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## Functional Requirements of the Active Site Position 185 in the Human Enzyme Galactose-1-phosphate Uridyltransferase\*

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**The active site of galactose-1-phosphate uridylyltransferase (GALT) includes a HPH sequence that has been conserved in all species examined from *Escherichia coli* to humans. The crystal structure of the *E. coli* enzyme suggests that this proline is important in positioning the active site histidine (His-166) near the substrate. To examine the role of this proline in the homologous human sequence, we have performed saturating mutagenesis at Pro-185 within human GALT and characterized each resultant mutant enzyme using a yeast expression system. Activity analyses in crude lysates indicated that only proline at position 185 produced wild-type levels of activity, although five other amino acids, Ala, Gly, Ser, Gln, and Glu, all produced partially active enzymes. Western blot analyses of the GALT proteins in these lysates demonstrated that abundance varied from 9–118% of wild-type and was independent of activity. All five active mutant proteins were purified and characterized with regard to specific activity, apparent  $K_m$  for both substrates, and temperature-dependence of activity. Finally, modeling of these mutations onto the conserved *E. coli* active site structure was performed. Together, these results provide functional evidence demonstrating the critical role of Pro-185 in facilitating the transferase reaction.**

One of the fundamental questions in biochemistry concerns the role of specific amino acids within a given polypeptide sequence in determining the structure and function of the encoded three-dimensional protein. This question is of particular interest for proteins associated with human genetic disease in which a multitude of different point mutations may be identified, all affecting the same gene and all ostensibly deleterious to function, but for unknown reasons. One such protein is galactose-1-phosphate uridylyltransferase (GALT,<sup>1</sup> EC 2.7.7.12), impairment of which results in the potentially lethal disorder galactosemia (1). To date more than 30 distinct point mutations have been identified in the GALT sequences of patients with galactosemia (2), raising the possibility that genotypic heterogeneity may constitute at least one factor contributing to the broad range of phenotypic severity observed in

these patients (1).

The most common mutation observed in association with classic galactosemia, Q188R (3–5), involves a residue only two positions from the putative active site nucleophile, histidine 186. The active site nucleophile for bacterial GALT, histidine 166, has been identified through a combination of chemical modification protocols (6), isolation and characterization of the covalent intermediate (6–8), selected site-directed mutagenesis of candidate residues (9), and most recently, x-ray crystallography of the dimeric enzyme complexed with UDP (10). The active site nucleophile is part of a HPH triad that has been conserved in all species examined from *Escherichia coli* to humans. X-ray crystallographic data from bacterial GALT further indicate that, in contrast to the catalytic role played by histidine 166, histidine 164 serves a structural role as a ligand to zinc (10). Indeed, site-directed mutagenesis indicates that both histidines are essential for function of the *E. coli* GALT enzyme (9). Clearly, a better understanding of the structure/function relationships within the human GALT active site should further our understanding not only of the wild-type enzyme but also of the Q188R mutation and its role in galactosemia.

The distinct and essential roles played by both active site histidines raise the obvious question: what is the role of the proline between them? Wedekind *et al.* (10) have proposed from their crystallographic studies that the existence of a metal ligand (His-164) followed by a proline may restrict the backbone dihedral angles to properly position the nucleophilic His-166. In the observed structure, the side chain of His-166 is further constrained by hydrogen bonding to the backbone carbonyl of His-164. These interactions presumably serve not only to help form the active site but also to stabilize the enzyme.

Closer examination of this structure, however, reveals that the active site His-166 is not properly positioned to attack the bound substrate (10). The authors attribute this apparent discrepancy to the presence of a disulfide bond formed between Cys-160 and 2-mercaptoethanol present in the crystallization medium. It is postulated that, in the absence of this artifactual disulfide, the bound substrate would be able to rotate around the C4'–C5' bond to generate a more relevant structure.

While x-ray crystallography and computer modeling can offer invaluable insight into the structure of a molecule, they cannot fully predict flexibility of the structure in solution or reveal dynamic features required for function. By determining experimentally which amino acids are able to function in place of proline in the human GALT active site, a rough consensus of structural requirements for the position may be derived. Toward this end, we have conducted saturating site-directed mutagenesis of codon 185 within the human sequence and characterized each of the resultant mutant enzymes using a yeast expression system. Our experiments were designed to ask three questions: 1) can any amino acids other than proline

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<sup>1</sup> The abbreviations used are: GALT, galactose-1-phosphate uridylyltransferase; Gal-1-P, galactose 1-phosphate; UDPG, UDP-glucose.

functionally replace proline in the HPH sequence? 2) what will be the properties of these resultant mutant proteins? and 3) will the effects of these mutations be consistent with the published structure for the bacterial enzyme? The results reported here indicate that although only proline at position 185 produced a fully functional enzyme, five other amino acids, Ala, Gly, Ser, Gln, and Glu, all produced partially active enzymes. This pattern of allowed substitutions is fully consistent with the published crystal structure.

#### EXPERIMENTAL PROCEDURES

**Site-directed Mutagenesis**—Site-directed mutagenesis of the sequence encoding Pro-185 was performed as described previously (11). The degenerate oligonucleotides (where N = G/A/T/C, W = T/A, H = A/C/T, K = T/G, and V = A/C/G) used to generate the Pro-185 mutations were 5'-GGCTGTTCTAACCCCCACNNNCACTGCCAGGTATGGGC-CAGC-3', 5'-GGCTGTTCTAACCCCCACAHCACCTGCCAGGTATGGGC-CAGC-3', 5'-GGCTGTTCTAACCCCCACKVWCACCTGCCAGGTATGGGC-CAGC-3', and 5'-GGCTGTTCTAACCCCCACAGACCTGCCAGGTATGGGC-CAGC-3' and were hybridized to a single-stranded template of M13mp19.hGALT. All Pro-185 mutants were sequenced using Sequenase 2.0 (U. S. Biochemical Corp.) after subcloning to verify the desired mutation and to ensure that no unintentional mutations were present.

**Yeast Strains, Plasmids, and Expression Studies**—All GALT expression studies were performed using yJFK1, a haploid strain of *Saccharomyces cerevisiae* deficient in endogenous GALT due to a large deletion in the endogenous *GAL7* locus (4). All procedures including yeast transformations, culture manipulations, extract preparations, and GALT enzymatic assays were performed as described previously (4, 12). Prior to harvest, all yeast cultures were grown in synthetic medium containing glycerol/ethanol rather than galactose as the carbon source so that no selective pressure associated with GALT activity was exerted on the cells. Protein concentrations of both crude yeast extracts and purified protein preparations were determined using the Bio-Rad protein assay kit, using bovine serum albumin as the standard.

The plasmids used in these studies, pLMY2 (13), a low-copy number CEN plasmid, and pJFY3, a high copy number 2  $\mu$  plasmid, both carry the *TRP1* nutritional marker to complement tryptophan auxotrophy in the host strain. pJFY3 was derived from pJFY1 (4) as follows. The unique *SalI* site in pJFY1 was eliminated by digestion with *SalI* followed by incubation with Klenow and religation. Then the *XhoI* site was converted into a *SalI* site by digestion with *XhoI*, incubation with Klenow, and ligation of synthetic *SalI* linkers.

All plasmid manipulations were performed using standard techniques (14) and bacterial strains (XL1-Blue from Stratagene, and RY2504 and 71.18, the generous gifts of Kathy Tatti and Dr. Charles Moran, Emory University).

**Western Blot Analysis**—Western blot analyses were performed as described previously (12, 13). GALT proteins were detected by incubation with each of the following: a 1:1,000 dilution (in IM2) of a rabbit polyclonal antiserum generated against native human GALT isolated from red blood cells (the generous gift of Drs. P. Dembure and L. J. Elsas, Emory University), a 1:4,000 dilution of a rabbit polyclonal antiserum generated against a synthetic C-terminal oligopeptide of human GALT (the generous gift of Drs. K. Lai and L. J. Elsas, Emory University), or a 1:10,000 dilution of a mouse polyclonal antiserum generated against nickel affinity purified HIS6 tagged human GALT protein isolated from yeast (generated in collaboration with Dr. P. Jensen and L. Westerman, Emory University). To control for loading of lanes, a 1:20,000 dilution of rabbit polyclonal antiserum generated against yeast cyclophilin A (15) was also included in some experiments.

**Enzyme Purification**—Yeast extracts were prepared from 6-liter cultures grown to midlogarithmic phase in YPGE (for yeast expressing the Pro-185 mutants) or YPGal (for yeast expressing wild-type GALT), harvested by centrifugation at 4 °C, resuspended in 100 ml of lysis buffer (20 mM HEPES-KOH, 200 mM NaCl, plus protease inhibitors: 1  $\mu$ g/ml pepstatin, 2.1  $\mu$ g/ml aprotinin, 0.5  $\mu$ g/ml leupeptin, 2.6  $\mu$ g/ml antipain, 0.6  $\mu$ g/ml phosphoramidon, 7.5  $\mu$ g/ml E64, 0.1  $\mu$ g/ml chymostatin, and 170  $\mu$ g/ml phenylmethylsulfonyl fluoride), and transferred to a Biospec Products bead beater containing 100 ml of pre-chilled, acid-washed 0.5-mm glass beads. The suspension was vortex-mixed at 4 °C for four cycles of 1 min at high speed alternating with 4 min on ice. Each lysed cell suspension was then centrifuged at 4 °C for 15 min at 15,000  $\times g$  to pellet insolubles.

Ten ml of crude extract were mixed with 1 ml of nickel-NTA resin (Qiagen) pre-equilibrated with binding buffer (20 mM HEPES, pH 7.5, 25 mM imidazole, and 200 mM NaCl), and 275  $\mu$ l of a 1 M solution of imidazole (pH 7.5) and incubated with gentle end-over-end rotation on a GLAS-COL Laboratory Rotator at 4 °C for 2–3 h. Resin was allowed to settle on ice for 10 min, then supernatant was removed using a LO-Dose 1/2 cc U-100 insulin syringe with a 28G1/2 Micro-fine IV needle (Becton Dickinson). The resin was washed three times with 10 ml of wash buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 30 mM imidazole, plus protease inhibitors), and on the final wash, the slurry was transferred to a fresh tube and the supernatant was removed as described above. Protein was eluted from the nickel resin by incubation with 1 ml of 100 mM elution buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 100 mM imidazole, plus protease inhibitors) rotating as described above for 20 min. The resin was allowed to settle on ice for 10 min, then the supernatant was removed as described above. This elution step was repeated once as described above and then a second time using elution buffer containing 200 mM imidazole. Eluted fractions were then dialyzed against 20 mM HEPES, pH 7.5.

**Enzyme Assays and Kinetic Analyses**—Enzymatic activities of GALT in crude cell lysates were determined using a direct assay as described previously (13). All assays were performed in quadruplicate involving samples prepared from two separate colonies, each analyzed at two different concentrations of extract. In all cases, assays were performed within the linear range as defined for that sample. Enzymatic assays performed on purified proteins at different temperatures (Fig. 7) also were performed according to this protocol. All other enzymatic analyses of purified proteins were performed using a spectrophotometric coupled assay, essentially as described previously (12). The standard reaction was performed at 37 °C and contained 0.1 M GLY-GLY buffer, pH 8.7, 5 mM dithiothreitol, 5  $\mu$ M glucose 1,6-diphosphate, 5  $\mu$ M  $MgCl_2$ , 0.8 mM NADP, 1.2 mM Gal-1-P, 0.6 mM UDPG, 0.06  $\mu$ g glucose-6-phosphate dehydrogenase, and 0.1  $\mu$ g of phosphoglucomutase in a total volume of 400  $\mu$ l. Kinetic constants were assayed in the same way except that to determine the apparent  $K_m$  for UDPG the concentration of Gal-1-P was held constant at 1.2 mM and the concentration of UDPG was varied from 0.02 to 1.0 mM, and to determine the apparent  $K_m$  for Gal-1-P, the concentration of UDPG was held constant at 0.6 mM and the concentration of Gal-1-P was varied from 0.05 to 2.0 mM. Each data point was determined in duplicate over at least six different substrate concentrations for each substrate. Control assays, lacking either one or both substrates, were routinely included and always gave the expected negative results. Kinetic constants were calculated by fitting the data to an Eadie-Hofstee plot using Cricket Graph.

**Computer Modeling**—All measurements, visualization, and modeling were done using either RasMol (Roger Sayles, Glaxo Research and Development) or SYBYL (Tripos Associates). Substitutions were examined for steric conflicts by replacing the active site proline with the desired residue and minimizing the side chain conformation while maintaining the conformation of the peptide backbone and histidines 115, 164 (zinc ligands), and 166 (active site nucleophile) of the *E. coli* enzyme. The Tripos60 force fields were used with the Kollman all atom charges using the Powell minimization scheme. After simplexing, 100 to 200 cycles of minimization were applied. In the case of Asp and Asn substitutions, the  $\epsilon$ -amino group of Lys-154 was repositioned before minimization.

#### RESULTS AND DISCUSSION

**Saturating Site-directed Mutagenesis of Codon 185 within the Predicted Active Site Region of Human GALT**—Sequences encoding each of the 20 naturally occurring amino acids, as well as a stop codon (Fig. 1), were introduced into codon position 185 of human GALT using site-directed mutagenesis with degenerate oligonucleotides, as described under "Experimental Procedures." Each of the resultant mutant GALT sequences was confirmed by dideoxy sequencing, subcloned into the low copy number yeast expression plasmid pLMY2 (13), and transformed into the GALT-deficient strain of yeast yJFK1, as described previously (4). Transformants were selected and grown as described under "Experimental Procedures."

**Activity of Mutant Human GALT Proteins Expressed in Yeast**—As an initial test of function, yeast whole-cell lysates from transformants expressing each of the substituted human GALT proteins were prepared and analyzed for GALT activity as described previously (13). Extracts from cells expressing



FIG. 1. Human GALT sequences expressed in yeast. Favored codons (in yeast) (16) are indicated in uppercase, and disfavored codons are indicated in lowercase.

NAME	AA	CODON(S)	NAME	AA	CODON(S)
P185A	ala	gca	P185N	asn	aat
P185C	cys	TGT	wildtype	pro	ccc
P185D	asp	GAT	P185P'	pro	cct
P185E	glu	GAA	P185Q	gln	cag
P185F	phe	TTC	P185R	arg	AGA
P185G	gly	GGT	P185S	ser	tcg, TCT
P185H	his	CAC	P185T	thr	acg
P185I	ile	ATT	P185V	val	GTT
P185K	lys	AAG	P185W	trp	TGG
P185L	leu	ctt	P185Y	tyr	TAC
P185M	met	ATG	P185X	STOP	TAA

wild-type human GALT and the truncated protein, P185X-GALT, were analyzed in parallel as positive and negative controls, respectively. Although only the proline-containing wild-type and P185P'-GALT (Fig. 1) proteins demonstrated full activity, extracts expressing each of the five amino acid substitutions P185A, P185G, P185S, P185Q, and P185E also demonstrated activity, albeit to a diminished extent (Fig. 2). Extracts expressing the P185A, P185G, and P185S-GALT proteins demonstrated between 11 and 14% wild-type activity, whereas the P185Q and P185E-GALT proteins demonstrated only 1–4% wild-type activity. All other substituted GALT proteins demonstrated no detectable activity, even under assay conditions involving 3 orders of magnitude more exact than is required to observe activity from the wild-type enzyme.

To confirm further the low levels of GALT activity observed in the five mutant strains listed above, studies parallel to those illustrated in Fig. 2 were performed using expression of the wild-type and relevant mutant GALT enzymes from the high copy number yeast expression plasmid pJFy3 (see "Experimental Procedures"), which typically expresses about 10-fold greater GALT per microgram of protein in a crude cell lysate than does pLMY2. In all cases, the results of these analyses (data not shown) were fully consistent with those derived from the low copy number pLMY2 expression studies (Fig. 2).

**Abundance of Mutant Human GALT Proteins Expressed in Yeast**—To distinguish mutational effects on GALT-specific activity from effects on steady-state abundance, whole-cell lysates from transformants expressing wild-type human GALT and each of the 21 substituted proteins were subjected to Western blot analyses with each of three independent anti-human GALT antisera. Analyses were repeated using each of the three different antisera, as described under "Experimental Procedures," to limit potential artifacts resulting from differential recognition of epitopes by any single antiserum. The values presented in Fig. 3 represent averages derived from six experiments involving all three antisera. As an additional control for loading of the lanes, some of the Western blot analyses were performed using a mixture of two antisera, one directed against human GALT and the other directed against endogenous yeast cyclophilin (15). As illustrated in Fig. 3B, cyclophilin signals varied insignificantly between different lanes, indicating that comparable amounts of protein were loaded in each lane.

To investigate further the possibility that one or more of the lowest abundance mutant GALT proteins failed to demonstrate detectable GALT activity because of its low abundance rather than impairment of specific activity, studies involving high

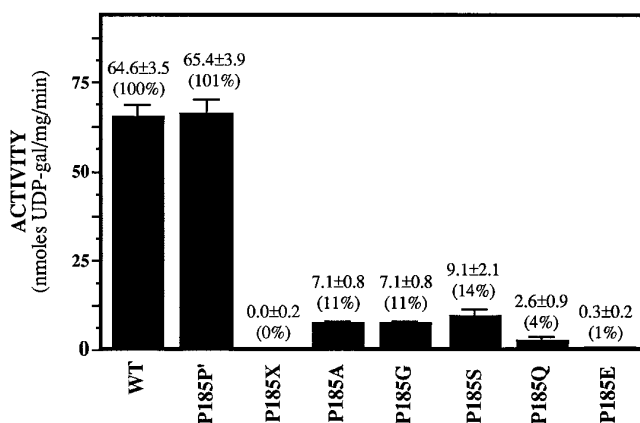


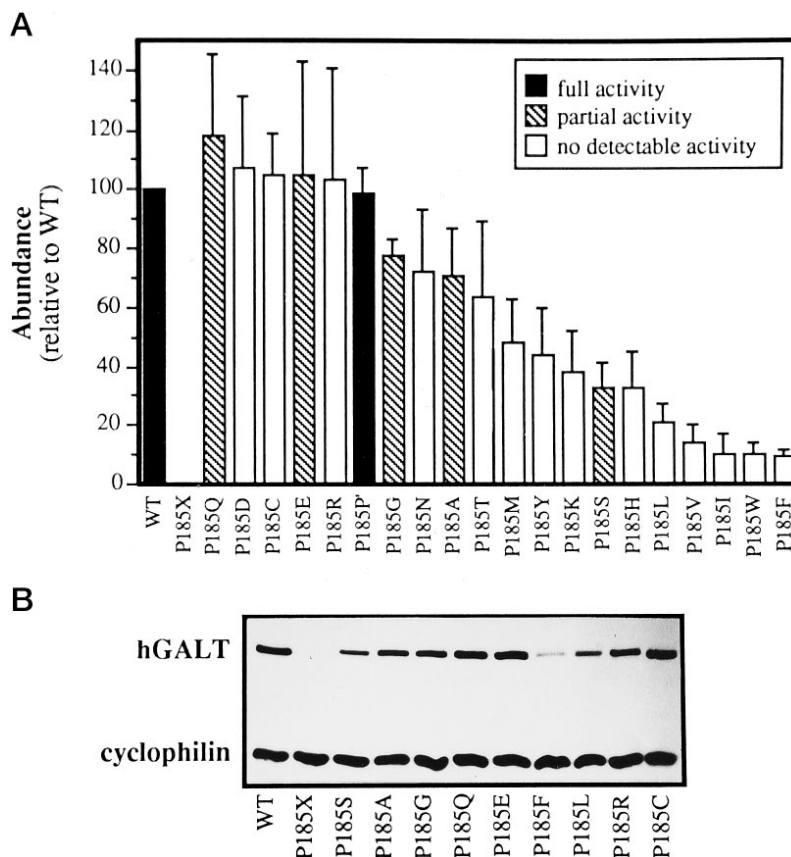
FIG. 2. Activities of mutant human GALT proteins expressed in yeast. Crude lysates prepared from yeast expressing either wild-type human GALT or each of the indicated mutant GALT enzymes were analyzed for GALT activity as described under "Experimental Procedures." Values plotted represent activity averages ( $n = 4$ ); bars, S.D. The values presented in parentheses represent activities normalized to the wild-type value.

copy number plasmid-based expression of several of these proteins also were performed. Wild-type human GALT and each of three of the lowest abundance inactive GALT mutants, P185W, P185I, and P185V, as well as two high or intermediate abundance GALT proteins, P185R and P185M, each were expressed in yeast from the plasmid pJFy3. As above, in all cases the results of these analyses were fully consistent with those derived from the low copy number expression studies (Fig. 2 and data not shown), indicating that protein abundance was not a confounding factor in determining GALT activity levels associated with these mutant proteins.

The apparent differences in steady-state abundance of the various mutant GALT proteins may in theory reflect differences either in rates of synthesis or rates of degradation. Considering that all alleles were expressed from the same plasmid backbone in the same strain of yeast, however, differential rates of synthesis seem unlikely. Two potential complications in interpreting the abundance data presented here stem from concerns about the possible effects of codon bias in yeast and from concerns about potential artifacts resulting from differences in antiserum affinity for the different mutant GALT proteins.

We have addressed the issue of codon bias by analyzing

**FIG. 3. Abundance of mutant human GALT proteins expressed in yeast.** A, values plotted represent averages  $\pm$  S.D. ( $n \geq 6$ ) of signal intensities observed on Western blot analyses of the indicated extracts probed with each of three different antisera directed against human GALT, as described under "Experimental Procedures." All values were normalized to the wild-type signal; bars, S.D. B, a representative Western blot analysis illustrating signals obtained with the indicated extracts. The antiGALT antiserum used in this experiment was the mouse polyclonal antiserum described under "Experimental Procedures." As a control for loading of lanes, a second antiserum directed against endogenous yeast cyclophilin (15) also was included in this experiment.



independent mutant sequences carrying, at position 185, either a highly favored codon for serine, TCT (16), or a disfavored codon for serine, TCG (16). Both Western blot analyses and activity assays involving each of these sequences demonstrated indistinguishable results (data not shown). Furthermore, no "low end" clustering was observed for mutant proteins carrying disfavored codons at position 185 (Fig. 3). Indeed, the wild-type sequence (Pro-185, 100%) and the most abundant mutant sequence (P185Q, 118%), both carried codons at position 185 that are considered disfavored in yeast (16). Nonetheless, differential expression of the mutant GALT proteins based on favored or disfavored codon usage at position 185 remains a formal possibility that may account for some of the variance observed with respect to abundance of these substituted proteins.

The concern regarding differential antiserum specificities was addressed by performing all Western blot analyses with each of three different polyclonal antisera, as described under "Experimental Procedures." Although most substituted GALT proteins gave very similar results with all three antisera, three (P185N, P185T, and P185K-GALT) gave disparate results in which the antiRBC-GALT antiserum reproducibly under-represented the amount of GALT protein present in a given sample relative to parallel experiments involving each of the other two antisera. The explanation for these discrepancies remains unknown. Abundance values derived from procedures using each of the three different antisera, therefore, were averaged in an effort to minimize any potential under- or overestimations of abundance (Fig. 3).

Absent synthesis or detection differences, there are two main parameters that may affect abundance: rate of folding, and stability of the folded protein. Although further experiments are required to distinguish formally between these two possibilities, it seems unlikely that the rate of folding is slowed in the mutants described here. Pro-165 in the wild-type bacterial protein exists in a *cis* conformation, and conversion from the

more stable *trans* conformation is a slow step that is often rate-limiting in folding (17). Because of greater conformational mobility, the substituted residues should be able to adopt the required rotational angles much more quickly than the wild-type proline, thereby speeding rather than slowing the folding process. Therefore, it seems much more likely that the reduced abundance observed for some of the mutants reported here reflects reduced stability of the folded proteins. Those mutants with the lowest observed levels of abundance carry substituted residues that are large and hydrophobic and may cause the greatest disruption of structure. This pattern is consistent with the published crystal structure.

**Kinetic Properties of Wild-type and Mutant Human GALT Enzymes**—To facilitate isolation of wild-type human GALT as well as each of the five active mutant GALT enzymes, each was expressed as a fusion with an N-terminal hexahistidine (HIS6) tag from the high copy number yeast expression plasmid pJFy3, as described previously (12). Activity assays of crude yeast lysates prepared from cells expressing either the wild-type or mutant HIS6-fusion proteins demonstrated that the HIS6 tag resulted in no significant change in relative activity associated with any of these proteins (data not shown). These results are fully consistent with our previous work involving use of the HIS6 tag with both wild-type and naturally occurring mutant alleles of human GALT (12).

HIS6 fusions of wild-type and mutant human GALT enzymes were purified to near homogeneity by nickel-affinity precipitation, as described under "Experimental Procedures" and illustrated in Fig. 4. Kinetic studies were performed using each preparation to define specific activity and apparent  $K_m$  values associated with each enzyme, as illustrated in Fig. 5, A and B; the results are summarized in Fig. 5C. We have calculated specific activity as the maximal velocity observed divided by the mass of enzyme present.

**Specific Activity**—The P185A, P185G, and P185S mutant

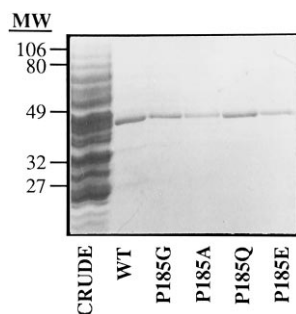


FIG. 4. Nickel-affinity purification of wild-type and mutant human GALT proteins overexpressed in yeast. Eighty  $\mu$ g of crude yeast lysate (from cells expressing P185Q-GALT) and 1.9  $\mu$ g of each of the indicated wild-type or mutant GALT proteins isolated as described under "Experimental Procedures" were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. Molecular weight markers are presented in thousands.

enzymes each exhibited a specific activity ranging from 5 to 8% of the corresponding wild-type value, whereas the P185Q and P185E mutant enzymes each demonstrated a specific activity that ranged from 1–2% of wild-type. This observation is not unexpected considering the position of the mutated proline: directly adjacent to the presumed active site nucleophile histidine 186. The kinetic parameters for mutated human GALT proteins must be interpreted, however, not only in comparison with values describing wild-type human GALT isolated from yeast (Fig. 5), but also in comparison with corresponding values that describe wild-type human GALT isolated from human cells. Indeed, these latter comparisons are fundamental to establishing the validity of the yeast expression system for detailed studies of the human enzyme.

Published values for the specific activity of human erythrocyte GALT range from 15  $\mu$ mol/min/mg (18) to 40  $\mu$ mol/min/mg (19); human placental GALT was reported to have a specific activity of 56  $\mu$ mol/min/mg (20). Presumably, this range of values reflects differences either in purity or partial inactivation of the human GALT enzyme during isolation. Wong *et al.* (6) noted from their work with *E. coli* GALT that the specific activities of different enzyme preparations varied significantly (e.g. from 56  $\mu$ mol/min/mg to 169  $\mu$ mol/min/mg), despite the apparent 90–95% electrophoretic homogeneity of all of their samples. These variations suggest that the reported activities likely reflect minimal rather than absolute values. This idea is supported by data reported here (Fig. 5) demonstrating a specific activity for wild-type human GALT of 122  $\mu$ mol/min/mg, more than two-fold greater than any previously published value for the human enzyme. It may be that our rapid, one-step isolation procedure simply avoided many of the complications resulting from the more traditional purification protocols used by other groups. It is unlikely that our increased specific activity is due to the presence of the HIS6 tag, considering that the tagged and native (untagged) human GALT proteins demonstrated indistinguishable levels of activity when assayed in crude lysates (12).

In defining the relative specific activities of the active mutant GALT proteins, we noted that some differences were apparent between values estimated from studies of these proteins in crude yeast lysates (Figs. 2 and 3) and values calculated from kinetic studies of the purified proteins (Fig. 5). Although we cannot fully explain these discrepancies, several possibilities deserve discussion. The first is that GALT activities were measured in the crude lysates and purified preparations using different methods (see "Experimental Procedures"). Because these two methods involved use of different amounts of substrates and even different pH values, the discordant activities

observed might reflect genuine differences in the behavior of the individual mutant enzymes under these different conditions. Alternatively, the abundance of some or all of the mutant proteins may have been either under- or overestimated relative to the wild-type due to differential antibody affinities. Finally, the different mutant proteins may have experienced differential degrees of inactivation during isolation.

**Kinetic Properties:  $K_m$** —Apparent  $K_m$  values of the mutant proteins for Gal-1-P varied from 16–74% of wild-type, and again clustering was evident; the P185A, P185G, and P185S-GALT enzymes all demonstrated values between 35–74% of wild-type, whereas the P185Q and P185E-GALT enzymes both demonstrated values between 16 and 26% of wild-type. Apparent  $K_m$  values for UDPG for all of the mutant GALT enzymes were only slightly different from those measured for the wild-type enzyme.

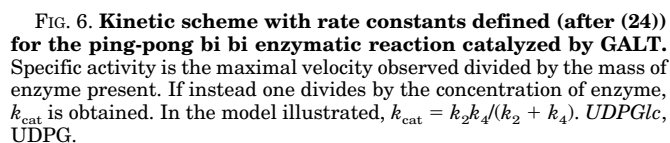
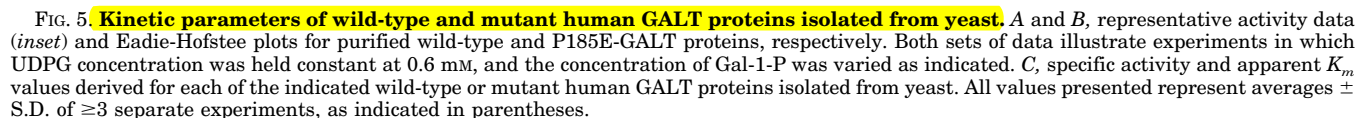
A range of apparent  $K_m$  values for the two natural substrates of human GALT, UDPG, and Gal-1-P have been reported (21–23). Because both substrates can be inhibitory at high concentrations, most authors have reported apparent  $K_m$  values for each substrate within the context of a given fixed concentration of the other substrate. Normal erythrocyte GALT assayed under conditions similar to those used here was reported to have an apparent  $K_m$  for UDPG of between 0.144 and 0.176 mM (21), a value very close to the 0.21 mM reported here. Similarly, normal erythrocyte GALT was reported to have an apparent  $K_m$  for Gal-1-P of between 0.389 and 0.392 mM, under conditions of fixed UDPG at 0.6 mM (21). The apparent  $K_m$  for Gal-1-P reported here is 0.57 mM. These values are also similar to those reported for the *E. coli* enzyme, 0.2 mM for UDPG and 0.3 mM for Gal-1-P. These similarities serve to strengthen the case for using yeast as a model system for the expression and study of human GALT.

To interpret the effects of mutation on apparent  $K_m$ , it is useful to consider the kinetic properties of the native enzyme. Evidence from the *E. coli* enzyme suggests a bi bi ping-pong mechanism with rate-limiting decay of the uridyl enzyme (*i.e.* the reaction of the uridyl enzyme with Gal-1-P in the direction assayed) (7). Values for  $K_m$  and  $K_i$  are similar for both substrates, implying rapid equilibrium binding and slower chemical or product release steps in each half reaction, such that  $k_{-1} \gg k_2$  and  $k_{-3} \gg k_4$ . Under these conditions: the apparent  $K_{m,A} = K_{i,A} \times [k_4/(k_2 + k_4)]$  and the apparent  $K_{m,B} = K_{i,B} \times [k_2/(k_2 + k_4)]$ , where  $k_2$  is the rate of product formation and release in the first half reaction ( $A = \text{UDPG}$ ) and  $k_4$  is the rate of product formation and release in the second half reaction ( $B = \text{Gal-1-P}$ ) (Fig. 6). Thus, the  $K_m$  is significantly changed only if the affinity for substrate ( $K_i$ ) is changed or if the catalytic rate constants ( $k_2$  and  $k_4$ ) are differentially affected (24).

The apparent  $K_m$  for UDPG was not significantly altered in most of the mutants. Nonetheless, it is interesting to note that for two mutants (P185G and P185Q), lowered apparent  $K_m$  for Gal-1-P was associated with a slightly increased apparent  $K_m$  for UDPG. This effect, although small, is most easily explained if one assumes that  $k_2$ , the catalytic rate constant for formation of the uridyl enzyme, is more severely compromised than is  $k_4$ , the catalytic rate constant for decay of the uridyl enzyme. This is supported by the  $k_{\text{cat}}/K_m$  results discussed below.

The apparent  $K_m$  for Gal-1-P for each of the active mutant GALT proteins ranged from 16 to 74% of wild-type (Fig. 5). This increase in apparent affinity is not completely unexpected and can be explained by either an increase in binding affinity or a differential effect on the catalytic rate constants. As described below, it appears that the majority of the effect is caused by differential effects on the catalytic rate constants.

**Kinetic Properties:  $k_{\text{cat}}/K_m$** —The kinetic constant  $k_{\text{cat}}/K_m$  is



studies reported here suggest that the  $k_{\text{cat}}/K_{m,A}$  for the wild-type enzyme is approximately  $4.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{\text{cat}}/K_{m,B}$  is approximately  $1.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ . Mutants showing  $k_{\text{cat}}/K_m$  values significantly less than this must be rate limited by the chemical steps or product release. All mutants show  $k_{\text{cat}}/K_m$  values for Gal-1-P of about  $1.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  ( $1.6 \pm 0.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ), whereas  $k_{\text{cat}}/K_m$  for UDPG were more variable, being 15- to 100-fold reduced. The ratio of the rate of uridyl enzyme formation ( $k_{\text{cat}}/K_{m,A}$ ) to the rate of its decay ( $k_{\text{cat}}/K_{m,B}$ ) are: wild type (2.7), P185S (2.2), P185A (1.8), P185G (0.6), P185E (0.4), and P185Q (0.4). As can be seen, the rate of uridyl enzyme formation becomes progressively more rate-limiting for the overall reaction in the order Pro, Ser, Ala, Gly, Glu, and Gln. This analysis predicts that the uridyl formation would be rate-limiting for the Gly, Glu, and Gln mutants, whereas the decay of the uridyl enzyme would be rate-limiting for the wild-type enzyme and Ser and Ala mutants. This is a testable hypothesis.

It is also obvious that the rate of uridylation is more generally affected than is the rate of its decay. This is consistent with the model which requires accurate positioning of the active site



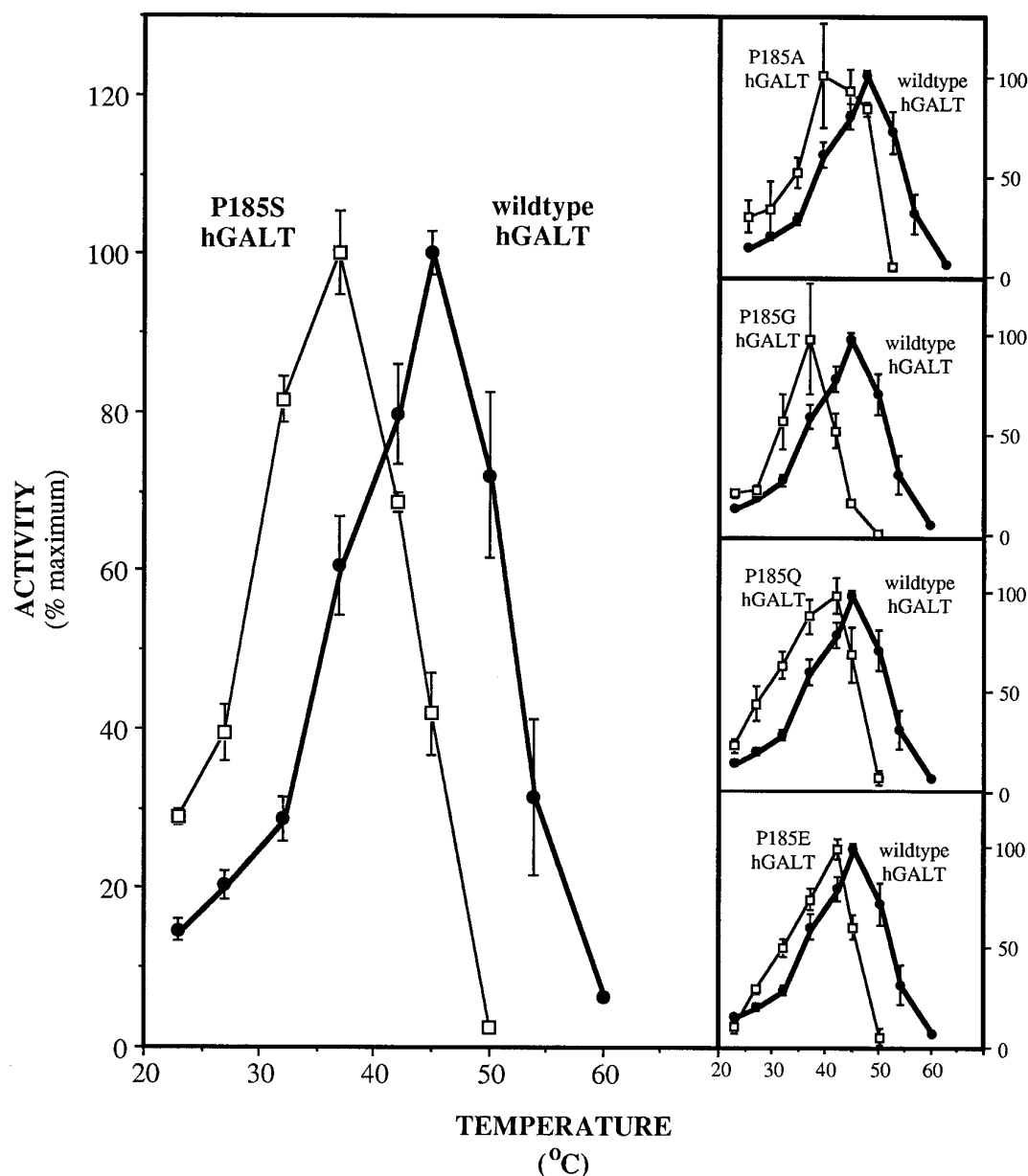


FIG. 7. Effects of temperature on activity of wild-type and mutant human GALT proteins isolated from yeast. Wild-type and mutant human GALT proteins were analyzed for activity at each of the temperatures indicated, as described under "Experimental Procedures." All values plotted represent averages (bars, S.D.) of four separate experiments.

histidine to attack UDPG. This sensitivity is reflected in the observation that  $k_{cat}/K_m$  for UDPG varies by nearly 100-fold upon substitution. After uridylation, the attack of Gal-1-P on the uridyl enzyme would be expected to be less affected since the specific binding of the uridyl group will help to position the intermediate for proper attack. Correspondingly,  $k_{cat}/K_m$  for UDPG is insensitive to the nature of the substituent.

**Impact of Temperature on Activity of Wild-type and Mutant Human GALT Enzymes**—The structural integrity of the active site regions of both the wild-type and partially active mutant human GALT enzymes were estimated by determining the effects of temperature on activity for each over the range of 20–60 °C. As illustrated in Fig. 7, wild-type human GALT showed a steep and steady rise in activity as the temperature was raised to 45 °C; activity dropped after that point with 72% residual activity at 50 °C and only ~6% residual activity at 60 °C. Parallel analyses of each partially active mutant enzyme produced activity profiles similar to that of the wild-type enzyme but shifted such that activity peaks were observed be-

tween 37–42 °C, with only between 1 and 7% activity remaining at 50 °C. As was observed with the kinetic values discussed above, the mutant enzymes appeared to cluster into two groups; the P185A, P185G, and P185S-GALT mutants all reached their optimum activities at about 37 °C, whereas the P185Q and P185E-GALT mutants both demonstrated optimum reaction temperatures around 42 °C (Fig. 7). This pattern is consistent with the idea that the smaller, less conformationally constrained residues (Ala, Gly, and Ser) offered the least resistance to thermal motion and denaturation of the enzyme. The relatively greater thermal stability associated with the Gln and Glu substitutions may reflect specific side chain interactions between the substituted residue and Thr-138 (homologous to *E. coli* position Thr-118) which stabilize the proteins. This is also consistent with the abundance data that shows relatively higher levels of Gln and Glu mutants than of the others.

**Computer Modeling of Mutations within the Active Site Region of Bacterial GALT**—To explain the effects of these muta-



tions on the structure and activity of the human enzyme, we have examined the corresponding area of the *E. coli* enzyme. The sequences of these enzymes are approximately 55% identical and have very similar physical and kinetic properties. The coordinates for the *E. coli* enzyme<sup>2</sup> were examined. Twenty-six residues were identified as being within 8 Å of the  $\alpha$  carbon of Pro-185 (amino acids 45, 55, 69, 111–122, 127, 130, 153–155, 164–166, and 275–277 of the *E. coli* enzyme). Of these, 16 are identical in the human sequence, and most are conservative substitutions; of those that are different, only Leu-122 of the *E. coli* sequence (Met in human) projects toward the side chain of Pro-165. These residues form a sphere around the active site HPH sequence with a pore extending from the proline side chain to the substrate binding cleft. Larger side chains could not be accommodated in the pocket surrounding proline and would have to extend into the substrate binding pocket, in some cases interfering with Thr-118 and Lys-154. Thus, Phe, His, Lys, Leu, Met, Arg, Trp, and Tyr cannot be accommodated in the pocket without significant rearrangements.

The HPH sequence contains a *cis*-proline with  $\phi/\psi$  angles similar to those observed in the collagen triple helix or a type II turn ( $\phi = -65.3^\circ$ ,  $\psi = 125.8^\circ$ ). Amino acids with branching at the  $\beta$  carbon would not be expected to adopt this conformation easily because of steric interference between the peptide carbonyl and the  $\beta$  substituent (25). Thus, one would not expect Ile, Thr, or Val to be able to adopt the correct conformation to maintain the positioning of the active site histidine.

Of the remaining eight possible substitutions, five show measurable activity, consistent with their steric, conformational, and polar characteristics. Cysteine might be expected to be similar to serine at this position, and indeed there is no obvious reason why this residue does not substitute. It may be that this thiol is easily oxidized, thereby increasing its charge and steric bulk. It is also possible that the larger size and softer electronic properties of S as compared to O allow it to ligand with the nearby zinc and interfere with the proper positioning of catalytic residues. Finally, it is curious that Asp and Asn do not show measurable activity, whereas the larger Glu and Gln are partially active. Glu and Gln can be accommodated in the proline pocket with the carbonyl functions forming close contacts to the backbone and sidechain of Thr-118 (Thr-138 in the human enzyme). Asp and Asn, however, do not make such good contacts but rather can form a strong H-bond with the  $\epsilon$ -amino group of Lys-154. This requires a rotation of  $120^\circ$  about the  $\gamma\delta$  bond of the lysine backbone and results in the movement of the amino group about 6 Å away from its position in the native structure. This residue has been implicated in binding of the phosphate moiety of the substrates, and this motion away from the phosphate binding site may well be detrimental to catalytic activity.

**Summary and Significance**—In summary, position 185 of human GALT is important for the rate of reaction of the enzyme with the first substrate, UDPG, but less so for the reaction of the uridyl enzyme with the second substrate, Gal-1-P. Substituting amino acids can be clustered into five groups: Pro (wild type); Ser and Ala; Gly; Glu and Gln; and all others. P185S and P185A are approximately 15-fold slower than wild-type at forming uridyl enzyme, have a temperature optimum of about  $37^\circ\text{C}$ , and approximately 30–50% wild-type abundance. The rate of uridylation of P185G is inhibited about 30-fold, with a temperature optimum and abundance similar to those seen

for P185S and P185A. P185E and P185Q are about 80-fold slower at uridyl enzyme formation, exhibit temperature optima of about  $42^\circ\text{C}$ , and accumulate to the same level as wild-type enzyme. Finally, no other mutants show detectable enzymatic activity, although the abundance of these proteins varies from ~10 to >100% of control. These results are fully consistent with the accepted catalytic model for this enzyme and are consistent with the published x-ray structure of the *E. coli* enzyme.

The results presented here are significant not only because they offer insight into the functional requirements of a key residue within the active site region of human GALT, but also because they serve as a functional test of the applicability of predictions derived from the crystal structure of the bacterial enzyme to the human sequence. Furthermore, these results demonstrate that absolute conservation of sequence across species and even kingdoms need not imply absolute functional intolerance to change. Finally, these results may serve as a model for future studies of structure and function involving human GALT or other proteins conserved between species and associated with human disease.

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<sup>2</sup> I. Rayment, privileged communication.