The Biochemical Role of Glutamine 188 in Human Galactose-1-phosphate Uridyltransferase*

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The substitution of arginine for glutamine at amino acid 188 (Q188R) ablates the function of human galactose-1-phosphate uridyltransferase (GALT) and is the most common mutation causing galactosemia in the white population. GALT catalyzes two consecutive reactions. The first reaction binds UDP-glucose (UDP-Glu), displaces glucose-1-phosphate (glu-1-P), and forms the UMP-GALT intermediate. In the second reaction, galactose-1-phosphate (gal-1-P) is bound, UDP-galactose (UDP-Gal) is released, and the free enzyme is recycled. In this study, we modeled glutamine, asparagine, and a common mutation arginine at amino acid 188 on the three-dimensional model of the *Escherichia coli* GALT-UMP protein crystal. We found that the amide group of the glutamine side chain could provide two hydrogen bonds to the phosphoryl oxygens of UMP with lengths of 2.52 and 2.82 Å. Arginine and asparagine could provide only one hydrogen bond of 2.52 and 3.02 Å, respectively.

To test this model, we purified recombinant human Gln<sup>188</sup>-Arg<sup>188</sup>-Asn<sup>188</sup>-GALT and analyzed the first reaction in the absence of gal-1-P by quantitating glu-1-P released using enzyme-linked methods. Gln<sup>188</sup>-GALT displaced 80 ± 7.0 nmol glu-1-P/mg GALT/min in the first reaction. By contrast, both Arg<sup>188</sup>-GALT and Asn<sup>188</sup>-GALT released more glu-1-P (170 ± 8.0 and 129 ± 28.4 nmol/mg GALT/min, respectively). The overall, double displacement reaction was quantitated in the presence of gal-1-P. Gln<sup>188</sup>-GALT produced 80,030 ± 5,910 nmol glu-1-P/mg GALT/min, whereas the mutant Arg<sup>188</sup>-GALT released only 600 ± 71.2 and 2960 ± 283.6 nmole glu-1-P/mg GALT/min, respectively. We conclude from these data that glutamine at position 188 stabilizes the UMP-GALT intermediate through hydrogen bonding and enables the double displacement of both glu-1-P and UDP-Gal. The substitution of arginine or asparagine at position 188 reduces hydrogen bonding and destabilizes UMP-GALT. The unstable UMP-GALT allows single displacement of glu-1-P with release of free GALT but impairs the subsequent binding of gal-1-P and displacement of UDP-Gal.

The enzyme galactose-1-phosphate uridyltransferase (GALT)<sup>†</sup> (EC 2.7.7.12) catalyzes the conversion of UDP-glucose (UDP-Glu) and galactose-1-phosphate (gal-1-P) to form glucose-1-phosphate (glu-1-P) and UDP-galactose (UDP-Gal) in the evolutionarily conserved Leloir pathway of galactose metabolism (1). The enzymology of GALT has been intensively studied using GALT purified from *Escherichia coli* (2, 3). Both *E. coli* and human GALT enzymes catalyze the conversion of UDP-Glu and gal-1-P to UDP-Gal and glu-1-P via a double displacement mechanism (2–4) (Fig. 1). Under normal physiological conditions, UDP-Glu binds to GALT to form a GALT-UDP-Glu intermediate. Glu-1-P is subsequently released, whereas the GALT enzyme remains bound to UMP. This GALT-UMP intermediate has been isolated and crystallized (5–8). Gal-1-P then reacts with the GALT-UMP complex to form UDP-Gal, freeing the GALT enzyme for continued catalysis. GALT does not use nucleoside di- or triphosphates as nucleotide donor substrates, making it unique among nucleotidyltransferases that utilize phosphates as acceptor groups (9). The GALT proteins are evolutionarily conserved; with an overall amino acid identity between the human and *E. coli* GALT proteins of 46%. There is near 100% conservation of the amino acid sequence in the catalytic domain studied here (10, 11).

The human GALT gene encodes 379 amino acids, contains 11 exons, and spans 4 kilobase pairs of genomic DNA (10). Exon VI is the most conserved domain, is composed of 19 amino acids, and is involved in these catalytic reactions (10). To date, 131 sequence changes have been identified in GALT genes from patients with galactosemia (12–15). The most common mutation is a substitution of arginine for glutamine at amino acid 188 (Q188R). This mutation is caused by an A to G transition, changing CAG to CGG in codon 188 of the highly conserved 11). A few human GALT mutations, including the Q188R mutation, have been characterized by various in vitro expression systems (12, 19–21, 36). These studies described some kinetic parameters (apparent <i>K<sub>M</sub></i> or <i>V<sub>max</sub></i>) of purified mutant proteins but did not explore the normal and mutant mechanisms involved in position 188 at the biochemical level. In this study, we

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The abbreviations used are: GALT, galactose-1-phosphate uridyltransferase; UDP-Glu, UDP-glucose; gal-1-P, galactose-1-phosphate; glu-1-P, glucose-1-phosphate; UDP-Gal, UDP-galactose; PAGE, polyacrylamide gel electrophoresis.
used purified, recombinant human GALT proteins, computer modeling, biochemical assays, and kinetic analyses to clarify the biochemical and molecular mechanisms by which glutamine 188 contributes to the overall reaction and how the Arg<sup>188</sup> and Asn<sup>188</sup> substitutions alter human GALT function.

**EXPERIMENTAL PROCEDURES**

**Computer Modeling of GALT Mutations**—The computer software SYBYL (Tripos Associates) was used to visualize and model human GALT at the active site of uridylated-GALT crystal of <i>E. coli</i> (6–8). At the beginning of this study, the Protein Data Base file of the <i>E. coli</i> GALT is deposited at the Brookhaven's National Laboratory was "on hold." We were graciously given the Protein Data Base file by the authors, Drs. J. Wedekind, Perry A. Frey, and Ivan Raymont from the Institute for Enzyme Research, University of Wisconsin. All computer modelings were performed at the Biomolecular Core Facility (Department of Biochemistry, Emory University) with consultation from Dr. Kim Gernert.

**Components of the Bacterial Expression System for GALT**—The bacterial host strain <i>E. coli</i> BL21(DE3) was used to express recombinant GALT from the recombinant plasmid pACWK containing the GALT gene, and the plasmid pACWK containing the GALT gene was transformed into the bacterial host strain <i>E. coli</i> BL21(DE3). The transformed bacteria were then harvested by centrifugation at 6000 × g for 10 min.

**Purification of the Recombinant His<sub>6</sub>-tagged Human GALT Proteins**—The bacterial expression vector, pTrcHisA cotranslates a hexa-amer of histidines (His<sub>6</sub>) in frame at the amino terminus of the cloned human GALT cDNA. A nickel-charged resin affinity column was used to purify recombinant GALT from bacterial cell lysates using modification of the manufacturer's method (Qiagen) (24, 25). Lysates of bacterial pellets were incubated with nickel-charged resin at 4 °C for 45 min with gentle shaking. Non-specific binding of non-His<sub>6</sub>-tagged proteins were eluted with wash buffer containing 20 mM imidazole. The His<sub>6</sub>-tagged, Gln<sup>188</sup>-, Arg<sup>188</sup>-, or Asn<sup>188</sup>-GALT proteins were then eluted with 100 mM imidazole. Eluates were concentrated and desalted using Centricon-300 and resuspended in a small volume of glycine buffer (100 mM, pH 8.7). Protein concentrations were determined by Bio-Rad assay with bovine serum albumin standards.

**Western Blot Analysis of Purified His<sub>6</sub>-tagged GALT Proteins**—GALT proteins were identified by a rabbit anti-human GALT polyclonal antibody using a Western protocol described previously (26).

**Assay of GALT Enzyme Activity**—Assays for the overall, double displacement reaction were carried out at 37 °C in 1 ml of glycine buffer (100 mM, pH 8.7) containing 0.6 mM UDP-Glu, 5 mM Mg<sub>2+</sub>, 5 mM dithiothreitol, 0.8 mM NADP, 1.2 mM gal-1-P, 5 μM glucose-α-1, 6-phosphate, phosphoglucomutase (0.5 IU/ml), glucose-6-phosphate dehydrogenase (0.5 IU/ml) (27). The formation of NADPH was quantitated by absorbance change at 340 nm. The quantitative relationship between increase in NADPH production and glucose-1-phosphate release was quantitated using the Beer-Lambert equation: Absorbance

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**TABLE I**

**List of oligonucleotide primers used in this study**

| Primer name | Primer sequence |
|-------------|-----------------|
| Q188RP1     | 5′-CCAACCCACTGCCGGGTATG-3′ |
| Q188RP2     | 5′-CAATTGGCGAGTGTTGCTATG-3′ |
| Q188KP1     | 5′-CCCCACCTGAAGAGTTG-3′ |
| Q188KP2     | 5′-CACAACCTGAGTGTTGCTATG-3′ |
| Q188NP1     | 5′-CCCTTACCTGAAGAGTTG-3′ |
| Q188NP2     | 5′-CAATTGGCGAGTGTTGCTATG-3′ |
| EXON3FOR    | 5′-TCTGGGATATGCTCTTTGCG-3′ |
| EXON3REV    | 5′-TCTGGGATATGCTCTTTGCG-3′ |
| EXON5FOR    | 5′-AAGGATCTCAACTTTCCGAG-3′ |
| EXON5REV    | 5′-AAGGATCTCAACTTTCCGAG-3′ |
| EXON6FOR    | 5′-CCATACCGAAGCTCAGTGGCGAG-3′ |
| EXON6REV    | 5′-AAAGAATCCCGAAGCTCAGTGGCGAG-3′ |
| EXON9FOR    | 5′-GACAAATGGCTAGAACAATCTTCT-3′ |
| EXON9REV    | 5′-GACAAATGGCTAGAACAATCTTCT-3′ |
| EXON10FOR   | 5′-GCGTCCCCCAACAGGGAGG-3′ |
| GALTPRO3    | 5′-AAGAAGATGCTCAGTGGCGAG-3′ |
| GALTPRO5    | 5′-AAATATGCTCAGTGGCGAG-3′ |

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**Fig. 1.** Double displacement reactions of GALT. Both <i>E. coli</i> and human GALT enzymes catalyze the conversion of UDP-Glu to a GALT-UDP-Glu intermediate via a double displacement mechanism (2–4). Under normal physiological conditions, GALT binds to UDP-Glu to form a GALT-UDP-Glu intermediate. glu-1-P is subsequently released, whereas the enzyme remains bound to UDP. gal-1-P then reacts with the enzyme-UMP intermediate to form UDP-Gal, freeing the GALT enzyme for continued catalysis.

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**Molecular Biochemistry of Galactosemia**
**RESULTS**

**Computer Modeling of Human Gln**<sup>188</sup>, Arg<sup>188</sup>, and Asn<sup>188</sup>-GALT—To develop an hypothesis for the structural role of the conserved amino acid Gln<sup>188</sup> in human GALT, we modeled *E. coli* GALT-UMP coordinates at amino acid Gln<sup>168</sup> (Fig. 2). There are 20 more amino acids at the amino terminus of human GALT protein as compared with the *E. coli* GALT (10, 11). Thus, human Gln<sup>188</sup> is equivalent to amino acid Gln<sup>168</sup> of *E. coli* GALT (Fig. 2). We found that the amide group of the glutamine side chain provided two hydrogen bonds to the phosphoryl oxygens of UMP with lengths of 2.52 and 2.82 Å (Fig. 3). When arginine was substituted for glutamine at position 188, asparagine stabilizes and properly orients the GALT-UMP mainly by replacing one hydrogen bond of 3.02 Å was formed (Fig. 3). So if glutamine 188 stabilizes the GALT-UMP intermediate, we would expect that asparagine or arginine substitution for glutamine would interfere with gal-1-P binding and the subsequent release of UDP-Gal in the second displacement reaction but not the release of glucose-1-phosphate in the first reaction. To further investigate these hypotheses, we dissected and analyzed the two displacement reactions in vitro using purified Gln<sup>188</sup>-Arg<sup>188</sup>, and Asn<sup>188</sup>-GALT proteins produced in the transformed bacteria. Purified GALT enzymes, instead of whole cell extracts from the bacterial expression system, were necessary because bacterial extracts contained abundant gal-1-P (data not shown). To isolate and examine the first displacement reaction (see Fig. 5A), we must deplete the reaction of gal-1-P. If gal-1-P is present in the reaction, the products of the first displacement reaction will instantaneously react with gal-1-P and complete the second displacement reaction (Fig. 1).

**Alignment of the predicted *E. coli* & human GALT protein sequences**

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**Mathematical Analyses of Rate Constants for the Single Displacement Reaction**—The microcomputer program, SSREG.BAS (steady-state rate equation generator) (28) composed and generously provided by Dr. Robert Gunh (Physiology Department, Emory University) was used to analyze the rate constants for the single displacement reaction. This program is based on the algorithm of Indge and Childs (29), which is a modification of King and Altman’s graphical method for derivation of rate equations (30).

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**Alignment of the predicted *E. coli* & human GALT protein sequences**

**Fig. 2.** Amino acid alignment of the predicted protein sequences of *E. coli* GALT (11) and human GALT (10). The alignment was performed by the modification of the method of Needleman and Wunsch (38). There is a 54% homology and a 46% identity between the two sequences. Two dots represent identical amino acids; one dot represents conservative substitutions.  

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**Fig. 3.** (A) Hydrogen bond of 2.52 Å from the amide side chain of arginine 188 of the human GALT protein, the model identified only one hydrogen bond of 3.02 Å was formed (Fig. 3A). When arginine was substituted for glutamine at position 188, the model identified only one hydrogen bond of 3.02 Å was formed (Fig. 3A). So if glutamine 188 stabilizes the GALT-UMP intermediate, we would expect that asparagine or arginine substitution for glutamine would interfere with gal-1-P binding and the subsequent release of UDP-Gal in the second displacement reaction but not the release of glucose-1-phosphate in the first reaction. To further investigate these hypotheses, we dissected and analyzed the two displacement reactions in vitro using purified Gln<sup>188</sup>-Arg<sup>188</sup>, and Asn<sup>188</sup>-GALT proteins produced in the transformed bacteria. Purified GALT enzymes, instead of whole cell extracts from the bacterial expression system, were necessary because bacterial extracts contained abundant gal-1-P (data not shown). To isolate and examine the first displacement reaction (see Fig. 5A), we must deplete the reaction of gal-1-P. If gal-1-P is present in the reaction, the products of the first displacement reaction will instantaneously react with gal-1-P and complete the second displacement reaction (Fig. 1).
FIG. 3. Computer modeling of human GALT mutations in the E. coli GALT-UMP crystal. Computer software SYBYL was used to model human Q188R mutation in the highly conserved E. coli UMP-GALT protein crystal. The UMP moiety in the uridylated GALT was complexed to histidine 166, which is the equivalent of histidine 186 of the human GALT (see text). In A, the phosphorus of the UMP moiety was colored orange-yellow, and the oxygens were red. The side chain of glutamine 168, which was colored in orange-yellow, formed two hydrogen bonds of lengths 2.52 and 2.82 Å with the phosphoryl oxygens of the intermediate. In B, the amino acid glutamine was replaced with arginine and its side chain was colored red. The arginine side chain could only form one hydrogen bond of length 2.52 Å with the phosphoryl oxygen atoms of the intermediate. In C, the amino acid glutamine was replaced with asparagine, and its side chain was colored blue.
from bacteria transformed with control plasmid pTrcHisA containing no GALT cDNA inserts (the negative controls).

To confirm the identity of the purified proteins, we blotted an identical SDS-PAGE gel to a nitrocellulose membrane and performed Western blot analysis using an anti-human GALT polyclonal antibody (26) (Fig. 4B). Two protein species that had molecular masses of the two protein bands on SDS-PAGE reacted with our anti-human GALT polyclonal antibody (26). Because the anti-human GALT polyclonal antibody was raised in rabbits injected with freshly prepared GALT protein isolated from human red blood cells, our Western blot results suggest that recombinant human Gln188-GALT protein shares at least two similar epitopes with native human GALT from human erythrocytes (26). In subsequent kinetic analyses, we assume that both dimeric and monomeric forms of GALT are active (32, 33).

The Specific Activity of the Purified GALT Proteins—Purified GALT proteins were assayed for their capacity to catalyze the overall double displacement reactions in the presence of 600 μM UDP-Glu and 1.2 mM gal-1-P. We found that the specific activity of the wild type Gln188-GALT was 80,300 ± 5,910 nmol glucose-1-phosphate/GALT/min (n = 3) (Table III). By contrast, the specific activities of mutant Arg188- and Asn188-GALT were 600 ± 71.2 (n = 3) and 2960 ± 283.6 (n = 3) nmol glucose-1-P/GALT/min, respectively (Table III). Apparently arginine substitution had a more deleterious effect than asparagine substitution on bi-bi molecular GALT reaction.

The First Displacement Reaction—We devised a strategy to separate the first displacement reaction from the overall double displacement reactions through omitting gal-1-P in the reaction. By depleting the reactions of gal-1-P, we initially predicted that each molecule of GALT enzyme would only catalyze the release of the gal-1-P and form the GALT-UMP intermediate. If no gal-1-P was available for the second displacement reaction, the overall reaction would stall without recycling the GALT enzyme for another round (Fig. 5A). If this prediction were true, only one molecule of glucose-1-phosphate would be displaced by each molecule of GALT. Because glucose-1-phosphate was removed from the reaction by our enzyme linked assay, quantitation of glucose-1-phosphate released and NADPH subsequently produced should be stoichiometric. We would then expect a ΔAbs540 = 0.0 to result from a change of NADPH concentration = 1.0/6220 μM = 0.16 μM in the reaction mixture, as dictated by the Beer-Lambert equation. Because this would predict only 1 mol of NADPH produced from each mol of glucose-1-phosphate released and because each mol of GALT could only displace 1 mol of glucose-1-phosphate, we would have to add 1.6 × 10^4 × 47,000 (molecular mass of His6-tagged GALT) = 7.56 mg of GALT enzyme to produce a change in absorbance of 1.0. However, if the GALT-UMP complex could dissociate to free UMP and GALT molecules and the free GALT enzyme could recycle only the first reaction with another UDP-Glu molecule, the single displacement of glucose-1-phosphate could continue (Fig. 5B). It would be possible under this second hypothesis to achieve ΔAbs540 = 1.0 with less than 7.56 mg of GALT enzyme.

From the results exemplified in Fig. 6, only microgram quantities of Gln188-GALT were required to quantitate the single displacement reaction. Each data point in Fig. 6 represents the mean value of three independent experiments where only 8 μg of purified Gln188-GALT protein was used to assay the first displacement reaction. There was a steady, significant release of glucose-1-phosphate over 3 h as compared with the “negative control” (Fig. 6). The negative control was column eluate of the bacterial extracts prepared from bacteria, which were transformed with the expression vector without any GALT cDNA insert. According to the Beer-Lambert equation, a ΔAbs540 of 0.697 at 175 min would equal to a ΔC = 11.2 × 10^−5 M. For ΔC = 11.2 × 10^−5 M; glucose-1-phosphate produced in the reaction = 11.2 × 10^−8 mol = 112 nmol. The 8 μg of purified GALT protein added to the reaction was equivalent to only 170 pmol of GALT monomeric subunits (8 × 10^−10/47,000 = 1.70 × 10^−10 mol = 170 pmol). Thus, some of the GALT-UMP intermediate must dissociate to free UMP and GALT and recycle a single displacement reaction releasing glucose-1-phosphate in the absence of gal-1-P. The rate of release of glucose-1-phosphate in the first displacement reaction was directly proportional to the amount of the wild type and mutant enzymes up

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**TABLE II**

**Purification scheme of GALT from 3 liters of bacterial cell culture**

| Step | Total protein | Total GALT activity | Specific activity | Increase in specific activity | Recovery |
|------|---------------|---------------------|-------------------|-------------------------------|---------|
| μg   | nmol glucose-1-phosphate released/min | nmol glucose-1-phosphate released/min/μg GALT | fold |
| Bacterial cell lysate | 10,338.8 | 12,406 | 1.2 | 80.38 | 67 |
| Nickel-charged resin | 7.0 | 566 | 1.0/6220 | 100 |

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**FIG. 4. Purification of the recombinant human His6-tagged GALT proteins. A, the left three lanes next to the molecular mass markers in the Coomassie blue-stained SDS-PAGE gel were loaded with the eluates eluted by buffers containing increasing concentrations of imidazole (100, 150, and 250 mM). Two prominent protein bands representing the recombinant His6-tagged Gln188-GALT proteins were seen in these three lanes. Two proteins bands seen on the left three lanes were not seen on the right three lanes, which were loaded with eluates prepared by the same purification procedures, except the bacterial extracts were prepared from a bacteria strain transformed with the control vector (no GALT cDNA insert). B, a SDS-PAGE gel identical to the one shown in A was blotted to a nitrocellulose membrane, and Western blot analysis was performed on the nytran blot using a polyclonal anti-human GALT polyclonal antibody (26).**

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The single displacement model. A, in the absence of gal-1-P, the conventional viewpoint for the first displacement reaction would have predicted that only 1 mol of glucose-1-phosphate could be formed from each mole of GALT. The GALT enzyme will stall at the point of GALT-UMP intermediate. B, however, if free GALT could be released from the GALT-UMP intermediate, it could catalyze further rounds of the first displacement reaction. As a result, more than 1 mol of glucose-1-phosphate would be generated from each mole of GALT. $k_2$ and $k_3$ denote rate constants of the forward and reverse reactions, respectively. Glucose-1-phosphate released was quantitated by an enzymatic linked assay by which 1 mol of NADPH was produced by each mole of glucose-1-phosphate release.

**Table III**

| Amino acid substitution | Bacterial extracts | Purified GALT proteins | Overall reaction |
|-------------------------|--------------------|------------------------|-----------------|
|                         | Overall reaction   | First displacement     | Overall reaction |
|                         | $\text{nmol glu-1-P/mg cell}$ | $\text{nmol glu-1-P/mg GALT}$ | $\text{nmol glu-1-P/mg GALT/min}$ |
| Wild type               | 1.338 + 0.032 (n = 6) | 80 + 7 (n = 3) | 80,030 ± 5,910 (n = 3) |
| Q188R                   | 3.2 ± 1.18 (n = 6)    | 170 ± 8 (n = 3) | 600 ± 71.2 (n = 3)  |
| Q188N                   | 18.75 ± 6.6 (n = 6)   | 129 ± 28.4 (n = 3) | 2,960 ± 283.6 (n = 3) |

**FIG. 5. The single displacement model.**

**DISCUSSION**

GALT is of considerable interest to fundamental biochemistry and provides a unique insight into a bi-bi molecular, double displacement reaction (2, 3). Earlier site-directed mutagenesis

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**Mathematical Analysis of Rate Constants for the Single Displacement Reaction**—We analyzed the rate constants of the first single displacement reaction for both Gln$^{188}$ and Arg$^{188}$ GALT in the absence of gal-1-P (Fig. 5B). We used a microcomputer program (28) based on the algorithm of Indge and Childs (29) and King and Altman's original graphical method for derivation of rate equations (39). From this analysis, the overall rate of product formation at steady state is as follows.

$$v = \frac{[\text{ENZ}][\text{UDP-Glu}]}{k_1 (k_2 + k_3 + [\text{UDP-Glu}](k_2 + k_3) + [\text{UMP}](k_1 + k_2)(k_3)}$$

(Eq. 1)

But free GALT will not bind UMP to form UMP-GALT (34, 35); therefore, $k_{-2} = 0$. Hence Equation 2 was proposed.

$$v = \frac{[\text{ENZ}][\text{UDP-Glu}]}{k_2 (k_3 + [\text{UDP-Glu}](k_2 + k_3) + [\text{UMP}](k_1 + k_2)(k_3)}$$

(Eq. 1)

By converting the above equation to a Michaelis-Menten equation form, we developed Equation 3.

$$V_{max} = \frac{[\text{ENZ}]}{k_2} (k_3/k_2)$$

(Eq. 2)

Equation 3 does not contain the term $k_{-2}$, which is zero because all glucose-1-phosphate released was converted to glucose-6-phosphate by phosphoglucomutase.

Although slower than the overall reaction, the single displacement reaction was saturable when catalyzed by either Gln$^{188}$ or Arg$^{188}$ GALT (Fig. 7). The $V_{max}$ for Arg$^{188}$ GALT was greater than that of Gln$^{188}$ GALT (294.1 nmol glu-1-P/mg GALT/min versus 149.3 nmol glu-1-P/mg GALT/min). From Equation 3, one sees that one criterion for the $V_{max}$ of Arg$^{188}$ GALT to be greater than that of Gln$^{188}$ GALT is that the rate constant $k_5$ of the conversion of the Arg$^{188}$ GALT-UMP intermediate to free Arg$^{188}$ GALT and free UMP would be greater than the $k_2$ of the conversion of the Gln$^{188}$ GALT-UMP intermediate to free Gln$^{188}$ GALT and free UMP. Thus by formal mathematical deduction, it takes longer for the Gln$^{188}$ GALT-UMP intermediate to produce free GALT than the Arg$^{188}$ GALT-UMP intermediate, and thus a lower capacity to recycle when the first displacement reaction is isolated.

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**Table III**

Relative specific activities of Gln$^{188}$, Arg$^{188}$, and Asn$^{188}$-GALT in its first displacement and overall reactions.
experiments concluded that amino acids His\textsuperscript{164} and His\textsuperscript{166} of the \textit{E. coli} GALT were crucial for initiating the first displacement reaction, but little is known regarding the requirements for the second displacement reaction (17). Meanwhile, a prevalent missense mutation, Q188R, found in the GALT genes of many galactosemic patients suggested an important catalytic role played by the highly conserved amino acid glutamine at position 188, which is equivalent to glutamine 168 in \textit{E. coli} GALT (10, 16, 18, 19).

Our initial examination of the structural role of the amino acid glutamine 168 (equivalent to glutamine 188 in human GALT) in the crystal structure of the \textit{E. coli} GALT-UMP (5–8) showed us that Gln\textsuperscript{188} in the human GALT could stabilize the GALT-UMP intermediate through two hydrogen bonds formed between the amide side chain of Gln\textsuperscript{188} and the phosphoryl oxygen of the UMP moiety (Fig. 3A). Because only one hydrogen bond could be formed in the cases of arginine or asparagine substitutions (Fig. 3, B and C), we subsequently proposed that the stabilization of the GALT-UMP intermediate is essential for the subsequent release of UDP-Gal in the second displacement reaction. This hypothesis was substantiated when we dissected and analyzed the two displacement reactions \textit{in vitro} using purified recombinant Gln\textsuperscript{188}-, Arg\textsuperscript{188}-, and Asn\textsuperscript{188}-GALT enzymes.

Our analysis of the first displacement reaction also showed that the GALT-UMP intermediate formed could undergo hydrolysis to form free GALT and UMP and recycle UDP-Glu to glucose-1-phosphate (Fig. 5B). Microgram quantities of GALT were capable of catalyzing this first displacement reaction, whereas milligram quantities would have been required if no
recycling occurred. These data supported the earlier studies of the E. coli GALT defining nucleophilic His\textsuperscript{166} Ne2 attack by E. coli GALT on the GALT-bound UDP-Glu substrate and consequent displacement of glucose-1-phosphate to form the GALT-UMP intermediate (5–8). A “covalent” bond was modeled between the UMP moiety and the amino acid His\textsuperscript{166} of the E. coli GALT in the uridy-GALT intermediate (5–8). These investigators emphasized that there was a substantial positive charge build-up on the His\textsuperscript{166} imidazolium ring (5–8), suggesting that uncatalyzed hydrolysis of imidazolium-UMP between pH 5 and pH 10 could occur, and such uncatalyzed hydrolysis was further enhanced by the positive charge build-up in the imidazolium group (37). Because we excluded gal-1-P, it was no longer available to this charged imidazolium group. Furthermore, glucose-1-phosphate was removed by the enzyme-linked reactions. Thus, the covalent bond formed by the zwitterionic UMP-imidazolium-1-phosphate was removed by the enzyme-linked reactions. Available to this charged imidazolium group. Furthermore, glutamine group (37). Because we excluded gal-1-P, it was no longer available to this charged imidazolium group. 

By contrast, when the overall reaction was analyzed through kinetic studies of the E. coli GALT crystal. We are also indebted to Drs. Robert Gunn and Kim Gernert (Emory University) for consultations on rate constants analyzes and computer modeling.

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