Impairment of the human enzyme galactose-1-phosphate uridylyltransferase (hGALT) results in the potentially lethal disorder classic galactosemia. Although a variety of naturally occurring mutations have been identified in patient alleles, few have been well characterized. We have explored the functional significance of a common patient mutation, F171S, using a strategy of conservative substitution at the defined residue followed by expression of the wild-type and, alternatively, substituted proteins in a null-background strain of yeast. As expected from patient studies, the F171S-hGALT protein demonstrated <0.1% wild-type levels of activity, although two of three conservatively substituted moieties, F171L- and F171Y-hGALT, demonstrated ~10% and ~4% activity, respectively. The third protein, F171W, demonstrated severely reduced abundance, precluding further study. Detailed kinetic analyses of purified wild-type, F171L- and F171Y-hGALT enzymes, coupled with homology modeling of these proteins, enabled us to suggest that the effects of these substitutions resulted largely from altering the position of a catalytically important residue, Gln-188, and secondarily, by altering the subunit interface and perturbing hexose binding to the uridylylated enzyme. These results not only provide insight into the functional impact of a single common patient allele and offer a paradigm for similar studies of other clinically or biochemically important residues, but they further help to elucidate activity of the wild-type human GALT enzyme.

The enzyme galactose-1-phosphate uridylyltransferase (GALT) catalyzes the second step of the Leloir pathway of galactose metabolism, converting UDP-glucose (UDP-glu) and galactose-1-phosphate (gal-1-P) to glucose-1-phosphate and UDP-galactose (1). Impairment of human GALT (hGALT) results in the potentially lethal disorder galactosemia (2).

Studies of hGALT alleles derived from patients with galactosemia have revealed extraordinary allelic heterogeneity in this disorder; the number of candidate mutations identified now exceeds 150, the majority of which are missense point mutations (3). Indeed, many if not most galactosemia patients studied are compound heterozygotes. Unfortunately, although many patient alleles have now been sequenced, few of the substitutions identified are well understood in terms of their impact on hGALT structure and/or function. Considering the marked variability of clinical outcome for patients with galactosemia (4) and the possibility that allelic heterogeneity may account for at least some fraction of this phenotypic variability, understanding the functional consequence of specific patient mutations is an issue of clinical as well as basic importance.

One of the most significant challenges to understanding the functional impact of patient mutations is not determining whether a given substitution in hGALT impairs activity, but why? No structure of the human enzyme is currently available, but extensive studies have been done with the highly homologous Escherichia coli enzyme (5–7, 18, 22, 24). Both the bacterial and human enzymes catalyze a double displacement reaction following classical ping-pong kinetics. The E. coli enzyme is uridylylated on the His-166 (His-186 in human), and this intermediate is catalytically competent (24). A step other than uridyl transfer is rate-limiting, possibly product release. In addition to these mechanistic studies, structural work has demonstrated that the substrate binding site is composed of residues from both subunits in the active dimer (7). Homology modeling of the human GALT sequence onto the available E. coli GALT crystal structure (5–7) should allow some predictions to be made regarding patient alleles, but if activity of a mutant protein is fully impaired, the possibilities for testing these predictions are limited.

One approach to address experimentally the impact of a “severe” naturally occurring substitution on hGALT function is to make and characterize more conservative substitutions at a given substitution in hGALT impairs activity, but why? No structure of the human enzyme is currently available, but extensive studies have been done with the highly homologous Escherichia coli enzyme (5–7, 18, 22, 24). Both the bacterial and human enzymes catalyze a double displacement reaction following classical ping-pong kinetics. The E. coli enzyme is uridylylated on the His-166 (His-186 in human), and this intermediate is catalytically competent (24). A step other than uridyl transfer is rate-limiting, possibly product release. In addition to these mechanistic studies, structural work has demonstrated that the substrate binding site is composed of residues from both subunits in the active dimer (7). Homology modeling of the human GALT sequence onto the available E. coli GALT crystal structure (5–7) should allow some predictions to be made regarding patient alleles, but if activity of a mutant protein is fully impaired, the possibilities for testing these predictions are limited.

One approach to address experimentally the impact of a “severe” naturally occurring substitution on hGALT function is to make and characterize more conservative substitutions at the same position. By carefully selecting the amino acids to be substituted, the importance of side chain properties such as charge, shape, aromaticity, or polarity can be tested. By studying the residual activity associated with these conservatively substituted hGALT proteins, a clearer image of the functional requirements of the natural residue may be inferred. By extension, the functional impact of the non-conservative patient mutation at that site also may be deduced. Thus, this approach is expected to yield insights into the specific role(s) of individual residues in the enzyme.

We have utilized this approach of directed conservative substitutions to investigate the functional significance of the patient-derived F171S allele of human GALT. F171S was selected for study for three reasons. First, like many patient alleles, F171S-hGALT has been associated with a complete loss of activity in patient hemolysates (8, 9) and with little if any activity in a COS cell expression system (10). Second, Phe-171...
in hGALT is predicted by homology modeling with the E. coli GALT crystal structure to participate in interactions with at least one and possibly both substrates of the enzyme (5–7). This prediction raises the intriguing possibility that F171S-hGALT may not function in patient cells because the substitution impairs interaction of the enzyme with UDP-glu and/or with gal-1-P. Finally, the F171S substitution accounts for approximately 4% of the African-American galactosemia patient alleles studied (8, 9), making it one of the more common naturally occurring mutations detected in a given ethnic group. By probing the functional requirements of Phe-171 in the experiments described here, we have attempted both to define further the nature of substrate binding and catalysis in wild-type human GALT and to shed light on the functional consequence of one important patient mutation.

We report here the construction, expression, and kinetic analyses of wild-type and F171S human GALT as well as three alternatively substituted alleles: F171L-, F171Y-, and F171W-hGALT. All five human proteins were expressed and studied using a previously described yeast expression system (11). The results demonstrate that position 171 is important in determining both catalytic efficiency and the ratio of $K_m/K_i$ for UDP-glu. Homology modeling suggests that the effects are modulated by interactions with Gln-188, another important active site residue that is often mutated in classic galactosemia (3), and/or with two loops on the other subunit involved in binding UDP-hexose. Each substitution at position 171 can result in a repositioning of the side chain of Gln-188 and the binding loops containing residues 50–60 and 335–340 from the other subunit in the dimer. These putative rearrangements fully explain the effects of the substitutions tested.

**Experimental Procedures**

**Plasmid Manipulation and Site-directed Mutagenesis**—All substitutions at codon 171 in human GALT were created by site-directed mutagenesis of the otherwise wild-type sequence in an M13mp19 vector using a mutagenic oligonucleotide degenerate at the last 2 positions of codon 171 (12). Specific substitutions were determined by sequence analysis of individual clones resulting from the mutagenesis procedure. The plasmids selected for further study contained sequences at codon 171: TCG (Ser, S), TTG (Leu, L), TAT (Tyr, Y), and TGG (Trp, W).

**Fragments of hGALT sequence containing these substitutions were then subcloned using BglII and NcoI into the corresponding sites in a vector encoding wild-type hGALT.** To facilitate isolation of these proteins, we also constructed plasmids encoding proteins with hexahistidine tags at their amino termini (12). All experiments described here were performed at 37°C. Experiments were performed with cultures growing at stationary phase. Yeast expressing wild-type hGALT was stable under these conditions, and hGALT protein (F171Y and F171L) was stable under these conditions, and hGALT was stable to freezing and so was often stored in 50% glycerol in water, charged with Ni$^{2+}$ and stored at $-80^\circ$C.

**Yeast Culture and Manipulation**—All yeast manipulations were performed according to standard techniques, as described previously (13). All YEP-GAP-derivative plasmids were transformed into sYFK1, a haploid strain of Saccharomyces cerevisiae deficient in GALT, the endogenous yeast GALT (11). Transformants were selected and maintained on the basis of tryptophan prototrophy, conferred by the plasmid. Before lysis, cultures were harvested at $A_600 \sim 2$. Extracts were prepared from 30-ml (small) and 6-liter (large) cultures essentially as described previously (11, 12). Briefly, cells from small cultures were harvested by centrifugation, resuspended in 500 μl of lysis buffer (20 mM HEPES, 1 mM dithiothreitol, 0.3 mg/ml bovine serum albumin), and transferred to 2-ml tubes. Acid-washed glass beads (500 μl of 0.5 mm) were then added to each tube, and the cells were disrupted by 6 cycles of 60-s agitation at high speed followed by 30-s vortex agitation at 4°C. Each disrupted cell suspension was then transferred to a 1.5-ml microcentrifuge tube and centrifuged at 14,000 × $g$ for 10 min at 4°C to pellet insoluble material. Finally, the clarified supernatant was transferred to a fresh 1.5-ml tube, and the protein concentration was determined using Bio-Rad protein assay reagent and bovine serum albumin as a standard as recommended by the manufacturer.

**Cell Purification**—Cells from 6-liter cultures were harvested by centrifugation at 7000 × $g$ (4°C) for 10 min and were resuspended in 150 ml of lysis buffer (20 mM HEPES-KOH, 200 mM NaCl, 1 mM NaF, 0.5 μg/ml leupeptin, 2.88 μM/ml E-64, 0.44 μg/ml phosphoramidon, 0.2 μg/ml chymostatin, 0.625 μg/ml pepstatin, 1 μg phenylmethylsulfonyl fluoride, 1 μM β-mercaptoethanol) and washed repeatedly with water until the background was clear and then finally resuspended in 100 ml 20 mM HEPES, 1 mM dithiothreitol, 0.5 μg/ml leupeptin, and 0.025 mg/ml glass beads. The suspension was agitated at 4°C for four cycles of 1 min at high speed alternating with 4 min on ice. The disrupted cell suspension was then centrifuged at 10,000 × $g$ for 10 min at 4°C. Finally, the clarified supernatant was stored until use at −85°C. Protein concentration was determined as described above.

**Western Blot Analysis and Quantitation**—Western blot analyses were performed as described previously (14). In all cases, 1 μg of total protein was loaded onto each lane of the gel. Both wild-type and mutant forms of human GALT protein were detected using a rabbit polyclonal antiserum (EU770) raised against hexahistidine-tagged human GALT at a dilution of 1:100,000. Parallel blots probed with a mouse polyclonal antiserum also directed against hGALT produced indistinguishable results (data not shown). As a control for comparable loading of lanes, all blots also were probed simultaneously with a rabbit polyclonal antiserum (at 1:20,000 dilution) directed against endogenous yeast cyclophilin (15). Signals were visualized using secondary antibodies (Amersham Pharmacia Biotech) and horseradish peroxidase conjugated to horseradish peroxidase and the ECL manufacturer.

**GALT Activity Assays from Crude Soluble Lysates**—GALT activity was determined in lysates of yeast expressing native and hexahistidine-tagged forms of the hGALT protein (11, 12). All assays were performed in quadruplicate (or greater) under initial rate conditions. For extracts containing wild-type hGALT, 0.1 μg and 0.5 μg of total protein were used per assay. Assays of extracts containing the substituted F171L and F171Y-hGALT proteins were performed using 3 μg and 6 μg of protein per assay to compensate for the impaired activities of these enzymes. Similarly, assays of extracts containing the substituted F171W- and F171S-hGALT proteins were performed using between 100 to 300 μg of protein per assay.

**His$_6$-hGALT Purification**—Hexahistidine-tagged forms of wild-type and F171L- and F171Y-hGALT were purified to near homogeneity for further kinetic analysis. Purifications were performed using Hi-Trap-chelating columns (Amersham Pharmacia Biotech), essentially as recommended by the manufacturer. In brief, each column was rinsed with wash buffer. The bound hGALT protein was then eluted using 250 mM imidazole in phosphate buffer. When 5-ml columns were used, all volumes were increased by a factor of five. Following isolation, each purified protein preparation was then passed over a Hi-Trap desalting column (Amersham Pharmacia Biotech) to remove the imidazole. Protease inhibitors (0.625 μg/ml leupeptin, 3.6 μg/ml E-64, 0.55 μg/ml phosphoramidon, 0.5 μg/ml chymostatin, 0.625 μg/ml pepstatin, 1.25 μg phenylmethylsulfonyl fluoride) and 0.5 μM β-mercaptoethanol were added to each sample, and the protein concentration was determined using Bio-Rad protein reagent. Purified wild-type hGALT was stable to freezing and so was often stored in 50% glycerol in liquid nitrogen until needed. Unfortunately, neither substituted hGALT protein (F171Y and F171L) was stable under these conditions, so both were always used fresh the same day that they were made.

**Colloidal Coomassie Blue Staining—SDS-PAGE** gels were run in 40% methanol, 10% acetic acid for 10 min, rinsed with water, and then stained with gentle rocking overnight in 40 ml of staining stock (1.17 mM Coomassie Brilliant Blue G-250, 0.8 mM ammonium sulfide, 0.1 mM phosphoric acid) plus 10 ml methanol (16). After staining, gels were washed repeatedly with water until the background was clear and then fixed in 50% methanol, 10% acetic acid, 1% glycerol. Finally, gels were air-dried between sheets of cellulose acetate and stored at room temperature.

**Kinetic Analyses**—Kinetic assays were carried out using a coupled, spectrophotometric assay as described previously (12, 17). The assay buffer contained 100 mM glycyl-glycine, pH 8.7, 6 mM dithiothreitol, 5 μM glucose-1,6-diphosphate, 5 mM MgCl$_2$, 0.8 mM NaDP, 0.1 μM of...
phosphoglucomutase, and 0.06 μg of glucose-6-phosphate dehydrogenase in a total reaction volume of 400 μL. For assays of wild-type hGALT activity, 300 ng of protein were used per reaction. Thirty assays were then performed in triplicate, with UDP-glu varying from 0.025 mM to 5 mM and gal-1-P varying from 0.075 mM to 1.2 mM. For assays of F171Y-hGALT, 3000 ng of purified protein were used per reaction, and activity was determined in triplicate with UDP-glu varying from 0.2 mM to 6.4 mM and gal-1-P varying from 0.3 mM to 4.5 mM. Finally, for F171L-hGALT, 3000 ng of purified protein were used per reaction, with UDP-glu varying from 0.0625 mM to 5 mM and gal-1-P varying from 0.6 mM to 9.6 mM. Negative control assays were routinely performed, leaving out one or both substrates or leaving out the enzyme. The expected negative result always was obtained. Values of $K_m$ of UDP-glu, $K_m$ of gal-1-P, $V_{max}$, $K_{cat}$ of UDP-glu and $K_{cat}$ of gal-1-P were determined for each hGALT enzyme tested. Briefly, the data for each enzyme were fitted to Equation 1 using a Microsoft Excel® spreadsheet programmed to perform a non-linear least squares fit.

$$V = \frac{V_{max}}{\frac{1}{K_m[A]} + \frac{1}{K_m[B]}} \\
\text{(Eq. 1)}$$

The estimates were further refined by fixing the invariant constants determined above and allowing the others to vary using the program MacCurve Fit (Kevan Ranier Software, Victoria, Australia). The goodness-of-fit was evaluated by calculating the correlation coefficient $r^2$ for each concentration of gal-1-P. In all cases, suitable fits were obtained using this model, and the kinetic constants did not vary significantly upon refinement. These constants are shown in Table I.

**Computer Modeling**—The effects of mutation were examined by homology modeling the human sequence onto the published structure of the uridylylated *E. coli* enzyme (17). The human sequence was submitted to the Swiss-Model automated protein modeler server. The resultant protein, and the human residues could be accommodated in positions similar to those observed in the *E. coli* crystal structure. Therefore, the coordinates of the *E. coli* enzyme were used with the program SwissPDBViewer. SwissPDBViewer's desired mutations were modeled using the MUTATE module of the program, and the optimal rotamers were selected such that steric clashes were minimized, and hydrogen bonds were maximized. The “best” rotomer was that with the lowest score $= (4 \times \text{number of clashes with backbone N, Ca, and C atoms}) + (2 \times \text{number of clashes with side-chain atoms}) + (4 \times \text{number of disulfide bonds}) - (4 \times \text{number of hydrogen bonds})$. In all cases, the rotamer selected had the β carbon of the mutated residue in the same position as the β carbon of Phe-171. Next, the side chain of Gln-188 was allowed to adopt all the common rotamer conformations, and the optimal rotamer was again selected. Finally, the side chain of each residue was manually rotated to allow any hydrogen bonds to form without introducing any steric clashes. The final adjustments leading to the structures shown in Fig. 4 were obtained with less than 20°s of rotation in all cases. The details of the algorithms used are further described at the SwissProt web site. Hydrogen bonds are automatically indicated and are shown in Fig. 4.

**RESULTS**

**Expression of Native and His<sub>6</sub>-tagged Wild-type and Phe-171-substituted hGALT Proteins in Yeast**—We have used site-directed mutagenesis of the otherwise wild-type hGALT sequence to recreate the naturally occurring substitution F171S as well as each of three more conservative substitutions at the same position: F171L, F171Y, and F171W. The substitution F171L was selected to preserve hydrophobicity while changing size and aromaticity, F171Y was selected to preserve aromaticity and size while changing hydrophobicity, and F171W was selected to preserve hydrophobicity and aromaticity while changing size. To facilitate the eventual rapid purification of each protein, all five sequences also were engineered to carry a hexa-histidine tag at the amino terminus. We have utilized this tag previously on the amino terminus of hGALT and demonstrated that it does not impair either expression or activity of the fusion protein (17).

All five hGALT sequences, with and without hexahistidine tags, were introduced into a previously described null-background strain of *S. cerevisiae*, yJFK1 (11), in the context of the yeast expression plasmid, YEP-GAP, as described under “Experimental Procedures.” Cultures were maintained in glucose-containing medium to avoid possible complications from growth under selective pressure for GALT (i.e. medium containing galactose).

Crude soluble extracts from each culture were analyzed to determine both abundance and activity of each substituted hGALT protein. As illustrated in Fig. 1, both tagged and untagged (native) forms of wild-type and F171S, F171L, and F171Y-hGALT proteins were abundant in these lysates; F171W-hGALT, in both tagged and native forms, was greatly decreased relative to the others. To control for comparable loading of lanes, filters were probed simultaneously with rabbit polyclonal antisera against both human GALT and the endogenous yeast protein, cyclophilin (15).

Because only soluble proteins were included in the preparations subjected to Western blot analysis in Fig. 1, the formal possibility remained that F171W-hGALT might be expressed at levels comparable with the other hGALT proteins, but that unlike the others, it preferentially partitioned to the insoluble fraction. To test this possibility, fresh cultures of yeast expressing each of the relevant hGALT proteins were grown, harvested, and prepared as before, except that both supernatant and pellet fractions were analyzed by Western blot analysis. Although some variability was seen from sample to sample, likely reflecting differential cell lysis efficiencies, the ratio of hGALT signals observed in supernatants and their corresponding pellets was not substantially different for any of the cultures tested (data not shown). Differential solubility of the various substituted hGALT proteins in yeast is therefore an unlikely explanation for the differential expression levels observed.

As expected, the wild-type protein was highly active under standard assay conditions, and the patient-derived F171S-hGALT was completely inactive (<0.1% wild-type activity). Of the three more conservative substitutions, F171L and F171Y demonstrated partial activity above background (Fig. 1). Whether the lack of detectable hGALT activity in extracts of cells expressing the F171W allele reflected catalytic impairment or diminished abundance of that substituted protein, or both, remains unclear. Nonetheless, substantial residual activity was retained by at least two conservative substitutions at the Phe-171 position, enabling further studies of those substituted enzymes and their catalytic properties relative to wild-type hGALT.
Purification and Kinetic Studies of Hexahistidine-tagged Wild-type and Substituted hGALT Proteins—To facilitate comparative kinetic studies of the wild-type and F171L- and F171Y-hGALT enzymes, all three proteins were purified to near homogeneity by nickel affinity chromatography, as described under “Experimental Procedures.” Fig. 2 presents colloidal Coomassie-stained SDS-polyacrylamide gel electrophoresis profiles of representative samples of all three proteins, both as crude (C) and purified (P) fractions.

Kinetic studies involving each purified protein were conducted at several concentrations of each substrate. The wild-type enzyme is subject to inhibition by excess substrates (18), and this is apparent in the decreased activity at high concentration of UDP-glu (Fig. 3A). Inhibition by gal-1-P is much less noticeable at these concentrations. The data were fit to Equation 1, describing the general case of a ping-pong mechanism with substrate inhibition caused by binding to the wrong form of the enzyme (18). Excellent fits were obtained (Table I), and the kinetic parameters were very similar to those published earlier for the human (19–21) and the E. coli (22) enzymes. Substitution of Phe-171 with Leu resulted in an enzyme with approximately 10-fold less activity and even more pronounced substrate inhibition (Fig. 3B). In contrast, there was little or no substrate inhibition observed with the F171Y mutant, and the maximal catalytic rate was about 4% that of the wild-type enzyme (Fig. 3C). In fact, aside from the maximal rate of catalysis, the most profound difference between the wild-type and mutant enzymes was the ratio of $K_{m}/V_{max}$ for UDP-glu. This difference was largely due to modulation of the inhibition constant $K_{in}$ describing the binding of UDP-glu to the uridylyl-enzyme intermediate. The $K_{in}$ for both substrates was relatively constant, varying by less than 3-fold for each, whereas $K_{m}$ varied by nearly 100-fold between wild-type and the F171Y mutant. The inhibition by excess gal-1-P was so weak that kinetic constants could not be accurately determined, but in all cases the inhibition constant was estimated to be above 20 mM.

Homology Model for Human GALT Based on the E. coli Crystal Structure—To examine the possible effects of these mutations on structure and function, it is necessary to have an accurate model for the human GALT structure. There is not yet a high resolution structure of the human enzyme; however, the E. coli enzyme has been extensively studied (5–8). These proteins are 55% identical over 344 amino acids and require only two gaps to align optimally. By the accepted standards of protein comparison, this should allow us to map the human sequence on the E. coli structure with considerable confidence (23). We have constructed this homology model using the automated modeling server at SwissProt. The human sequence was homology modeled onto the five different known E. coli GALT monomer conformations. These differ largely by the ligands bound and by the construction of residues 28–45 of the E. coli enzyme. This loop is partially disordered in most structures and adopts somewhat different positions in the different structures, possibly due to interactions with the uridyl group and crystal contacts (5–8). GALT is a dimeric enzyme with a substrate binding site at the interface of the dimer. In the bacterial enzyme, residues Val-61, Asn-77, Asp-78, Gln-168, Asn-153, Gly-159, and Ser-161 of one subunit have been implicated in binding to the UMP portion of the substrate, whereas residues Arg-28, Arg-R31, Lys-311, Phe-312, Tyr-316, Glu-317, and Gln-323 from the other subunit interact with the hexose 1-phosphate portion (7).

The homology model of the human enzyme closely resembles the templates with a root mean squared deviation between the model and the E. coli protein complexed to UDP-galactose (1gup.pdb) of 0.61 Å for 1292 backbone atoms corresponding to residues 8–343 of the E. coli sequence (residues 22–310 and 314–367 of the human protein). Fits to all the other templates were similarly good when adjusted for the different extents of disordered residues. Notably, all the residues that make contacts with the substrates are conserved in the human enzyme and can adopt a similar side chain conformation. To emphasize the chemical nature of the covalent intermediate, the coordinates of the uridylylated E. coli enzyme (1hxq.pdb) were used in the analysis described below.

Several close contacts are apparent between the human model and the uridyl group, including hydrogen bonds between N3 of the ring and Asp-98, and between O2 of the ribose and Asn-97. Asp-98 is also hydrogen-bonded to Arg-80. As these positions are far removed from the site of mutation, we did not further investigate these contacts. Other hydrogen bond inter-
actions appear important in stabilizing the covalent uridyl group, including hydrogen bonds between the γ sulfur of Cys-180 and the non-bridge phosphoryl oxygen of the covalent intermediate and between the epsilon O of Gln-188 and both the bridge and non-bridge oxygens of the covalent intermediate. Gln-188 also makes hydrogen bonds between the side chain amide and the indole nitrogen of Trp-190 and between both the backbone carbonyl and amide nitrogen and the corresponding backbone groups of Phe-171. Finally, Phe-171 is at the dimer interface, within 5 Å of the substrate binding loop on the other subunit consisting of residues 335 to 343. Some or all of these interactions are probably perturbed upon mutation of Phe-171 and are summarized in Fig. 4.

**DISCUSSION**

To characterize further the impact of mutation on structure and function of human GALT, we have taken the approach of conservative substitution at selected positions. The principle applied is that we learn more about the molecular impact of a mutation if the resultant enzyme is impaired but not totally inactive. By systematically altering the physical properties of the selected side chain we can begin to look at structure/activity relationships and infer the effects of the more dramatic mutations. Unless otherwise noted, all residue numbers discussed below refer to the human enzyme.

**Effects of Mutation on Expression and Abundance**—Looking first at the effects of mutation on expression levels in yeast, Fig. 1 demonstrates that most mutations are tolerated. Only the F171W mutation dramatically reduces expression levels. This is obvious in constructs encoding both the native and the His6-tagged versions of hGALT. When the homology modeled structure is examined and the F171W mutation is modeled, all rotamers have unfavorable steric interactions. The least disruptive rotomer places the indole ring of Trp-171 in the same position as the benzene ring of Phe-171, and there is an unfavorable steric clash with the indole ring of Trp-190. To relieve this clash, the side chain of Trp-171 would have to rotate to positions with even more unfavorable interactions. Since this side chain is in the dimerization domain, it is possible that the clash is resolved by interfering with dimerization and that the resultant monomeric GALT is not properly folded or stable in the absence of these interactions. The levels of F171L, and to a lesser extent F171Y, are reduced but only a few fold. Interestingly, F171S appears to be a very stable protein in yeast, albeit inactive.

**Effects of Mutation on Enzymatic Activity**—The activity of these mutations can be estimated in crude extracts by comparing observed activities and expression levels. The F171S and F171W mutations show very low levels of activity, although the latter mutant appears to be marginally active despite its low level of expression. As these activities are near the lower limits of detection, it is difficult to know whether they represent real activity. Because the expression levels were so low, the F171W mutation was not further characterized. In contrast, both F171L and F171Y mutants showed activities at least several percent that of those seen with wild-type GALT. As has been observed previously, the addition of the His6 tag did not significantly alter this conclusion.

To quantitate the consequences of these substitutions, the wild-type and mutant proteins were purified and characterized. Fig. 2 demonstrates that essentially pure proteins were obtained from the nickel chelate chromatography. The substituted proteins were somewhat unstable on storage, and so for these samples all characterizations were done with freshly purified proteins. Fig. 3 shows that all three versions of GALT were significantly active, although the mutant proteins were present at 10 times the level of the wild-type protein in these assays. It is also apparent that the wild-type and F171L proteins were subject to inhibition by excess UDP-glu, whereas the F171Y protein was much less inhibited. This inhibition has been observed many times before and results from the binding of UDP-glu to the wrong stable form of the enzyme, i.e. to the uridylylated enzyme. It is not clear how a second uridyl group is simultaneously bound, but it must bind in such a way as to block the binding of the incoming second substrate, gal-1-P. An examination of the known crystal structures suggests that, with some adjustment of the conformation of bacterial residues 30–40 and 75–80 (human residues 50 to 60 and 95 to 100, respectively), the hexose portion of UDP-galactose could bind in the previously described hexose binding pocket, with the uridine positioned in such a way as to allow ring stacking inter-

**TABLE I**

**Kinetic constants for the wild-type and mutant enzymes**

Data were fit to Equation 1 as described under “Experimental Procedures.” The correlation coefficient ($r^2$) was calculated for the fits to each concentration of gal-1-P, and the average of the five fits is given here.

|           | Wild type | F171L | F171Y |
|-----------|-----------|-------|-------|
| $K_\text{m}$ (mM UDP-glu) | 0.6       | 0.9   | 0.3   |
| $K_\text{m}$ (mM UDP-glu) | 0.2       | 0.1   | >20   |
| $K_\text{m}$ (mM gal-1-P) | 0.4       | 0.4   | 0.8   |
| $K_\text{m}$ (mM gal-1-P) | >20       | >20   | >20   |
| $k_{\text{cat}}$ (s$^{-1}$) | 98        | 9.3   | 3.6   |
| $r^2$ (average ± S.D.) | 0.92 ± 0.03 | 0.95 ± 0.02 | 0.79 ± 0.03 |

**FIG. 4. Homology models of wild-type (WT) human GALT and the Phe-171 mutations.** The view shown is from the subunit interface looking into the substrate binding cleft. Details are given under “Experimental Procedures.”
actions with the covalent uridyl group. The latter motions are quite reasonable in light of the significant degree of disorder seen in this region of the known structures. It may also be that a motion in this loop is required to allow access of the substrate to the active site. Nevertheless all the kinetic results can be fitted to a simple model for ping-pong kinetics with dead-end inhibition due to binding of excess substrates to the wrong stable form of the enzyme (equation 1). Satisfactory fits to the data are obtained (Fig. 3), and the kinetic values obtained are shown in Table I. It should be noted that all kinetic parameters for the wild-type enzyme are within 3-fold of those estimated by careful studies of the E. coli enzyme (22) and are consistent with less detailed studies on the human enzyme (19–21).

The maximal velocity of the wild-type enzyme is estimated to be 98 s\(^{-1}\). Substitution of Phe-171 with Leu results in a 10-fold decrease in maximal velocity, whereas substitution with Tyr reduces the maximal velocity to about 4% that of the wild-type enzyme. Although we were unable accurately to measure the velocity of the reaction catalyzed by the F171S mutant, the limits of detection in our assay suggest that it is reduced at least 1000-fold.

Michaelis constants for each substrate are similar in all cases, varying only 2–3-fold. The constant for inhibition by excess gal-1-P was indeterminate but exceeded 20 mM in all cases. By contrast, the constant for inhibition by excess UDP-glu was 0.25 mM for wild-type, 0.12 mM for F171L, and greater than 20 mM for F171Y. This is apparent in the shapes of the curves shown in Fig. 3 and must result from alterations in the binding mode or affinity for UDP-glu binding to the uridylyl-enzyme intermediate.

**Modeling the Effects of Mutation on Structure**—To understand the results, we have modeled the human sequence onto the E. coli GALT crystal structure. These two proteins are 55% identical at the amino acid level, and a high quality model is expected from these studies. This model predicts that all the residues that contact the uridyl group in the covalent intermediate are absolutely conserved between these two species, and this is consistent with the remarkably similar properties of the wild-type and mutant versions of these enzymes. Addressing first the effects on \(V_{\text{max}}\), we note that Phe-171 is hydrogen-bonded through its backbone atoms to the backbone of Gln-188 (Fig. 4). The side chain of Gln-188 is in turn hydrogen-bonded to the indole N of Trp-190 and the bridge and non-bridge oxygens of the phosphate group of the uridyl enzyme. Substitution of Gln-188 with Arg results in the almost complete inactivation of the human (11), yeast (11), and E. coli (24) enzymes and is a common human mutation causing galactosemia (3). Mutation of the E. coli Gln-168 to Asn, His, or Gly reduced catalytic activity by about 40-fold, suggesting that this position is important but not absolutely required for catalytic activity. As this residue only marginally stabilizes the uridylylated enzyme intermediate, the role of this residue is likely to polarize the phosphate group to increase the vulnerability of the phosphorus to nucleophilic attack (24).

We can estimate the effects of mutation at position 171 of the human enzyme by examining the homology model. This procedure involves substituting the appropriate side chain in the model and examining a library of common rotamers for the conformation of the side chains that give the fewest unfavorable interactions. Likewise, those giving additional favorable interactions (hydrogen bonds) are then selected. After applying this protocol, we find that in all cases, the optimal rotamers have their \(\beta\) carbon superimposed on the \(\beta\) carbon of Phe-171 in the wild-type enzyme. Finally, the side chain of Gln-188 is then optimized by the same procedure. It should be noted that the algorithm used to rank the rotamers is only a crude energetic approximation based on avoidance of steric clashes and maximizing potential hydrogen bonds. It does not take into account subsequent rearrangements of other side chains in the neighborhood and affords only one description of the minimal perturbations that might be expected on mutation. A more sophisticated analysis would require inclusion of explicit solvent waters, the effects of the two required metal ions, and computationally expensive molecular dynamics. Because of the uncertainty associated with force fields involving metals and the fact that molecular dynamics gives an indication of motions that are not necessarily equilibrium positions, these calculations were considered to be of marginal value. A true test of our hypotheses or any of the more sophisticated models would still require the determination of a crystal structure for the human enzyme. This structural determination is currently in progress.

All of our models explaining the altered catalytic efficiency can be summarized by pointing out that these mutations may alter the position of Gln-188 and therefore prevent it from participating in catalysis. When Phe-171 is mutated to Leu, the model predicts that a void will be created due to the smaller side chain (Fig. 4). The best fit conformation of the amide side chain of Gln-188 is now rotated from its wild-type position to form a hydrogen bond with the backbone N of Trp-190 and to occupy partially the void created by this substitution (Fig. 4). In this conformation, Gln-188 would be removed from its catalytically important position and result in a significant decrease in catalytic rate. When the F171Y mutation is examined by this same protocol, the phenolic oxygen is able to hydrogen bond with the indole N of Trp-190 but makes unfavorable contacts with the side chain of Gln-188. Allowing the side chain of Gln-188 to move away and relieve this steric clash results in disruption of the hydrogen bonds to the uridylyl phosphate. The new conformation results in the side chain pointing out toward a loop at the surface of the protein formed by residues 80 to 100. Again this would result in displacement of the catalytically important residue. In the case of the F171S mutation, rotation of the side chain toward Ser-171 can result in a new hydrogen bond between the amide group of Gln-188 and the oxygen of Ser-171 while retaining the hydrogen bond between Gln-188 and Trp-190.

Thus, the effects of mutation of Phe-171 on \(V_{\text{max}}\) can be understood if alternative conformations of Gln-188 are induced. The substitution of Gln-188 with Gly, His, or Asn in the E. coli protein results in about 2.5% activity (24), consistent with the activity of the F171Y mutation. The additional activity of the F171L (10% of wild type) may reflect the equilibrium between the new catalytically inactive conformation(s) and the original active conformation. F171Y is more severely affected than F171L because the former cannot readily adopt the catalytic conformation without displacing the tyrosine side chain. F171S may be significantly inactivated because two hydrogen bonds possible in the inactive conformation would greatly stabilize this inactive form.

**Effects of Mutation on Inhibition by UDP-glu**—The results with excess substrate inhibition by UDP-glu strongly support the above conclusions. The wild-type enzyme is subject to dead-end inhibition. If the sugar portion of the inhibitory nucleotide occupies the same position as the incoming gal-1-P, then the side chain of Gln-188 should be near this inhibitor. Movement of the side chain away from the uridyl group (as is predicted to occur with F171L and F171S) would be expected to lessen this interference. This is exactly what is seen with F171L, and the inhibition by excess UDP-glu is more severe in that mutation. In contrast, the F171Y mutation blocks inhibition caused by binding of UDP-glu to the uridylylated enzyme. As mentioned above, binding of UDP-glu to the uridylylated enzyme is expected to occur with the hexose binding near the true hexose
binding site, composed in part of residues 334–340 of the other subunit in the dimer. Phe-171 is located at the interface between subunits and is 4–5 Å from a turn consisting of residues 337–340. In the E. coli enzyme, these residues (314–317) make extensive hydrogen bonds with the C3,4, and 6 hydroxyls of the bound hexose. Thus, minor readjustments of the subunit contact and side chain positions at position 171 might sufficiently disrupt these interactions in the uridylyl enzyme and significantly destabilize binding of UDP-glu to the intermediate. As a consequence, the inhibition by UDP-glu would be less severe, and this is what is observed.

Conclusion—The effects of mutation at position 171 of human GALT appear to result from two phenomena: 1) altering the position of a catalytically important residue, Gln-188 and 2) destabilizing the active dimer, perhaps by altering the dimer interface. The effects of these mutations depend on the size and hydrophobicity of the substituted side chains and may reflect the equilibria reached between wild-type and mutant conformations. In the case of F171S, the naturally occurring substitution, the altered interactions are predicted to be so strong as to almost completely preclude the wild-type conformation of Gln-188, and this stability is due in part to the two backbone hydrogen bonds that hold Gln-188 and Ser-171 in close proximity. The effects on catalysis and the dimer interface suggest that mutations in the hexose binding loop might modulate the activity of the F171S mutation in compound heterozygotes. These conclusions can best be tested by crystalizing the mutant proteins and determining the actual structures involved.

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