiary structure and sheds new light on the structure-function relationship of the enzyme.

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Structural and Functional Analysis of Missense Mutations in Fumarylacetoacetate Hydrolase, the Gene Deficient in Hereditary Tyrosinemia Type 1
Anne Bergeron, Myreille D'Astous, David E. Timm and Robert M. Tanguay

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