Kinetic Mechanism of UDP-Hexose Synthase, a Point Variant of Hexose-1-Phosphate Uridylyltransferase from *Escherichia coli*†

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ABSTRACT: Galactose-1-phosphate (galactose-1-P) uridylyltransferase from *Escherichia coli* catalyzes the interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P by a double-displacement mechanism through a uridylyl−enzyme intermediate, in which the uridine-5′-phosphoryl group is covalently bonded to N^{ϵ} of His 166. The point variant H166G displays a UDP-hexose synthase activity, in that it catalyzes the reaction of uridine 5′-phosphoimidazolide (UMPIm) with glucose-1-P to form UDP-glucose and imidazole. Inasmuch as the wild-type uridylyltransferase catalyzes its cognate reaction with ping-pong kinetics, an intrinsically ordered substrate binding mechanism, the kinetic mechanism of the UDP-hexose synthase activity of H166G became of interest. The synthase activity follows sequential kinetics [Kim, J., Ruzicka, F., and Frey, P. A. (1990) *Biochemistry 29*, 10590−10593]. In this work, product inhibition patterns for the synthase activity of H166G indicate random equilibrium binding of substrates. Comparison of the synthase activities of the variants H166G and H166A showed that the glycine variant is about 340- and 600-fold more active than the alanine variant in the forward and reverse directions, respectively. The kinetic consequences of varying the amino acid at position 166 were largely k_{cat} effects, with more modest K_m effects. Comparison of the synthase activities of these variants with that of the wild-type enzyme in the production of glucose-1-P showed that the loss of the β-carbon of His 166 in the complex H166G−UMPIm increases the activation energy for uridylyl group transfer by 2.4 kcal mol^{-1}, and the presence of two additional hydrogen atoms in the complex H166A−UMPIm increases the activation energy by 6.2 kcal mol^{-1}. It is concluded that the active site is much less tolerant of additional steric bulk in the locus of the β-carbon of His 166 than it is of the loss of the β-carbon. The sensitivities to additional steric bulk around other positions of the His 166−imidazole ring are much less severe, as indicated by the reactivities of methylated analogues of UMPIm in the synthase reaction of H166G. Uridine 5′-phospho-*N*-methylimidazolide is more reactive as a synthase substrate than UMPIm, and this is attributed to the positive charge of the imidazole ring. The fact that the imidazole ring of the wild-type covalent uridylyl−enzyme retains its proton and is positively charged is supported by the pH−rate profile for hydrolysis of the intermediate.

Galactose-1-phosphate uridylyltransferase (hexose-1-phosphate uridylyltransferase, EC 2.7.7.12) is an important enzyme in the Leloir pathway for galactose metabolism. The Leloir pathway is the sole means by which galactosyl and glucosyl groups are interconverted in nature. Galactose-1-phosphate uridylyltransferase, hereafter referred to as uridylyltransferase,[1] ensures the appropriate balance of UDPGal, UDPGlc, Glc1P, and Gal1P, which are required in several important biochemical processes, including glycolysis and the synthesis of disaccharides, glycoproteins, and glycogen (*2*). Galactosemia is an inherited disease of humans that is characterized as an autosomal recessive trait and results from a deficiency in the activity of this enzyme (*3*−*6*).

Uridylyltransferase catalyzes the reaction of Glc1P with UDPGal to form Gal1P and UDPGlc by a double-displacement mechanism involving an enzyme−substrate covalent intermediate (*7*−*11*). A histidyl residue in the enzyme from *Escherichia coli* serves as a nucleophile by transferring the uridine 5′-phosphoryl group according to eqs 1 and 2.

$$\text{E-His 166} + \text{UDPGlc} \rightleftharpoons \text{E-His 166−UMP} + \text{Glc1P} \quad (1)$$

$$\text{E-His 166−UMP} + \text{Gal1P} \rightleftharpoons \text{E-His 166} + \text{UDPGal} \quad (2)$$

The mechanism of action of uridylyltransferase from *E. coli* has been established by the observation of ping-pong kinetics (*7*), the isolation and characterization of the covalent

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[1] Abbreviations: uridylyltransferase, galactose-1-phosphate uridylyltransferase; UDPGlc, uridine diphosphoglucose; UDPGal, uridine diphosphogalactose; Glc1P, α-D-glucose 1-phosphate; Glc6P, D-glucose 6-phosphate; Glc1,6dP, α-D-glucose 1,6-diphosphate; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; UMPIm, uridine 5′-phosphoimidazole; UMP-2-MeIm, uridine 5′-phospho-2-methylimidazole; UMP-3-MeIm, uridine 5′-phospho-3-methylimidazole; UMP-4-MeIm, uridine 5′-phospho-4-methylimidazole; UMP-5-MeIm, uridine 5′-phospho-5-methylimidazole; UMP-2,4,5-Me_3Im, uridine 5′-phospho-2,4,5-trimethylimidazole; 2-MeIm, 2-methylimidazole; 4-MeIm, 4-methylimidazole; dATPαS, 2′-deoxyadeosine 5′-1-thiotriphosphate; Im, imidazole; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; LB, Luria broth; MC, main chain; SC, side chain.

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intermediate (*8*, *12*, *13*), and the observation of stereochemical inversion at phosphorus in each step with retention of configuration in the overall reaction (*14*, *15*).

The essential histidyl nucleophile of uridylyltransferase from *E. coli* was identified by site-directed mutagenesis and chemical rescue. The activities of His → Asn point variants revealed that of the 15 His residues, only His 164 and His 166 were essential for activity (*16*). Chemical rescue experiments showed His 166 to be the essential nucleophile (*1*).

In the chemical rescue, the two point variants H164G and H166G of the uridylyltransferase were tested for activity in the reaction of eq 3.

$$\text{UMPIm} + \text{Glc1P} \rightleftharpoons \text{UDPGlc} + \text{imidazole} \qquad (3)$$

The rationale was that uridine 5′-phosphoimidazolate (UMPIm) might bind to the active site and function in place of the uridylyl−enzyme intermediate, with the imidazole ring occupying the imidazole binding site vacated in one of the point variants. In the reverse direction, imidazole itself would bind to the site and cleave UDPGlc. The variant H166G catalyzed reaction 3, and H164G did not, which led to the assignment of His 166 as the nucleophilic catalyst. Reaction 3 was not catalyzed by the wild-type enzyme, and the variant H166G did not catalyze reaction 1 or 2. The variant also catalyzed the similar reaction of UMPIm with Gal1P. In recognition that H166G was a new enzyme, it was named UDP-hexose synthase. The assignment of His 166 as the nucleophilic catalyst was verified by the crystal structures of the uridylyltransferase and the covalent uridylyl−enzyme intermediate (*17*, *18*). The structures also showed that His 164 was a ligand to Zn(II), which had been found to be required for activity but not to participate in the chemical mechanism (*19*).

In this paper, we examine two issues regarding the activity of UDP-hexose synthase in reaction 3. The ping-pong kinetic mechanism of the parent uridylyltransferase necessarily entails ordered binding of substrates. The kinetic mechanism for reaction 3 is not ping-pong, however, but rather sequential, which raises the question of whether the mechanism requires ordered binding of substrates and release of products. This issue has been addressed in product inhibition studies. Second, the design of the point variant as H166G rather than H166A for chemical rescue experiments was rationalized on the basis that the putative substrate complexes of H166A would be sterically crowded, with two additional hydrogen atoms compared to the corresponding complexes of the parent enzyme−substrate complexes. Here we examine the issue of crowding in the imidazole binding site, both by testing H166A for activity in reaction 3 and by evaluating the efficacy of various isomers of uridine 5′-phosphomethylimidazolide and of methylated imidazole itself as substrates for H166G (UDP-hexose synthase).

EXPERIMENTAL PROCEDURES

Materials. Imidazole, 1-methylimidazole, 2-methylimidazole, 4-methylimidazole, triphenylphosphine, and 2,2′-dipyridyl disulfide were obtained from Aldrich. UMP (free acid), UDP-glucose dehydrogenase (type VI), phosphoglucomutase (rabbit muscle), glucose-6-phosphate dehydrogenase (type VII, baker's yeast), α-D-glucose-1-P, UDPGlc, NAD^+, $NADP^+$, bicine, Mes, Ches, Mops, Hepes, EDTA, PMSF, carbenicillin, and chloramphenicol were purchased from Sigma Chemical Co. Ampicillin was purchased from Boehringer Mannheim. All the necessary components required for site-directed mutagenesis were obtained from the Muta-Gene Phagemid *In Vitro* Mutagenesis Kit (version 2) from Bio-Rad. For sequencing, [α-^{35}S]dATPαS and the T7 Sequenase version 2.0 DNA sequencing kit were purchased from Amersham Life Science. Bactotryptone and Bacto yeast extract were obtained from Difco.

UMPIm and the isomers of UMPMeIm were synthesized with the procedure of Ruzicka and Frey (*20*). 2,4,5-Trimethylimidazole was synthesized with the method of Cowgill and Clark (*21*).

Bacterial Strains, Plasmids, and Bacteriophage. E. coli BL21(DE3)plysS was obtained from Novagen. *E. coli* strain CJ236 (dut^-, ung^-), MV1190, and helper phage M13K07 were purchased from Bio-Rad. Plasmid pTZ18R was obtained from U.S. Biochemicals, while plasmid pTZ18ROT was a gift from J. Wedekind (this laboratory).

Preparation of Mutant Enzymes H166G and H166A. The mutant gene specifying the H166G mutant of *E. coli* uridylyltransferase was prepared by site-directed mutagenesis of the replicative form of M13 containing the wild-type gene (*1*). The mutant gene was transferred to the expression vector, pKK222-3 (Pharmacia), and expressed as the pKF6 vector in *E. coli* CA13 (galT118, relA1, 1-spoT1) cells (*1*). The mutant gene specifying H166A was made from the wild-type *E. coli* uridylyltransferase gene contained in the expression plasmid pTZ18ROT (*17*) by site-directed mutagenesis using the method of Kunkel (*22*) with the Muta-Gene Phagemid *In Vitro* Mutagenesis Kit (version 2). The H166A gene was expressed as the pTZ18ROT H166A vector in *E. coli* BL21(DE3)pLysS cells. Both mutant genes were sequenced in their entirety either by the dideoxy NTP method (*23*) using [α-^{35}S]dATP and the Sequenase version 2.0 DNA sequencing kit or by the ABI Prism Dye Termination Cycle Sequencing method (Perkin-Elmer) at the University of Wisconsin Biotechnology Center (Madison, WI).

Cell Culture. E. coli cells, CA13 cells with the plasmid pKF6 (H166G), and BL21(DE3)pLysS cells with the plasmid pTZ18ROT (H166A) were grown at 37 °C from seed stocks prepared from frozen cells plated on LB agar (10 g of bactotryptone, 5 g of Bacto yeast extract, 10 g of NaCl, and 15 g of Bacto agar per liter of distilled water) and carbenicillin (100 μg/mL) (CA13) and LB, carbenicillin (100 μg/mL), and chloramphenicol (34 μg/mL) [BL21(DE3)pLysS]. Seed stocks were used to prepare 500 mL batch stocks in 2 L shake flasks containing 2× YT medium (16 g of bactotryptone, 10 g of Bacto yeast extract, and 5 g of NaCl per liter of distilled water) with either 100 μg/mL ampicillin (CA13 cells) or 100 μg/mL ampicillin and 34 μg/mL chloramphenicol [BL21(DE3)pLysS cells]. Cells were allowed to grow to a density corresponding to 1−1.5 absorbance at 600 nm, after which they were harvested by centrifugation at 6000*g* for 20 min, frozen in liquid nitrogen, and stored at −70 °C.

Purification of UDP−Hexose Synthase (H166G) and Mutant (H166A). UDP-hexose synthase from *E. coli* CA13 cells and the mutant protein (H166A) from BL21(DE3)plysS

cells were purified essentially according to the method of Arabshahi et al. (*15*) with several modifications. All buffers contained 10 mM β-mercaptoethanol and 1 mM PMSF. Q-Sepharose Fast Flow (Pharmacia) was substituted for DEAE-Sephadex A-50 in the last chromatographic step, and a linear salt gradient was instituted consisting of 1 L each of 0.05 M NaCl and 0.25 M NaCl in 0.01 M Na-Hepes buffer (pH 7.5). The purified enzyme was concentrated in an Amicon ultrafiltration stirred cell with a PM 30 membrane prior to drop-freezing in liquid nitrogen. The frozen droplets were stored in liquid nitrogen. The protein was analyzed by measurement of the absorbance at 280 nm ($\epsilon_{280} = 7.24 \times 10^4$ M^{-1} cm^{-1} subunit) (*19*).

Polyacrylamide Gel Electrophoresis. Native and SDS−polyacrylamide gel electrophoresis of purified proteins were conducted on mini gels either with the Phast Gel Electrophoresis system (Pharmacia) or with the Mini-PROTEAN II cell (Bio-Rad). Proteins for SDS−PAGE were denatured by treatment with 0.5 M β-mercaptoethanol and 1% SDS at 95 °C for 5 min. The purity of the isolated proteins was estimated to be greater than 95% by native and SDS−polyacrylamide gel electrophoresis.

Metal Analysis. The zinc and iron content of UDP-hexose synthase (H166G) and the mutant protein (H166A) were measured by ICP mass spectrometry at the University of Wisconsin Department of Soil Sciences (Madison, WI) facility.

Assay of UDP-Hexose Synthase. UDP-hexose synthase activity was routinely measured either in the forward direction (UDPGlc production from UMPIm and Glc1P) or in the reverse direction (Glc1P production from UDPGlc and Im) during purification of the enzyme. In the forward direction, enzyme activity was measured spectrophotometrically at 340 nm (NADH) under the following conditions: 1.0 mM UMPIm, 1.0 mM Glc1P, 1.25 mM NAD^+, 10 mM β-mercaptoethanol, 0.04 unit/mL UDPGlc dehydrogenase, and 90 mM sodium bicinate at pH 8.5 and 27 °C. When 1 mol of UDPGlc is oxidized to UDP-glucuronic acid, 2 mol of NADH is produced. In the reverse direction, enzyme activity was measured at 340 nm (NADPH) under the following conditions: 0.4 mM UDPGlc, 0.2 M imidazole, 330 μM $NADP^+$, 10 mM β-mercaptoethanol, 4 units/mL phosphoglucomutase, 0.3 unit/mL Glc6P dehydrogenase, 10 μM Glc1,6dP, and 90 mM sodium bicinate at pH 8.5 in a total volume of 1 mL at 27 °C. When 1 mol of Glc1P is oxidized to 6-phosphogluconate, 1 mol of NADPH is produced.

The concentrations of substrates, except for the imidazoles, were established by enzymatic assays as follows. UMPIm was assayed as NADH in the presence of UDP-hexose synthase and UDPGlc dehydrogenase. Glc1P was assayed as NADPH in the presence of phosphoglucomutase and Glc6P dehydrogenase. UDPGlc was assayed as NADH in the presence of UDPGlc dehydrogenase. The purity of UMPIm was further assessed by HPLC (*20*), and it was found to contain less than 3% UMP. Imidazoles were recrystallized from benzene, dried in vacuo under phosphorus pentoxide, and weighed.

Steady-State Kinetic Measurements. Initial forward rates were measured under the same conditions described for assaying the enzyme, except that the concentration of UMPIm was varied from 0.05 to 0.5 mM and Glc1P from 0.05 to 0.5 mM. Concentrations of methylated imidazolides were varied as above with the exception of UMP-2MeIm for which the range was 0.2−2 mM. Initial reverse rates were also measured under the assay conditions, except that the concentration of UDPGlc was varied from 0.03 to 0.4 mM and imidazole from 0.05 to 0.2 M. Complications arose because of secondary effects of several substrates on the coupling enzymes. In the forward reaction, UDPGlc dehydrogenase is the coupling enzyme, but UMPIm is a potent inhibitor ($K_i = 36 \pm 2.8$ μM), and the value of K_m for UDPGlc is 13.5 ± 1.3 μM. Therefore, at the concentrations of UMPIm used to measure the activity of UDP-hexose synthase (0.1−1 mM), the activity of the coupling enzyme is inhibited. To overcome inhibition, large amounts of UDPGlc dehydrogenase with a high specific activity were used. Furthermore, measured rates were routinely checked for proportionality to the amount of UDP-hexose synthase used at all concentrations of UMPIm. In the reverse reaction, imidazole, at concentrations at or below K_m, inhibits the coupled phosphoglucomutase−Glc6P dehydrogenase activity. At 0.5 M imidazole, the coupled enzymes are 85% inhibited. Therefore, the reverse rates were measured at imidazole concentrations of ≤0.2 M, and with sufficiently high concentrations of coupling enzymes to overcome inhibition.

Initial rates were fitted to eq 4

$$v = V_{max}[A][B]/(K'_A[B] + K'_B[A] + [A][B] + K_A K'_B) \quad (4)$$

where A and B represent substrates, K'_A and K'_B are the Michaelis constants of the substrates, and K_A is the dissociation constant of the substrate. Data were computer fitted by the nonlinear least-squares method (*24*).

Equilibrium constants were calculated from the respective Haldane equations for the equilibrium random bi-bi mechanism. The following equations were used, and the result was reported as an average of the calculated K_{eq} of each equation:

$$K_{eq} = \frac{k_{cat(r)}K'_{UMPIm}K_{Glc1P}}{k_{cat(f)}K_{UDPGlc}K'_{Im}} = \frac{k_{cat(r)}K_{UMPIm}K'_{Glc1P}}{k_{cat(f)}K'_{UDPGlc}K_{Im}} = \frac{k_{cat(r)}K'_{UMPIm}K_{Glc1P}}{k_{cat(f)}K'_{UDPGlc}K_{Im}} = \frac{k_{cat(r)}K_{UMPIm}K'_{Glc1P}}{k_{cat(f)}K_{UDPGlc}K'_{Im}}$$

Product Inhibition Studies. Determining the effect on the rate of varying the concentration of each product versus each of two substrates was required to establish a complete product inhibition pattern. This necessitated changes in the manner in which some rates were measured. When appropriate, the rates were measured by the coupled enzyme assay procedures described above. The following measurements were made with the variable and fixed substrate and the product inhibitor using the coupled enzyme assays: forward direction (varied substrate UMPIm), UMPIm (0.09−0.35 mM), Glc1P (0.2 mM), and Im (50−215 mM); forward direction (varied substrate Glc1P), Glc1P (0.046−0.51 mM), UMPIm (0.2 mM), and Im (50−215 mM); reverse direction (varied substrate UDPGlc), UDPGlc (0.043−0.47 mM), Im (83 mM), and UMPIm (0.6−6 mM); and reverse direction (varied substrate Im), Im (18−165 mM), UDPGlc (0.3 mM), and UMPIm (0.58−6.4 mM). The temperature and assay

components, including buffers, coupling enzymes, and cofactors, were as described above for steady-state kinetic assays.

The other product inhibition experiments required the use of fixed time assay methods, either because of severe inhibition of coupling enzyme by product inhibitors or because of incompatibiity of the product inhibitor with a coupled assay. Each 6 mL reaction mixture contained 10 mM β-mercaptoethanol and 90 mM sodium bicinate at pH 8.5. The varied substrate and product inhibitors were as follows: forward reaction (varied substrate Glc1P), Glc1P (0.13−0.56 mM), UMPIm (0.4 mM), and UDPGlc (0.06−0.5 mM); forward reaction (varied substrate UMPIm), UMPIm (0.19−2.1 mM), Glc1P (0.4 mM), and UDPGlc (0.06−0.27 mM); reverse reaction (varied substrate UDPGlc), UDPGlc (0.09−0.8 mM), Im (170 mM), and Glc1P (0.3−2.4 mM); and reverse reaction (variable substrate Im), Im (76−280 mM), UDPGlc (0.3 mM), and Glc1P (0.3−4.8 mM). At time intervals of 0.25−2 min, 0.5 mL of the reaction solution was mixed with 2.5 mL of boiling 1 mM Na_2-EDTA and the mixture heated for an additional 5 min at 100 °C and cooled to room temperature. For the measurement of UDPGlc, NAD^+ (100 μM) and UDPGlc dehydrogenase (0.015 unit) were added. After 1 h at room temperature, the increase in A_{340} was measured. For measurement of Glc1P, the following reagents were added: $NADP^+$ (30 μM), Glc1,6dP (3 μM), $MgCl_2$ (2 mM), phosphoglucomutase (1.4 units/mL), and Glc6P dehydrogenase (0.1 unit/mL). The samples were incubated for 1 h at room temperature, and the increase in A_{340} was measured.

Product inhibition patterns were obtained by fitting the rate data to two product inhibition models based on the following rate equations:

$$\text{competitive product inhibition: } v = V_{max}[A]/[K_m(1 + [I]/K_i) + [A]] \quad (5)$$

$$\text{noncompetitive product inhibition: } v = V_{max}[A]/[K_m(1 + [I]/K_{is}) + [A](1 + [I]/K_{ii})] \quad (6)$$

in which [A] is the concentration of variable substrate, [I] is the concentration of inhibitor, K_m is the Michaelis constant for the variable substrate, and K_{is} and K_{ii} are inhibition constants for the competitive and noncompetitive binding, respectively. The data were computer fitted by use of nonlinear least-squares fitting programs (*24*). The inhibition type for each assay was determined both by visual inspection of the fitted curve with the experimental data and by an assessment of the error produced in the estimation of the various kinetic parameters.

Rate Measurements for the Hydrolysis of the Uridylyl−Enzyme Intermediate. Wild-type uridylyl enzyme (E−UMP, subunit concentration of 77.3 μM) was incubated at 27 °C in 0.1 M buffer and 10 mM β-mercaptoethanol adjusted to an ionic strength of 1.0 by NaCl addition. Buffers and pH ranges used were as follows: Mes for pH 5.5−7.0, Mops for pH 6.5−8.0, and Ches for pH 8.5−9.8. Aliquots were removed periodically and added to an assay mixture containing Glc1P (4.5 mM), UDPGlc dehydrogenase (0.03 unit/mL), β-mercaptoethanol (10 mM), and NAD^+ (1.25 mM). E−UMP groups not hydrolyzed were converted to UDPGlc which allowed the measurement of E−UMP hydrolysis over time. Pseudo-first-order kinetics were observed for hydrolysis up to four half-lives, except when rates were very slow. Rate constants were calculated by fitting rate data to the first-order rate equation. First-order rate constants for the hydrolysis of the uridylyl−enzyme measured as a function of pH were fitted to the equation

$$k_{obs} = k_1 + k_2 a_{OH}$$

using the HBBELL program written by W. W. Cleland.

Table 1: Zinc and Iron Content of UDP-Hexose Synthase (H166G) and Mutant H166A

preparation	Zn (mol/mol)[a]	Fe (mol/mol)[a]
H166G (1)	1.16 ± 0.04	0.70 ± 0.04
H166G (2)	0.99 ± 0.04	0.76 ± 0.03
H166A	1.33 ± 0.05	0.59 ± 0.11
wild type[b]	1.21 ± 0.09	0.67 ± 0.14

[a] Moles per mole of enzyme subunits ± SD. [b] Gal1P uridylyltransferase data from ref *19*.

RESULTS

Metal Analyses. The wild-type uridylyltransferase is a metalloenzyme that contains both zinc and iron (*19*). Enzymatic activity requires the presence of these metal ions, which serve to maintain the structure of the active enzyme. The X-ray crystal structure showed that His 164, the other essential histidine, was a ligand for zinc (*17*). To properly interpret the rate data on point variants at position 166, two residues away from His 164, it was important to measure the Zn and Fe content. The data are given in Table 1 for variants H166G and H166A in comparison to the values for the wild-type enzyme (*19*). The Zn and Fe content of the variant enzymes is essentially the same as that of the wild-type enzyme. Therefore, the differences in kinetics and reaction specificity cannot be attributed to any difference in the structural metal ions.

Product Inhibition Patterns. It is known that the substrate concentration dependence for the reaction of UDP-hexose synthase is consistent with eq 3 (*1*). Both the steady-state ordered and random equilibrium kinetic mechanisms are compatible with eq 3 (*25*). The two mechanisms can in principle be distinguished by product inhibition patterns. If the inhibition patterns of both products with respect to both substrates are competitive, the kinetic mechanism is most likely equilibrium random. However, one or two noncompetitive inhibition patterns may be observed in an equilibrium random mechanism if a product also engages in dead-end inhibition, that is, if it binds to the wrong complex to form an unproductive ternary complex. If, however, six out of the eight possible product inhibition patterns are noncompetitive, a steady-state ordered substrate binding mechanism must be considered.

The results of the product inhibition studies are given in Table 2. All but one of the product inhibition patterns are competitive. Inasmuch as seven of the eight patterns are competitive, the basic kinetic mechanism is likely to be random equilibrium. The deviation from purely random equilibrium behavior is noncompetitive inhibition by UMPIm with imidazole as the variable substrate. The simplest rationale for the inhibition pattterns in Table 2 is that the

Table 2: Product Inhibition of UDP-Hexose Synthase

	product inhibitor	variable substrate	K_i (mM)	inhibition type
A	UDPGlc	Glc1P	0.066 ± 0.005	C
B	UDPGlc	UMPIm	0.088 ± 0.005	C
C	imidazole	Glc1P	96.2 ± 5.3	C
D	imidazole	UMPIm	78.5 ± 6.1	C
E	Glc1P	UDPGlc	0.16 ± 0.05	C
F	Glc1P	imidazole	1.18 ± 0.05	C
G	UMPIm	UDPGlc	3.09 ± 0.11	C
H	UMPIm	imidazole	10.1 ± 0.74	NC[a]

[a] K_{ii} = 3.9 ± 0.61 mM.

Table 3: Kinetic Parameters for UDP-Hexose Synthase

parameter	H166G	H166A
K'_{UMPIm} (mM)	0.26 ± 0.033	0.78 ± 0.16
K_{UMPIm} (mM)	0.60 ± 0.083	1.63 ± 0.30
K'_{Glc1P} (mM)	0.21 ± 0.033	0.30 ± 0.07
K_{Glc1P} (mM)	0.49 ± 0.061	0.63 ± 0.089
K'_{UDPGlc} (mM)	0.34 ± 0.03	0.65 ± 0.19
K_{UDPGlc} (mM)	0.051 ± 0.003	0.093 ± 0.008
K'_{Im} (mM)	970 ± 110	1090 ± 290
K_{Im} (mM)	150 ± 10	160 ± 0.02
$k_{cat(f)}$ (s^{-1})	5.52 ± 0.52	0.0162 ± 0.001
$k_{cat(r)}$ (s^{-1})	13.5 ± 1.3	0.0215 ± 0.005
K_{eq}	0.00617	0.00634

basic random equilibrium mechanism allows the additional formation of a dead-end complex, in which UMPIm binds to two forms of the enzyme, the free enzyme and the enzyme−Im complex. The steady-state ordered binding mechanism is ruled out by the results in Table 1.

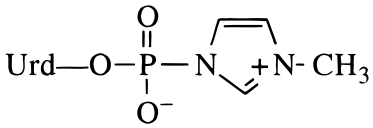
Kinetics of UDP-Hexose Synthase Variants H166G and H166A. The Michaelis constants in Table 3 for UDPGlc (0.34 mM) and Glc1P (0.21 mM) in H166G catalysis of reaction 3 are comparable to the corresponding constants for wild-type uridylyltransferase, which are reported to be 0.20 and 0.16 mM, respectively (*7*). Furthermore, the K_m of 0.26 mM for UMPIm as a substrate for H166G indicates a good fit of UMPIm in the active site. The high value of K_m for imidazole suggests that imidazole is weakly bound to the cavity left by the substitution of glycine for His 166. The k_{cat} of 5.5 s^{-1} for H166G is approximately $^1/_{200}$ of that for the wild-type uridylyltransferase. The 200-fold smaller activity of H166G in reaction 3 relative to that of wild-type uridylyltransferase in reactions 1 and 2 suggests that the positioning of the imidazole ring in His 166 relative to the α-phosphorus of UDPGlc, which is fixed by covalent bonding to the peptide backbone, facilitates high catalytic activity in uridylyltransferase. The 200-fold difference in activities of H166G and wild-type uridylyltransferase for their cognate reactions, while substantial, is modest in comparison to the absence of any detectable activity of H166G in the wild-type reactions 1 and 2 or of the wild-type uridylyltransferase in reaction 3.

The kinetic parameters of the UDP-hexose synthase variant H166A are also given in Table 3 for comparison with those of H166G. The Michaelis constants for UMPIm and Glc1P in the forward direction and UDPGlc in the reverse direction are approximately 1.5−2-fold larger for H166A than for H166G, and the K_M values for imidazole are similar. However, the values of k_{cat} for H166G are 340- and 600-fold larger for the forward and reverse reactions, respectively, than those for H166A. The β-methyl group of Ala 166 in the variant H166A dramatically interferes with UDP-hexose synthase activity (reaction 3).

Steric Effects of Methyl Groups in the Imidazole Binding Cavity. To evaluate the sensitivity of the imidazole cavity to steric bulk at other positions around the ring, a series of methylated imidazoles and UMP 5′-phosphomethylimidazolides were studied as substrates for H166G−UDP-hexose synthase in reaction 3. The methyl groups appended to the imidazole rings in the methylated substrates allowed the sensitivity of the reaction to moderate increases in bulk around the imidazole ring to be assessed. The results are displayed in Table 4. Modest increases (2−3-fold) in the Michaelis constants were observed with the methylimidazole substrates. The addition of a single methyl group to position 4 or 5 on the imidazole ring lowers k_{cat} by 2−3-fold. When the additional methyl group is in position 2 of the imidazole ring, k_{cat} is decreased 15-fold relative to that of UMPIm. However, when methyl groups are introduced at all three positions in the compound UMP 5′-phospho-2,4,5-trimethylimidazole, the value of k_{cat} decreases an additional 60-fold compared to that of UMP-2-MeIm and a total of 900-fold compared to that of UMPIm.

In the reverse direction, only minor changes, no greater than 2-fold in the values of K_m, were observed for methylimidazoles relative to imidazole. However, there were 6- and 300-fold decreases in k_{cat} for 4-MeIm and 2-MeIm, respectively. Although at very high enzyme concentrations and long incubation times (>24 h) at 27 °C, a small conversion to product occurred with 1-MeIm, the rate was too slow to be practical for enzymatic rate measurements.

Protonation State of the His 166−Imidazole Ring. It is a matter of mechanistic significance that the value of k_{cat} for the reaction of UMP-3-MeIm as a substrate in reaction 3 is significantly larger than that for UMPIm. The methyl substituent in this substrate is on N3, and its presence has the electronic effect of a proton, in that it results in the imidazole ring being positively charged, as shown.



UMP-3-MeIm

Because their pK_as lie between 5.68 and 6.4 (*20*), the imidazole rings of UMPIm and the other methylated analogues are not protonated in solutions above pH 7. Inasmuch as H166G−UDP-hexose synthase accepts UMP-3-MeIm as a better substrate than UMPIm, at least in terms of maximum velocity, it seems that a positive charge in the ring may be the normal state of the uridylyl group in the covalent intermediate of the wild-type uridylyltransferase (E-His 166−UMP of eq 1).

A further implication of imidazolyl protonation in the uridylyl−enzyme intermediate involves the pH−rate profile for the hydrolysis of the covalent intermediate, as shown in Figure 1. The uridylyl−enzyme intermediate is normally a free intermediate in the mechanism for the action of the wild-type uridylyltransferase in catalyzing reactions 1 and 2. However, in the absence of hexose 1-phosphate, the uridylyl−enzyme intermediate will gradually undergo hydrolysis to the free enzyme and UMP at a slow rate. The first-order rate constant for hydrolysis is plotted as a function of pH in

Table 4: Kinetic Parameters for UDP-Hexose Synthase with Methylated UMP Imidazolates and Imidazoles

	substrate A/B	K'_A (mM)	K_A (mM)	K'_B (mM)	K_B (mM)	k_{cat} (s^{-1})
A	UMPIm/Glc1P	0.26 ± 0.03	0.60 ± 0.08	0.21 ± 0.033	0.49 ± 0.06	5.52 ± 0.52
B	UMP-3-MeIm/Glc1P	0.23 ± 0.10	1.03 ± 0.15	0.42 ± 0.055	1.83 ± 0.40	10.50 ± 2.78
C	UMP-4-MeIm/Glc1P	0.29 ± 0.11	0.67 ± 0.20	0.74 ± 0.33	1.71 ± 0.43	2.63 ± 0.59
D	UMP-5-MeIm/Glc1P	0.13 ± 0.05	0.37 ± 0.03	0.26 ± 0.05	0.74 ± 0.03	1.17 ± 0.14
E	UMP-2-MeIm/Glc1P	0.56 ± 0.07	2.45 ± 0.23	0.20 ± 0.024	0.86 ± 0.08	0.35 ± 0.018
F	UMP-2,4,5-MeIm/Glc1P	0.49 ± 0.29	5.98 ± 0.80	0.43 ± 0.082	5.33 ± 2.95	0.006 ± 0.00059
G	imidazole/UDPGlc	970 ± 110	150 ± 10	0.34 ± 0.03	0.051 ± 0.003	13.5 ± 1.3
H	4-MeIm/UDPGlc	539 ± 93	76 ± 7	0.38 ± 0.07	0.054 ± 0.003	2.06 ± 0.32
I	2-MeIm/UDPGlc	802 ± 220	79 ± 12	0.46 ± 0.14	0.046 ± 0.005	0.041 ± 0.010

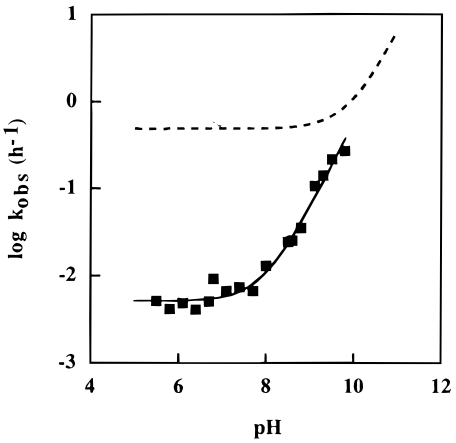


FIGURE 1: pH−rate profile for the hydrolysis of the uridylyltransferase−UMP (E−UMP) complex. The observed first-order rate constants (k_{obs}) for the hydrolysis of E−UMP, measured at 27 °C as described in Experimental Procedures, are plotted as closed squares vs pH. The line is calculated from the parameters obtained by fitting the data points to the equation $k_{obs} = k_1 + k_2a_{OH}$. The upper curve (dashed) is the pH−rate profile for the hydrolysis of UMP-3-MeIm at 27 °C (*20*).

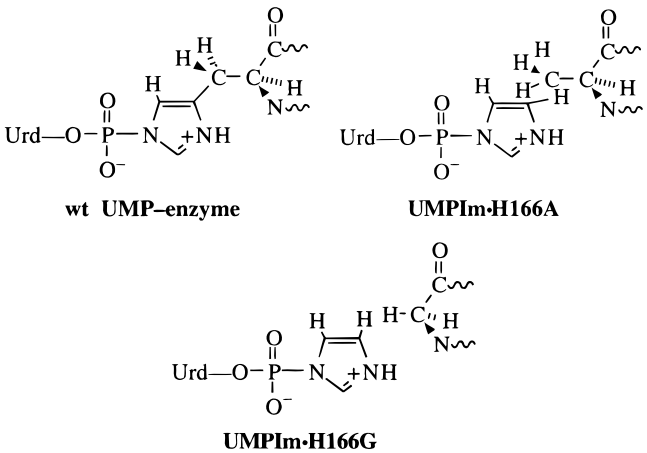
Figure 1, which shows that the hydrolysis is pH-independent at low to neutral pHs and increases at higher pHs. The profile is similar to that for the hydrolysis of UMP-3-MeIm (Figure 1), in which the increased rate at higher pHs was attributed to hydrolytic cleavage by hydroxide ions. At lower pHs, the reaction rate is dominated by the reaction of water with the protonated imidazolide or, in the case of UMP-3-MeIm, with the N-methylated imidazolide. The pH−rate profile for hydrolysis of the uridylyl−enzyme intermediate is analogous to that for UMP-3-MeIm but slightly displaced to the acid side, indicating a slightly greater sensitivity of the reaction to hydroxide ion. The profile is very different from those for UMPIm and the other UMP methylimidazolides, which display acid dependence in their pH−rate profiles (*20*).

DISCUSSION

Kinetic Mechanism of the Action of H166G−UDP-Hexose Synthase. The ping-pong bi-bi kinetic mechanism of uridylyltransferase is intrinsically ordered with respect to substrate binding and product release. The point variant H166G catalyzes the UDP-hexose synthase reaction, which is similar to one step of the ping-pong mechanism, reaction 3, differing in the fact that the uridylyl group is not covalently bonded to the protein, and the kinetics is sequential (*1*). The question of whether substrate binding would be ordered or random in the UDP-hexose synthase activity of H166G arose in connection with the compulsory orderedness of the parent reaction (eqs 1 and 2). These results show that the kinetic mechanism can be described by Scheme 1, random equilibrium binding of substrates and products with one dead-end complex. The rate law for the mechanism is given by eq 7 in Scheme 1, where A is UMPIm, B is Glc1P, P is Im, and Q is UDPGlc. The parenthetical term $(1 + [A]/K_A^I)$ in the denominator accounts for noncompetitive product inhibition by UMPIm with respect to Im in the reverse of reaction 3. Equation 7 reduces to eq 4, 5, or 6 under the appropriate conditions, that is, in the absence of one or both products.

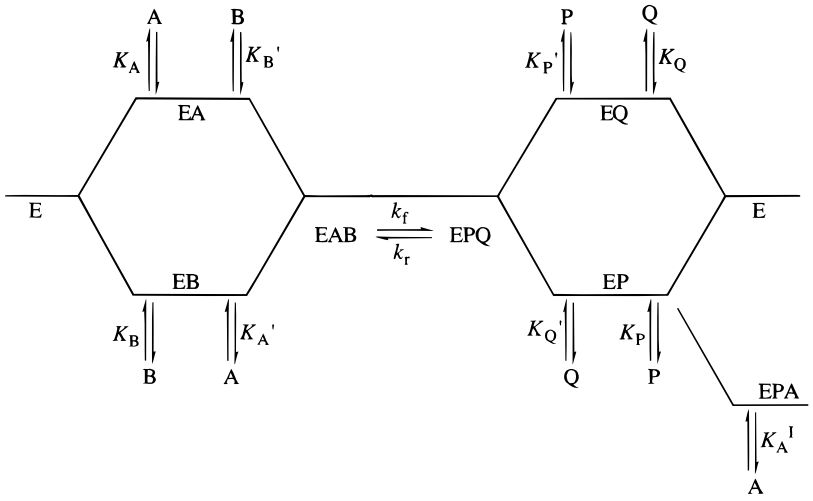
UDP-Hexose Synthase Activity of H166A. The calculated value of k_{cat} for the production of Glc1P by the wild-type uridylyltransferase is 780 s^{-1} at 27 °C and pH 8.5. The calculated value is based on a maximum rate of 6.5 μmol min^{-1} $unit^{-1}$ (*7*), a specific activity of 180 units mg of $protein^{-1}$ (*19*), and a subunit molecular weight of 40 000. The value of k_{cat} for the production of Glc1P by the H166G−UDP-hexose synthase is 13.2 s^{-1} so that the wild-type enzyme is 58 times more active than H166G. The value of k_{cat} for H166A−UDP-hexose synthase in the same reaction is 0.0215 s^{-1} so that the wild-type enzyme is 36 000 times more active than H166A.

The difference between H166G and H166A in UDP-hexose synthase activity is of interest. In the original work to determine which of the two essential histidine residues, His 164 and His 166, was the nucleophilic catalyst, the decision was made to prepare the point variants H164G and H166G and test them for activity in reaction 3 (*1*). H166G was active, and H164G was not; therefore, His 166 was assigned as the nucleophilic catalyst. The glycine variants were chosen for testing instead of the alanine variants for steric reasons. A significant difference between the Michaelis complex H166A−UMPIm and the wild-type UMP−enzyme intermediate is the absence of a covalent bond and the presence of two additional hydrogen atoms in the variant, as illustrated below.



The steric requirements of two additional hydrogen atoms in the complex UMPIm−H166A relative to the wild-type

Scheme 1



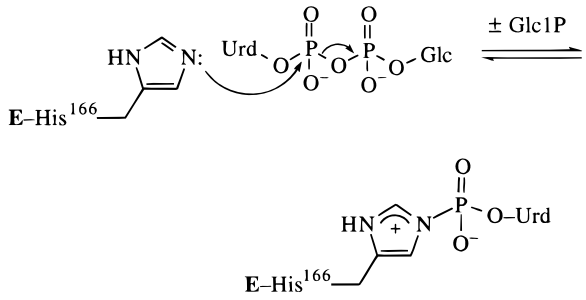
$$\frac{v_f}{[E_o]} = \frac{k_f[A][B] - \dfrac{k_r K_A K_B'[P][Q]}{K_P' K_Q}}{[A][B] + K_A'[B] + K_B'[A] + K_A K_B' + \dfrac{K_A K_B'[P][Q]}{K_P' K_Q} + \dfrac{K_A K_B' K_Q'[P]}{K_P' K_Q}\left(1 + \dfrac{[A]}{K_A^I}\right) + \dfrac{K_A K_B'[Q]}{K_Q}} \quad (7)$$

uridylyl−enzyme intermediate were expected to work against the expression of activity in reaction 3. The complex UMPIm−H166G would not include added bulk but would differ sterically from the wild-type complex in the absence of the β-carbon atom of His 166. These results are consistent with the original expectation; that is, H166G is 340 times more active in producing UDPGlc and 600 times more reactive in producing Glc1P than is H166A, and the difference resides mainly in the values of k_{cat} for these variants rather than in the Michaelis constants.

These results allow us to estimate the effects of varying steric bulk at the His 166−β-methylene locus on the activation energy for production of Glc1P by the wild-type and variant uridylyltransferases. The complex UMPIm−H166G illustrated above differs from the UMP−enzyme in the absence of a covalent bond and the β-carbon atom. The 58-fold difference in k_{cat} for Glc1P production corresponds to an activation energy for the reaction of H166G 2.4 kcal mol^{-1} higher than for that for the wild-type enzyme. The complex H166A−UMPIm differs from the wild-type UMP−enzyme in the absence of a covalent bond and the presence of two additional hydrogen atoms. The 36000-fold difference in k_{cat} corresponds to an activation energy 6.2 kcal mol^{-1} higher than that for the wild-type enzyme. The steric requirements of two additional hydrogen atoms in H166A exert a dramatically greater effect on catalysis and activation energy than the loss of the β-carbon atom in H166G.

Effects of Methyl Substituents at Other Imidazolyl Positions. To further examine the steric sensitivity around the imidazole ring of His 166, we evaluated methylated substrates for the UDP-hexose synthase activity of H166G kinetically. As shown in Table 4, the effects of methyl substituents at N3, C4, and C5 of the imidazole ring in UMP 5′-imidazolides were modest, with respect to both k_{cat} and K_m, in the forward and reverse reactions. The effects of methyl substitution at C2 were larger in both directions, but still not comparable to the difference between the activities of H166G and H166A. However, the three methyl substituents in UMP-2,4,5-Me$_3$Im decreased the value of k_{cat} by about 1000-fold and increased the value of K_m by 10-fold relative to those of UMPIm. In the reaction of the trimethylated substrate, one of the methyl groups can be expected to be forced into the site normally occupied by the β-methylene group of His 166 in the wild-type uridylyltransferase, and this seems to be the most sterically restricted position around the imidazole ring.

Mechanistic Significance of Imidazolyl Protonation in the Uridylyl−Enzyme Intermediate. The reactivity of UMP-3-MeIm as a substrate for the UDP-hexose synthase activity of H166G (Table 4), together with the pH−rate profile for the hydrolysis of the uridylyl−enzyme intermediate (Figure 1), indicates that the imidazole ring in the intermediate is protonated. The propensity of the imidazole binding locus to accommodate a positive charge is accented by the fact that, as shown in the crystal structure of the complex H166G−UDPGlc, the cavity normally occupied by the imidazole ring of His 166 is occupied by potassium hexahydrate [$K^+(H_2O)_6$] (*26*). It has been pointed out, on the basis of the structures of H166G−UDPGlc and the uridylyl−enzyme intermediate, that the imidazole ring is likely to be protonated (*26*). The present chemical and kinetic evidence supports the conclusion that the imidazole ring is protonated in the intermediate. Retention of the imidazole proton upon formation of the intermediate, as indicated in eq 8, appears to be an important aspect of the mechanism. The protonated



(8)

intermediate exists in a chemically poised and activated state for the subsequent uridylyl group transfer in the second step.

Removal of the imidazole proton, which might be expected to promote uridylyl−enzyme intermediate formation, would not necessarily promote the overall reaction. Retention of the proton on the imidazole ring at pHs well above its pK_a must be made possible by stabilization through binding interactions between the uridylyl group and the active site. The uridylyl group is engaged in close, apparently hydrogen-bonded interactions with Ser 161 (SC), Cys 160 (SC), Gln 168 (SC), Asn 77 (SC), Asp 78 (SC and MC), and Val 61 (MC). His 164 (MC C═O) forms a potential H bond interaction with the His 166 N^{δ} proton (*18*, *26*) which may help stabilize the protonated form of E−UMP. Further studies of the structural and functional aspects of point variants at these amino acid positions may provide information about the stabilization of the uridylyl−enzyme intermediate.

SUPPORTING INFORMATION AVAILABLE

Six figures (Figures 2−7) of double-reciprocal plots of the kinetic and product inhibition data of UDP-hexose synthase (6 pages). Ordering information is given on any current masthead page.

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