Transient Kinetics of Formation and Reaction of the Uridylyl-Enzyme Form of Galactose-1-P Uridylyltransferase and Its Q168R-Variant: Insight into the Molecular Basis of Galactosemia[†]

Sandaruwan Geeganage and Perry A. Frey*

Institute for Enzyme Research, The Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin−Madison, 1710 University Avenue, Madison, Wisconsin 53705

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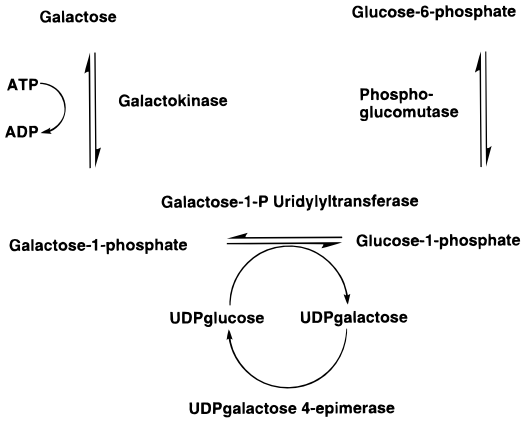
ABSTRACT: Galactose-1-phosphate uridylyltransferase catalyzes the reaction of UDP-glucose with galactose 1-phosphate (Gal-1-P) to form UDP-galactose and glucose 1-phosphate (Glc-1-P) through a double displacement mechanism, with the intermediate formation of a covalent uridylyl-enzyme (UMP enzyme). Gln 168 in *E. coli* uridylyltransferase engages in hydrogen bonding with the phosphoryl oxygens of the UMP moiety, which is bonded to His 166 in the intermediate [Wedekind, J. E., Frey, P. A., and Rayment, I. (1996) *Biochemistry 35*, 11560−11569]. In humans, the point variant Q188R accounts for 60% of galactosemia cases. The corresponding *E. coli* variant Q168R has been overexpressed and purified. In preparation for kinetic correlation of Q168R and wild-type uridylyltransferases, we tested the kinetic competence of the wild-type UMP-enzyme. At 4 °C, the first-order rate constant for uridylylation by UDP-glucose is 281 ± 18 s^{-1}, and for deuridylylation it is 226 ± 10 s^{-1} with Glc-1-P and 166 ± 10 s^{-1} with Gal-1-P. Inasmuch as the overall turnover number at 4 °C is 62 s^{-1}, the covalent intermediate is kinetically competent. The variant Q168R is uridylylated by UDP-glucose to the extent of about 65% of the potential active sites. Uridylylation reactions of Q168R with UDP-glucose proceed with maximum first-order rate constants of 2.2 × 10^{-4} s^{-1} and 4.2 × 10^{-4} s^{-1} at 4 and 27 °C, respectively. In experiments with uridylyl-Q168R and glucose-1-P, the mutant enzyme undergoes deuridylylation with maximum first-order rate constants of 4.8 × 10^{-4} s^{-1} and 1.68 × 10^{-3} s^{-1} at 4 and 27 °C, respectively. The value of K_m for uridylylation of Q168R is slightly higher than for the wild-type enzyme, and for deuridylylation it is similar to the wild-type value. The wild-type enzyme undergoes uridylylation and deuridylyation about 10^6 times faster than Q168R. The wild-type activity in the overall reaction is 1.8 × 10^6 times that of Q168R. The wild-type enzyme contains 1.9 mol of Zn+Fe per mole of subunits, whereas the Q168R-variant contains 1.36 mol of Zn+Fe per mole of subunits. The mutation stabilizes the uridylyl-enzyme by 1.2 kcal mol^{-1} in comparison to the wild-type enzyme. These results show that the low activity of Q168R is not due to overstabilization of the intermediate or to the absence of structural metal ions. Instead, the main defect is very slow uridylylation and deuridylation.

Galactose-1-phosphate uridylyltransferase (EC 2.7.7.12; uridylyltransferase) is an enzyme in the Leloir pathway of galactose metabolism (Scheme 1). It catalyzes the nucleotide exchange between UDP-glucose[1] and Gal-1-P to produce UDP-galactose and Glc-1-P by a ping-pong kinetic mechanism (Scheme 2). In the first step, His 166 in the active site of the enzyme from *E. coli* attacks the α-phosphorus of UDP-glucose, displaces Glc-1-P, and forms the covalent uridylyl-enzyme (UMP-enzyme) intermediate. The uridylyl-enzyme reacts with Gal-1-P in the next step to produce UDP-galactose.

Scheme 1



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* To whom correspondence should be addressed.

[1] Abbreviations: UDP-glucose, uridine-5′-diphosphate glucose; Gal-1-P, galactose 1-phosphate; UDP-Gal, uridine-5′-diphosphate galactose; Glc, glucose; Glc-1-P, glucose 1-phosphate; UMP-Im, uridine 5′-phosphoimidazolate; NAD^+, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PMSF, phenylmethylsulfonyl fluoride; BICINE, *N,N*-bis(2-hydroxyethyl)glycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ICP-MS, inductively coupled plasma mass spectrometry.

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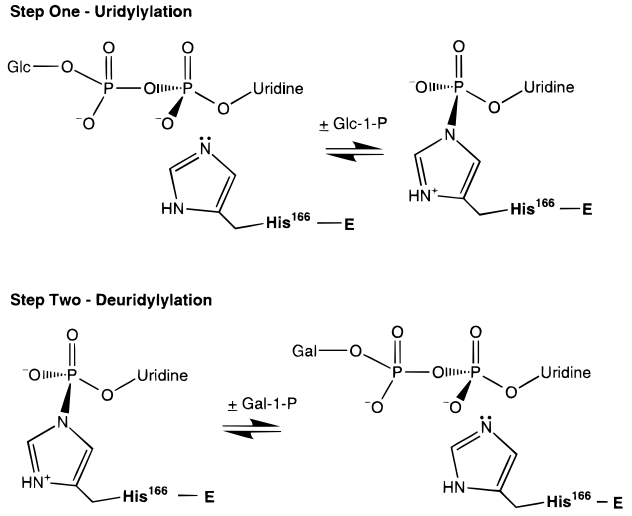
Scheme 2



Uridylyltransferase plays a major role in galactose metabolism, in which galactose is converted into Glc-1-P. It also provides the means for equilibrating uridylylated sugars in the cell (*1*). The UDP-hexoses are needed in the synthesis of disaccharides, glycoproteins, glycolipids and cellulose in the cell (*2*).

The human autosomal recessive genetic disease galactosemia results from the impairment of uridylyltransferase activity, leading to high levels of galactose, and Gal-1-P (*3*). The symptoms include cataracts, jaundice, cirrhosis, and brain damage and neurodevelopmental problems (*4*). These symptoms can be alleviated by placing patients on a galactose-restricted diet, but a number of secondary complications such as mental retardation, learning disorders, ovarian cancer, and decreased fertility often develop (*5*−*7*).

Several mutations in human uridylyltransferase that result in galactosemia have been identified. Such mutations are seen in 1 in every 40 000−60 000 newborn infants. Of these mutant enzyme forms, Q188R (Gln 168 in *E. coli*) accounts for 70% of galactosemia cases among the Caucasian population (*8*, *9*). Fridovich-Keil and co-workers have expressed this variant of human uridylyltransferase enzyme and discovered that it displays no detectable overall enzyme activity (*10*). They have also investigated its effect on enzyme heterodimer formation and have shown that this mutation causes a partially dominant negative effect (*11*).

The loss of enzyme activity could result from an impairment of the first step (uridylyl group transfer from UDP-glucose to form the uridylyl-enzyme intermediate) or an impairment of the second step (transfer of the UMP group to Gal-1-P acceptors). Alternatively, both steps could be impaired, contributing cumulatively to a major loss in enzyme activity. In addition, structural Zn^{2+} or Fe^{2+}, which are essential constituents of the *E. coli* enzyme, may be absent; or the substitution of the positively charged arginine for glutamine in the active site might overstabilize the uridylyl-enzyme intermediate. We were interested in finding out which of the above possibilities contributed to the dramatic loss of activity in this variant enzyme. To answer these questions, we used the well-characterized *E. coli* uridylyltransferase to model the Q188R mutation and carried out a detailed mechanistic investigation. *E. coli* uridylyltransferase has a 72% sequence similarity with the human enzyme and is a reasonable model for this study.

The enzyme from *E. coli* is a dimer of molecular mass 80 kDa and contains 348 amino acids per subunit (*12*−*14*). It contains 1.21 mol of Zn/mol of subunits and 0.67 mol of Fe/mol of subunits. These metals do not participate in catalysis; however, their presence is critical to maintain the enzyme activity (*15*). Recently Wells and Fridovich-Keil have shown that the human enzyme also contains zinc (*16*).

The crystal structure of uridylyltransferase with UDP bound at the active site and the structure of the uridylyl-enzyme intermediate have been solved recently and provided information about residues interacting closely with His 166 and the uridine 5′-phosphoryl group (*17*, *18*). Gln 168, which is conserved in all known uridylyltransferases, faces the active site, and is near His 166. In the uridylyl-enzyme structure, Gln 168 interacts closely with phosphoryl oxygens, indicating that it may be important in catalysis. In this paper, we describe the kinetic and thermodynamic properties of the *E. coli* uridylyltransferase point variants Q168R and Q168N.

MATERIALS AND METHODS

Materials. Glc-1-P, Gal-1-P, UDP-glucose, NAD^+, NADP, glucose-6-phosphate dehydrogenase, phosphoglucomutase, UDP-glucose dehydrogenase, β-mercaptoethanol, PMSF, BICINE, and HEPES were from Sigma. Affi-Gel Blue was from Bio-Rad. Amicon membranes and Microcon Concentrators were from Amicon. Q-Sepharose was from Pharmacia. All molecular biology supplies were from Promega and Fisher.

Protein Purification. Wild-type galactose-1-phosphate uridylyltransferase was purified as described previously (*19*). The Q168R-variant was purified by a minor modification of the same procedure. The lysis buffer contained 5 mM UDP-glucose, and ammonium sulfate precipitation steps were excluded. The supernatant fluid from the streptomycin sulfate precipitation was diluted to 4 mg·mL^{-1} protein and applied directly to the Affi-Gel Blue affinity column. UDP-glucose was added to a final concentration of 1 mM to the pooled fractions from the affinity column prior to Amicon filtration. Fractions from the Q-Sepharose column were pooled, concentrated, and stored in liquid nitrogen. SDS/PAGE analysis indicated that the Q168R protein was approximately 95% pure.

Metal Analysis. All metal analyses were performed at the University of Wisconsin−Madison Plant and Soil Analysis Laboratory by ICP-MS. Highly purified uridylyltransferase was diluted in Nanopure water and submitted for Fe and Zn analysis. Each analysis was complemented by appropriate control experiments. Protein concentrations were measured spectrophotometrically using the extinction coefficient for uridylyltransferase at 280 nm (*15*).

Spectroscopy. UV−visible spectrophotometry was performed either on a Hewlett-Packard Model 8452A diode array spectrophotometer or a Shimadzu Model 1601PC spectrophotometer. Fluorescence spectra were obtained on a Perkin-Elmer Model MPF-3 spectrofluorometer. Circular dichroism spectra were obtained on an Aviv Model 62ADS CD spectrometer at the Biophysics Instrumentation Facility of the Department of Biochemistry at the University of Wisconsin−Madison.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by using the Bio-Rad Mutagene site-directed

mutagenesis kit which utilizes the method of Kunkel (*20*). The *gal*T gene has previously been cloned into the high-expression pTZ18ROT vector (*21*). All oligonucleotide primers were purchased from Cruachem. The mutagenic primers were 22 bases in length and contained the mutation (CAG→CGC in Gln→Arg; CAG→AAC in Gln→Asn) in the center. These codons allowed the placement of an *Nde*I restriction site for facile screening. Mutant colonies were identified by restriction digestion, and the presence of the mutation was confirmed by DNA sequencing. The Q168R mutant gene was sequenced in its entire length twice, first using Sequenase (from USB) and then by ABI Prism Ampli Taq sequencing in the University of Wisconsin−Madison Biotechnology Center to confirm that there were no adventitious mutations. The Q168N mutant gene was sequenced in its entire length in the University of Wisconsin−Madison Biotechnology Center. Mutant plasmids pTZ18ROQ168R and pTZ18ROQ168N were transformed into BL21(DE3)-pLysS cells and overexpressed as described previously (*21*).

Enzymatic Assays. Uridylyltransferase activity was measured by a standard coupled assay (*22*). The formation of Glc-1-P was coupled to NADPH formation by phosphoglucomutase and Glc-6-phosphate dehydrogenase. The formation of the uridylyl-enzyme was observed and quantitated spectrophotometrically under the conditions of the standard assay with omission of Gal-1-P. Under these conditions, the amount of NADPH formed is equal to the amount of uridylyl-enzyme formed. The uridylyl-enzyme content of uridylyltransferase was measured and quantitated spectrophotometrically by adding Glc-1-P to the uridylyl-enzyme. The UDP-glucose formed was coupled to NADH production by UDP-glucose dehydrogenase. Under these conditions, 2 mol of NADH is produced per mole of UDP-glucose.

Equilibrium constant measurements and free energy calculations were performed as described previously (*19*). The wild-type and Q168R-uridylyltransferase equilibrium constant measurements were independently confirmed by reactions of enzyme with UDP-[^{14}C]glucose of known specific activity. Aliquots (50 μL) from the reaction were taken periodically, and the reaction was stopped by adding to 500 μL of boiling water with 2 mM EDTA. Precipitated enzyme was removed by centrifugation. Sodium bicinate buffer, pH 8.5, was added to a final concentration of 20 mM to the supernatant, along with 0.102 mM unlabeled Glc-1-P, 2 mM $MgCl_2$, and 1 unit of calf intestinal alkaline phosphatase. The phosphatase reaction to convert Glc-1-P to Glc was performed at 37 °C for 1 h and terminated by adding 2 mM EDTA. The reaction mixture was passed through Dowex AG1-2x anion-exchange resin in a mini column, and [^{14}C]Glc was eluted with 1.5 column volumes of water. Eluted [^{14}C]Glc was quantitated by counting in a Beckman LS6500 liquid scintillation counter. The amount of Glc-1-P formed was calculated from the known specific activity of UDP-[^{14}C]glucose. Appropriate control reactions were performed to ensure that all Glc-1-P was converted into Glc, and that UDP-glucose was not hydrolyzed by any contaminants possibly present in alkaline phosphatase.

Steady-state kinetic studies for wild-type enzyme at 4 °C were performed by using ^{14}C-labeled UDP-glucose in a timed point assay as described previously (*22*).

Rapid Mix Quench Experiments. Rapid mix quench experiments were performed by use of an Update Instruments (Madison, WI) apparatus equipped with a Model 745 Syringe-Ram Controller. Syringes, tubes, mixers, and all solutions were kept at 4 °C throughout the experiment. The syringe chamber was cooled to 4 °C, and the packed syringes were assembled in the chamber. The first syringe contained wild-type uridylyltransferase (129 μM) with 10 mM β-mercaptoethanol in 100 mM Na-bicinate buffer, pH 8.5. The second syringe contained UDP-glucose at 10.2 mM in Na-bicinate buffer. The third syringe contained the quenching acid (0.5 M HCl). Once the syringes were assembled, the computer-controlled ram device mixed the enzyme and substrate and then quenched the reactants at times ranging from 3 to 100 ms. The quenched samples were collected and frozen in liquid nitrogen. The temperature at each time point was checked immediately after mixing to ensure that it remained at 4 °C. Initially, the points were obtained in triplicate at a ram mixing velocity of 2.5 cm s^{-1}. The complete time course was repeated at a mixing velocity of 5 cm s^{-1} to determine whether there were mixing artifacts. The rate constants were independent of mixing velocity, indicating the absence of mixing artifacts in the data. UDP-glucose concentration was varied from 0.2 to 10.2 mM, and the respective time courses were obtained and fitted to the first-order rate equation to obtain the pseudo-first-order rate constants. Deuridylylation rates were measured in an identical procedure except that uridylyl-enzyme (129 μM) and Glc-1-P (10.2 mM) or Gal-1-P (10.2 mM) were used as the reactants.

The frozen samples were thawed, and the denatured enzyme was removed by membrane ultrafiltration using Microcon 10 microconcentrators. The enzyme-free filtrate was collected and neutralized with 1 M Tris base. The amount of Glc-1-P (uridylylation) or UDP-glucose (deuridylylation) in each sample was measured by an enzymatic fluorometric method (*23*). The amount of UDP-galactose (deuridylylation with Gal-1-P) was measured by the same method as UDP-glucose, with the addition of 0.005 unit mL^{-1} UDP-galactose-4-epimerase to convert UDP-galactose to UDP-glucose. The amount of Glc-1-P, UDP-galactose, or UDP-glucose formed was plotted as a function of time and fitted to a first-order rate equation using Kalaidagraph (Abelbeck Software, Reading, PA).

RESULTS

Preparation and Preliminary Assays of Q168R and Q168N Uridylyltransferases. The variants Q168R and Q168N of uridylyltransferase were produced by changing the CAG codon for Gln to CGC for Arg and AAC for Asn using the Kunkel method for site-directed mutagenesis (*20*). The resulting mutated genes were overexpressed in *E. coli.* The specific activity of the Q168R-cell extract was 4.0×10^{-4} units (mg of protein)$^{-1}$, whereas a similar extract of cells containing the wild-type uridylyltransferase gene in the same plasmid displayed a specific activity of 16.3 units (mg of protein)$^{-1}$. An extract of cells that did not contain the mutant or wild-type plasmid displayed a specific activity of 8.82×10^{-5} units (mg of protein)$^{-1}$ under the same conditions. These results indicated that the Q168R enzyme has lost most of its catalytic activity compared to the wild-type enzyme. However, the presence of activity above the background activity levels indicates that the Q168R-variant retains very low

activity. Under the same conditions, Q168N displays a specific activity of 0.19 unit (mg of protein)$^{-1}$.

The Q168R-variant of uridylyltransferase was purified as described under Materials and Methods. The purified enzyme displayed a specific activity of 6.6×10^{-4} units (mg of protein)$^{-1}$. Wild-type enzyme purified has a specific activity of 180 units (mg of protein)$^{-1}$. The Q168R mutation has caused a (2.7×10^5)-fold loss in uridylyltransferase activity. In contrast, the purified Q168N-variant displayed a specific activity of 3.4 units (mg of protein)$^{-1}$, which is only a 50-fold loss in activity.

Uridylylation and Deuridylylation of the Q168R-Uridylyltransferase. Because UDP-glucose was added to the enzyme at cell lysis and during the purification, the active sites may contain uridylyl groups. We measured the extent of uridylylation by adding Glc-1-P to the enzyme and measuring the amount of UDP-glucose released. We observed that on average about 45% of the active sites contained uridylyl groups. Reaction of the enzyme with UDP-glucose led to the uridylylation of an additional 20% of sites. Therefore, approximately 35% of all the active sites are nonfunctional in the Q168R-variant of uridylyltransferase. This raised the question why only 45% of the active sites remained uridylylated during purification despite the presence of high levels of UDP-glucose in the purification buffers. This matter will be addressed in a later section.

We also measured the uridylylation and deuridylylation of wild-type enzyme. All active sites in the wild-type enzyme can be uridylylated to give 100% uridylylated enzyme. Uridylylated wild-type enzyme can be isolated, and all the uridylyl groups can be removed to give 100% deuridylylation. The major differences in uridylylation and deuridylylation between the wild-type and Q168R-variant enzymes are the rates at which they are uridylylated and deuridylylated. As seen in Figure 1A, the wild-type enzyme undergoes uridylylation very quickly at a rate faster than that can be captured by the coupling enzyme system. In contrast, the Q168R-variant enzyme can be uridylylated to a total of 65% of potential active sites over a period of 200 min. Deuridylylation follows the same pattern as seen in Figure 1B. Deuridylylation of the wild-type enzyme is almost instantaneous, while that of the Q168R occurs over a period of 20 min.

These experiments show that the Q168R-variant enzyme can carry out the uridylylation reaction and the deuridylylation reaction and most of its active sites are functional. The reason for its overall loss of activity is the kinetic effect of slow uridylylation and deuridylylation. This stands in contrast to previous observations on the human enzyme that the Q188R-uridylyltransferase has no detectable activity (*7*). The activity we observed here cannot be attributed to contamination by wild-type activity because the experiments are single-turnover reactions. Any trace contamination by wild-type enzyme in single-turnover reactions would not be detectable. We were unable to detect any wild-type contamination in our preparations and are confident that the activities reported here are the intrinsic activities of the Q168R-enzyme.

Metal Content and Extent of Uridylylation for Q168R-Uridylyltransferase. Ruzicka et al. have previously shown that the presence of Zn and Fe is critical for maintaining uridylyltransferase activity (*15*). To understand why only

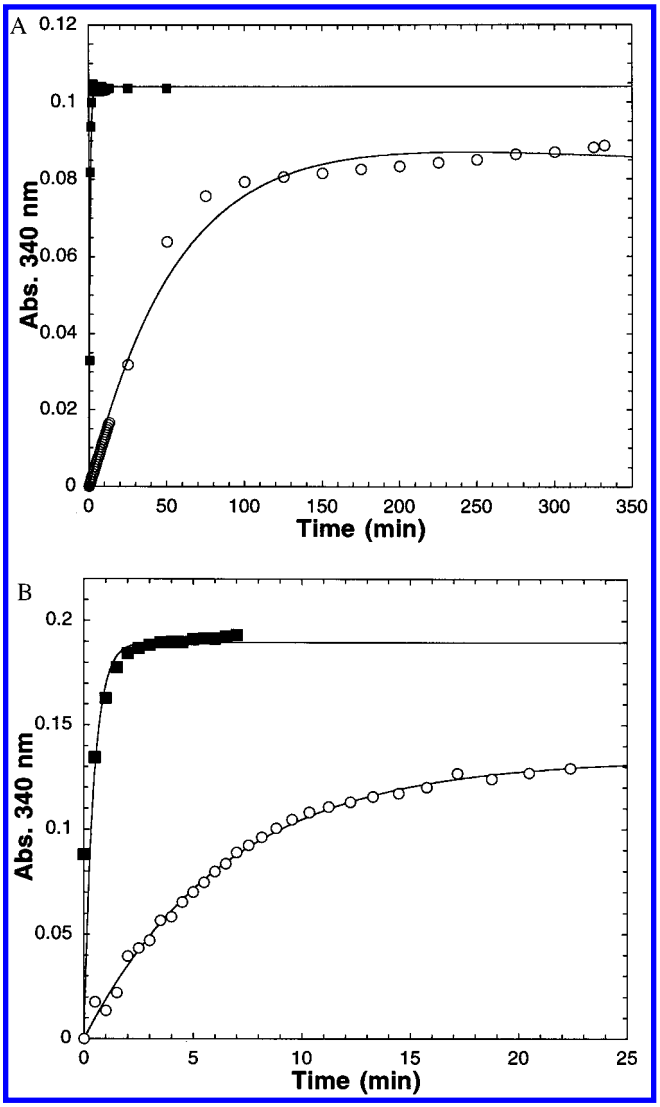


FIGURE 1: Uridylylation and deuridylylation time courses for wild-type and Q168R-variant uridylyltransferases. Figure 1A shows the uridylylation time course for wild-type (closed squares) and variant (open circles) enzymes. Approximately 20 μM wild-type and 20 μM Q168R (uridylyl-enzyme content less than 2%) were mixed with 1.02 mM UDP-glucose in 100 mM Na-bicinate, pH 8.5, at 27 °C. The production of Glc-1-P was monitored by coupling to NADPH production with phosphoglucomutase and Glc-6-P dehydrogenase. While the wild-type enzyme uridylylated completely almost instantaneously, Q168R enzyme undergoes uridylylation to 65% of completion over a period of 100 min. Uridylylation of Q168R-variant also shows biphasic kinetics. Figure 1B shows the deuridylylation time course for wild-type (closed squares) and variant (open circles) enzymes. Approximately 20 μM wild-type uridylyl-enzyme and 20 μM Q168R (total enzyme concentration 20 μM, uridylyl-enzyme content 45%) were mixed with 1.5 mM Glc-1-P in 100 mM Na-bicinate, pH 8.5, at 27 °C. The production of UDP-glucose was monitored by coupling to NADH production with UDP-glucose dehydrogenase. While the wild-type enzyme undergoes deuridylylation very quickly, Q168R-variant undergoes deuridylylation (45%) over a period of 20 min.

65% of the active sites in Q168R are uridylylated, we decided to take three preparations of Q168R and analyze them for their Zn and Fe contents and the extent of total active sites uridylylated. The results in Table 1 show that the Q168R-variant contains less Zn and Fe than the wild-type uridylyltransferase. Active sites in wild-type enzyme can be almost completely uridylylated, whereas only 65% of the potential active sites of Q168R-enzyme can be uridylylated. The

Table 1: Comparison of Wild-Type and Q168R Metal Contents and Active Sites Uridylylated

	Zn (mol/mol)[a]	Fe (mol/mol)	Zn+Fe (mol/mol)	% metal in enzyme[b]	% enzyme uridylylated[c]
Q168R prep I	0.80	0.33	1.13	60.31	64.49
Q168R prep II	0.94	0.62	1.55	82.58	66.92
Q168R prep III	1.01	0.38	1.38	73.56	64.44
Q168R mean ± SD	0.91 ± 0.10	0.44 ± 0.15	1.36 ± 0.21	72.15 ± 11.2	65.28 ± 1.4
wild-type mean ± SD	1.21[d] ± 0.09	0.67[d] ± 0.14	1.88[d] ± 0.20	100.00	92.00 ± 9.3

[a] Moles per mole of enzyme subunits. [b] Percentage of Zn+Fe content assuming wild-type Zn+Fe content is 100%. [c] Active sites uridylylated upon reacting with UDP-glucose as a percent of total enzyme subunit concentration. This indicates the amount of active sites that is involved in catalysis. [d] From reference (*15*).

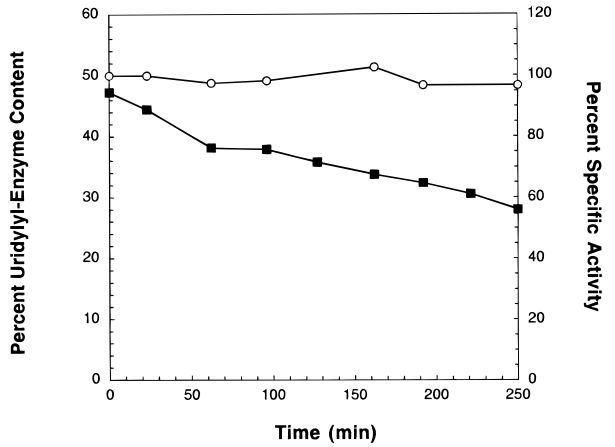


FIGURE 2: Stability of Q168R uridylyl-enzyme intermediate. Q168R enzyme as purified (45% active sites uridylylated) was incubated in 100 mM, pH 8.5, Na-bicinate buffer at 27 °C. Aliquots were taken periodically and were added to an assay mixture containing Glc-1-P and the UDP-glucose dehydrogenase/NAD coupling system to measure the amount of UDP-glucose produced. Over a 250 min period, the amount of UDP-glucose produced upon addition of Glc-1-P is decreased (squares). The control reaction where the overall activity of Q168R was measured showed that it remains unchanged during this period under similar conditions (open circles). The first-order rate constant for this hydrolysis is 1.48×10^{-3} min^{-1}.

extent of uridylylation for Q168R is comparable within error to its total Zn+Fe content. We suggest that there is a mixed population of Q168R enzyme molecules, 65% of which contain the Zn+Fe while the rest may not contain any metal ion or may contain only one metal ion in a subunit. Attempts to reconstitute uridylylation sites in the Q168R-variant uridylyltransferase by dialysis against Zn^{2+} and Fe^{2+} have been unsuccessful.

Stability of the Uridylyl-Enzyme Intermediate. The Q168R enzyme as purified (45% active sites uridylylated) was incubated in pH 8.5 Na-bicinate buffer at 27 °C. Aliquots were removed periodically and added to an assay mixture containing Glc-1-P and the coupling system to measure the amount of UDP-glucose produced. We observed that over a 250 min period the amount of UDP-glucose produced upon addition of Glc-1-P decreased (Figure 2). The control reaction, in which the overall activity of Q168R-enzyme was measured, showed that it remained unchanged during this period under the same conditions. This indicates that the uridylyl group on the enzyme active site undergoes hydrolysis under these conditions and gives an explanation for why 20% of the available active sites in the purified Q168R did not contain uridylyl groups. The observed first-order rate constant for hydrolysis is 2.47×10^{-5} s^{-1} at 27 °C. Hydrolysis is also evident in experiments monitoring the slow uridylylation of the Q168R-enzyme. The uridylylation time course shows biphasic kinetics. An initial burst can be assigned to the uridylylation of the available sites, and the subsequent steady-state rate can be assigned to the reuridylylation of sites vacated by hydrolysis of the uridylyl-Q168R. This property is not unique to the Q168R-enzyme. The wild-type enzyme shows hydrolysis of the uridylyl-enzyme at an even faster rate under the same conditions (6.5×10^{-5} s^{-1}). Interestingly, UMP-5′-phosphoimidazolate, which is chemically similar to the uridylyl-enzyme intermediate, undergoes a much slower hydrolysis at pH 8.5 (8×10^{-8} s^{-1}) (*25*). The molecular basis of this difference may be that the imidazole ring of His 166 is protonated at pH 8.5 in the uridylyl-enzyme (*26*) and presumably also in uridylyl-Q168R. The imidazole ring of UMP-5′-phosphoimidazolate is not protonated at pH 8.5 and so is less hydrolytically reactive.

Circular Dichroism Spectra of the Q168R-Uridylyltransferase. To assess whether there are major secondary structural differences between Q168R and the wild-type enzymes, CD spectra were obtained. The spectra of the variant and wild-type enzymes appeared identical. The Q168R change in the active site may cause a structural perturbation at the enzyme active site that cannot be detected in the CD spectrum.

Energetics of the Uridylyl-Q168R-Uridylyltransferase. Measurement of the equilibrium constant for uridylylation allows the free energy change associated with the uridylylation reaction to be calculated. Moreover, the results can be used to calculate the free energy for the hydrolysis of the uridylyl-enzyme intermediate. Arabshahi et al. have previously carried out these measurements and calculations for the uridylylation of wild-type uridylyltransferase at pH 7.0 (*19*). We performed similar measurements for the Q168R enzyme to investigate the effect of the mutation on the thermodynamics of uridylylation. The equilibrium constant value for Q168R uridylylation measured in triplicate is 0.032 ± 0.002 and the corresponding free energy for uridylylation at pH 8.5 is 2.04 ± 0.04 kcal mol^{-1}. The equilibrium constant for uridylylation of wild-type enzyme was previously calculated using Haldane relationships to be 2.1 (*22*).[2] We performed a direct measurement of the equilibrium constant as described under Materials and Methods, and it turned out to be 0.005 ± 0.002 in 6 trials. This value was

[2] The equilibrium constant calculated from the Haldane relationship in ref (*22*) is subject to propagation of error because of being evaluated as the ratio of products of several kinetic parameters, each of which contains significant error. The equilibrium constant measured directly in the present work is regarded as the more reliable value.

Table 2: Uridylyl Transfer Rates

	4 °C		27 °C	
	WT	Q168R	WT	Q168R
		Uridylylation		
$K^{UDP-glucose\ a}$ (mM)	0.5 ± 0.1	—[b]	—[c]	2.6 ± 0.2
k (s^{-1})	281 ± 18	$(2.2 \times 10^{-4}) \pm (0.4 \times 10^{-4})$	—[c]	$(4.2 \times 10^{-4}) \pm (0.1 \times 10^{-4})$
		Deuridylylation		
$k^{Glc-1-P}$ (s^{-1})	226 ± 10	$(4.8 \times 10^{-4}) \pm (0.5 \times 10^{-4})$	—[c]	$(1.68 \times 10^{-3}) \pm (0.04 \times 10^{-3})$
$K^{Glc-1-P\ a}$ (mM)	0.37 ± 0.18	—[b]	—[c]	0.194 ± 0.014
$k^{Gal-1-P}$ (s^{-1})	166 ± 13	$(3.3 \times 10^{-4}) \pm (1.2 \times 10^{-4})$	—[c]	—[b]
$K^{Gal-1-P\ a}$ (mM)	0.061 ± 0.020	—[b]	—[c]	—[b]

[a] Observed rate constants at various substrate concentrations were fitted to the equation: $k_{obs} = k[S]/(K + S)$. [b] Not determined. [c] Too fast to measure.

Table 3: Free Energies for Uridylyl-Enzyme Hydrolysis

reaction	$\Delta G°$(pH 8.5) (kcal mol^{-1})[a]
E-HisWild-Type-UMP + H_2O ⇌ E-HisWild-Type + UMP	−13.5
E-HisGln168Arg-UMP + H_2O ⇌ E-HisGln168Arg + UMP	−12.3
E-HisGln168Asn-UMP + H_2O ⇌ E-HisGln168Arg + UMP	−12.9

[a] All equilibrium constant values were measured at pH 8.5 at 27 °C. Free energy values were calculated as described previously (*19*).

confirmed by a different method using radiolabeled UDP-[^{14}C]glucose. The free energy value associated with wild-type uridylylation is 3.2 kcal mol^{-1}. These results indicate that Q168R uridylyl-enzyme is slightly more thermodynamically stable than the wild-type uridylyl-enzyme.

Free energies for uridylyl-enzyme hydrolysis calculated from the above free energy values are given in Table 3. These results indicate that there is only a small thermodynamic stabilization of the uridylyl-enzyme intermediate by the Arg side chain at pH 8.5. This stabilization is not significant enough to cause a 10^5-fold loss in activity. Interestingly, Q168N uridylyl-enzyme also shows a minor stabilization (Table 3).

Kinetic Parameters for Uridylylation and Deuridylylation of Q168R-Uridylyltransferase. The high sensitivity of the coupled spectrophotometric assay allowed the measurement of kinetic parameters for the uridylylation and deuridylylation reactions for the Q168R-uridylyltransferase. The results, along with wild-type values, are shown in Table 2. Uridylylation and deuridylylation rates for the wild-type enzyme were measured at 4 °C since they were too fast to be measured at 27 °C. Q168R-uridylyltransferase uridylylation and deuridylylation rates were also measured at 4 °C to facilitate comparison.

Steady-state kinetic parameters for the overall reaction of Q168R were not measured because the low activity of Q168R-uridylyltransferase necessitates high concentrations of enzyme at low substrate concentrations to obtain detectable initial rates. The steady-state approximation may not be valid under these conditions. Nevertheless, the comparison of single-turnover kinetic parameters at 27 °C for Q168R-uridylyltransferase and wild-type overall k_{cat} provides interesting insights.

The most striking effects of the Q→R mutation are on the rate constants for uridylylation and deuridylylation at 27 °C compared to the overall k_{cat} (780 s^{-1}) for the wild-type enzyme at 27 °C (22). The uridylylation rate is decreased by (1.9×10^6)-fold while the deuridylylation rate is decreased by (4.6×10^5)-fold. For the wild-type enzyme, the actual uridylylation and deuridylylation rates have to be faster than 780 s^{-1} because Wong and Frey have shown that uridylyl transfer is not rate-limiting (*22*). Our work also confirms that uridylylation and deuridylylation with UDP-glucose and Glc-1-P, respectively, for wild-type enzyme are not rate-limiting. Therefore, the decrease in uridylylation and deuridylylation rates measured here is a lower limit. The actual effect by the mutation has to be higher than (1.9×10^6)-fold for uridylylation and (4.6×10^5)-fold for deuridylylation. The loss of activity of Q168R galactosemia mutant therefore can be directly attributed to the effect on uridylylation and deuridylylation rates.

Kinetic Competence of the Wild-Type Uridylyl-Enzyme. The kinetic competence of wild-type uridylyl-enzyme formation has not been directly established previously. One obstacle in establishing kinetic competence is that the value of k_{cat} for the wild-type enzyme at 27 °C is 780 s^{-1}. This amounts to a lower limit for half-time for uridylylation of 0.8 ms. Such a rate cannot be measured by available rapid mixing techniques. Therefore, we decided to carry out transient kinetic studies at 4 °C where the rate is at least 4-fold slower. Uridylylation of wild-type enzyme occurred at a first-order rate constant of 281 ± 18 s^{-1} when monitored by the appearance of Glc-1-P as described under Materials and Methods (Figure 3). The time points for this number were obtained in triplicate at a mixing velocity of 2.5 cm s^{-1}. We also performed a single time course at a mixing velocity of 5 cm s^{-1} to ensure that there were no mixing artifacts and obtained a first-order rate constant of 275 ± 28 s^{-1}. These values agree within error.

To establish the kinetic competence of uridylyl-enzyme formation, the uridylylation rate has to be compared to the overall reaction rate. Inasmuch as the uridylylation rate was measured at 4 °C, we repeated the steady-state kinetic studies for the wild-type enzyme at 4 °C. We obtained parallel-line ping-pong double-reciprocal plots for five UDP-glucose and five Gal-1-P concentrations. The K_m for Gal-1-P was 0.50 ± 0.09 mM, and the K_m for UDP-glucose was 0.098 ± 0.018 mM at 4 °C. The k_{cat} value at 4 °C was found to be 62 ± 8 s^{-1}. Therefore, the uridylylation rate is approximately 4 times faster at 4 °C than the overall rate, and we conclude that uridylyl-enzyme formation in uridylyltransferase is kinetically competent.

We also measured the deuridylylation rates for wild-type uridylyltransferase with both hexose 1-phosphate acceptors

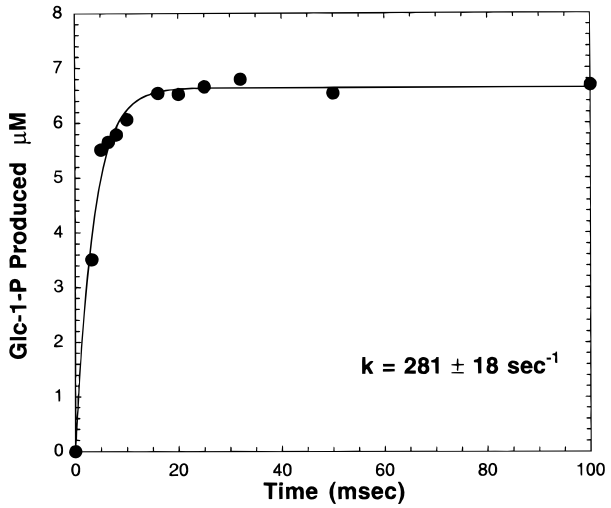


FIGURE 3: Time course for wild-type enzyme uridylylation observed by transient phase kinetics. 129 μM wild-type and 10.2 mM UDP-glucose were reacted at 4 °C and quenched at given times using 0.5 M HCl. The amount of Glc-1-P produced at each time point is plotted as a function of time. The time points were fitted to a first-order rate equation which gave a first-order rate constant of 281 ± 18 s^{-1}. The overall turnover number for the enzyme at 4 °C is 62 ± 8 s^{-1}. This indicates that uridylylation of wild-type uridylyltransferase is kinetically competent.

(Table 2). Deuridylylation rates with Gal-1-P and Glc-1-P are 166 ± 13 and 226 ± 10 s^{-1}. These data suggest that deuridylylation with Gal-1-P occurs at a rate faster than the overall turnover rate and is kinetically competent. Interestingly, at 4 °C, neither uridylylation with UDP-glucose nor deuridylylation with either Gal-1-P or Glc-1-P is rate-limiting. The only possibilities for a rate-limiting step are a product release step at 4 °C or a conformational change following product formation. UDP-glucose binding, Gal-1-P binding, or any conformational changes before substrate binding cannot be rate-limiting as such a rate-limiting step would be detected in our rates. But, our chemical quench method will not detect events following product formation; such events include the dissociation of the product being measured. Therefore, by process of elimination, only the above two possibilities exist for rate-limiting steps.

Comparison of Uridylylation and Deuridylylation Rates for Wild-Type and Q168R-Uridylyltransferases. To compare the uridylylation rates for wild-type and Q168R enzymes, we measured the uridylylation rate for Q168R at 4 °C, which turned out to be 2.2×10^{-4} s^{-1}. Comparison with the transient kinetic uridylylation rate constant of 281 s^{-1} for wild-type enzyme shows that the rate is 1.3×10^6 times faster for the wild-type enzyme. The rate difference corresponds to an activation energy 7.7 kcal mol^{-1} higher for the variant Q168R than the wild-type enzyme. The deuridylylation rate for the wild-type enzyme with Glc-1-P was found to be 226 s^{-1} at 4 °C (Table 2), and the deuridylylation rate for the Q168R-variant enzyme with Glc-1-P was 4.8×10^{-4} s^{-1}. This accounts for a rate difference of 4.7×10^5 for deuridylylation. This translates to an activation energy difference of 7.2 kcal mol^{-1}. Therefore, at 4 °C, both uridylylation with UDP-glucose and deuridylylation with Glc-1-P reactions for Q168R-variant have significantly higher kinetic barriers than that for wild-type enzyme.

Other Mutations at Gln 168. We have constructed and expressed three other variants of uridylyltransferase at the Gln 168 position. Q168N, Q168H, and Q168G all show comparable activity in crude cell extracts. We have purified the Q168N-variant enzyme and found a specific activity of 3.4 units (mg of protein)$^{-1}$. Steady-state kinetic analysis of the Q168N-variant shows an overall k_{cat} of 19.0 ± 1.0 s^{-1} and comparable K_m values with the wild-type enzyme ($K_{UDP-glucose} = 0.200 \pm 0.016$ mM; $K_{Glc-1-P} = 0.293 \pm 0.023$ mM). The overall k_{cat} is decreased by 40-fold for Q168N, but K_m values remain unchanged from those of wild-type enzyme (*22*). It can uridylylate all its active sites, and the isolated uridylyl-enzyme can undergo efficient deuridylylation. The Q168G and Q168H mutant enzymes were not purified.

DISCUSSION

The human and *E. coli* galactose-1-P uridylyltransferases are homologous, with substantial identities (56%) and similarities (72%) in their aligned amino acid sequences. In particular, the amino acid sequences in the active sites of the human and *E. coli* enzymes are conserved. The principal variant of uridylyltransferase in human galactosemia is the Q188R form of the enzyme (*8*, *9*), and this corresponds to Q168R in the *E. coli* enzyme. Structures of the *E. coli* enzyme show that the side chain carboxamide group of Gln 168 is within hydrogen bonding distance of the α-phosphoryl group of the substrate bound at the active site and of the phosphoryl group of the uridylyl-enzyme intermediate (*17*, *18*). The human Q188R uridylyltransferase is essentially inactive (*7*), and, as shown in the present work, the activity of the corresponding *E. coli* Q168R is very difficult to detect.

Because of the double-displacement mechanism, low activity can arise from a kinetic barrier to either one of the two steps or from barriers to both steps. In the case of galactose-1-P uridylyltransferase, the two mechanistic steps are uridylyl-group transfer from a UDP-hexose to His 166 and transfer of the same group from the covalent intermediate to a hexose-1-P to form UDP-hexose. We have shown that the Q168R-variant of the *E. coli* uridylyltransferase undergoes both steps of the mechanism at very low rates, and that it displays very low activity in the standard assay. The low rates for the individual steps account for the low overall activity of Q168R-uridylyltransferase. A slow rate for either one of the two steps could have accounted for the low overall activity; however, the rates of both steps are very slow.

The reason for the low reactivity of Q168R in the two mechanistic steps is not known at this time. Arginine in place of glutamine will introduce additional steric bulk into the active site, and it is expected also to introduce a positive charge, which would alter the electrostatic balance. Either of these two effects can be significant. One possibility that existed at the start of the present work was that the positively charged guanidinium group introduced by the Gln→Arg change might stabilize the covalent uridylyl-enzyme electrostatically. In the wild-type enzyme, the neutral Gln 168 is hydrogen bonded to the presumably negatively charged phosphoryl group of the uridylyl-enzyme. An electrostatic contact between Arg 168 of Q168R and the phosphoryl group might stabilize the uridylyl-enzyme and block the deuridylylation step. However, the present results rule out the possibility that the impaired activity of Q168R could be explained by overstabilization of the uridylyl-enzyme. First,

both uridylylation and deuridylylation are slow with the Q168R-variant. Second, $\Delta G°$ for the hydrolysis of the uridylyl-Q168R enzyme has been compared with that of the wild-type uridylyl-enzyme and found to be only 1.2 kcal mol^{-1} more negative. Third, the uridylylation rate difference between the wild-type and Q168R enzymes corresponds to a 7.7 kcal mol^{-1} higher activation energy for reaction of the mutated enzyme. Therefore, the physicochemical basis for impaired activity by Q168R cannot be simple electrostatic stabilization of the uridylyl-enzyme intermediate.

The exact relationship between the catalytic properties of the *E. coli* Q168R-uridylyltransferase and the human Q188R variant is not known with certainty; however, the reaction mechanisms of the two wild-type enzymes are the same, their kinetic parameters are remarkably similar (*7*), there is a very high degree of overall amino acid sequence identity between them, and the active site amino acid sequences are the same. All things considered, the two glutamine variants are likely to behave in a similar fashion.

The kinetic analysis of the individual uridylyltransfer steps of Q168R and comparison with the wild-type rates offered the opportunity of examining the kinetic competence of the covalent uridylyl-enzyme in the wild-type enzyme. Extensive data previously published strongly supported the ping-pong kinetic mechanism (*22*). However, the kinetic competence of the covalent intermediate had never been directly verified. The present results clearly prove the kinetic competence of the uridylyl-enzyme.

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