Remodeling Hexose-1-phosphate Uridylyltransferase: Mechanism-Inspired Mutation into a New Enzyme, UDP-hexose Synthase†

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ABSTRACT: Hexose-1-phosphate uridylyltransferase catalyzes the interconversion of UDP-galactose and glucose-1-P with UDP-glucose and galactose-1-P by a double-displacement mechanism through a covalent intermediate (E–UMP), in which UMP is bonded to one of two histidine residues at the active site, H164 or H166. To identify which histidine is the nucleophilic catalyst, we prepared two specific mutants of the enzyme from *Escherichia coli*, H164G and H166G, in each of which the imidazole ring and methylene carbon of one histidine are deleted. To determine whether the function of the deleted imidazole in these mutants could be carried out by the imidazole ring in uridine 5′-(phosphoimidazolate) (UMP-Im), we examined the mutant proteins for catalytic activity in the reaction of UMP-Im with glucose-1-P to form UDP-glucose and imidazole. The mutant H166G catalyzes this reaction, as well as the reverse reaction, by a sequential kinetic mechanism involving ternary complexes as intermediates. The mutant enzyme also accepts galactose-1-P as a substrate to form UDP-galactose. Hexose-1-P uridylyltransferase does not catalyze these reactions, and H166G does not catalyze the wild-type reaction. The substrate K_m values for the mutant enzyme are similar to those for hexose-1-P uridylyltransferase. The value of k_{cat} in the direction of UDP-glucose formation is 1.31 ± 0.01 s^{-1}, compared with 350 s^{-1} for hexose-1-P uridylyltransferase, and in the reverse direction k_{cat} is 4.8 ± 0.4 s^{-1}, compared with 960 s^{-1} for the wild-type enzyme. Thus, the kinetic "price" that is paid for removing the covalent bond and a methylene group linking the enzyme and the nucleophilic imidazole in the parent enzyme is an approximately 250-fold decrease in turnover number. The mutant H164G has no detectable catalytic activities. We conclude that the imidazole ring of H166 is the nucleophilic catalytic group of hexose-1-P uridylyltransferase. The mutant protein H166G is a *new enzyme* that catalyzes a new reaction by a kinetic mechanism different from that of its parent enzyme. We suggest the name *UDP-hexose synthase* for this new enzyme.

Hexose-1-phosphate uridylyltransferase, also known as galactose-1-phosphate uridylyltransferase, is a key enzyme in the Leloir pathway for galactose metabolism and one of the three enzymes in the *gal* operon of *Escherichia coli*. The activity of this enzyme is deficient in galactosemia, an inherited disease of humans that is transmitted as an autosomal recessive trait (Kakckar, 1960; Levy & Hammersen, 1978). Hexose-1-P uridylyltransferase catalyzes the reaction of glucose-1-P[1] with UDP-galactose to form galactose-1-P and UDP-glucose by a double-displacement mechanism, in which the imidazole ring of a histidine residue in the active site serves as a nucleophilic catalyst to transfer the uridine 5′-phosphoryl group according to eqs 1 and 2. the uridine 5′-phosphoryl group of UDP-

$$\text{E-His} + \text{UDP-galactose} \rightleftharpoons \text{E-His·UDP-galactose} \rightleftharpoons \text{E-His–UMP} + \text{galactose-1-P} \quad (1)$$

$$\text{E-His–UMP} + \text{glucose-1-P} \rightleftharpoons \text{E-His–UMP·glucose-1-P} \rightleftharpoons \text{E-His} + \text{UDP-glucose} \quad (2)$$

galactose is transferred to the imidazole N^3 of a histidine in the active site, and galactose-1-P is released from the enzyme. In the second step (eq 2) the uridylyl enzyme (E-His–UMP) binds glucose-1-P and produces UDP-glucose by transferring the UMP group to glucose-1-P. This mechanism is securely established by the observation of ping pong kinetics according to the rate law of eq 3, the isolation and partial characterization of the covalent intermediate, and the observation of stereochemical inversion at phosphorus in each step and retention of configuration in the overall reaction (Wong & Frey, 1974a,b; Yang & Frey, 1979; Sheu et al., 1979; Arabshahi et al., 1986).

$$v = \frac{V[A][B]}{[A][B] + K_{mA}[B] + K_{mB}[A]} \quad (3)$$

forward reaction: A = UDP-galactose; B = glucose-1-P

reverse reaction: A = UDP-glucose; B = galactose-1-P

In order to determine which histidine or histidines of hexose-1-P uridylyltransferase are essential for activity, Field et al. (1989) mutated each in turn to asparagine and measured the activities of the mutant enzymes. They found that, of the 15 histidine residues in the enzyme from *E. coli*, only two, H164 and H166, are essential for activity, and they concluded that both are at the active site and have essential functions.

In approaching the problem of assigning nucleophilic function to one of the histidine residues, we were guided by the active site model advanced earlier to explain the evolution of those phosphotransferases and nucleotidyltransferases that

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[1] Abbreviations: UDP-glucose, uridine 5′-(diphosphoglucose); UDP-galactose, uridine 5′-(diphosphogalactose); glucose-1-P, glucose 1-phosphate; galactose-1-P, galactose 1-phosphate; glucose-6-P, glucose 6-phosphate; UMP, uridine 5′-phosphate; UMP-Im, uridine 5′-(phosphoimidazolate); NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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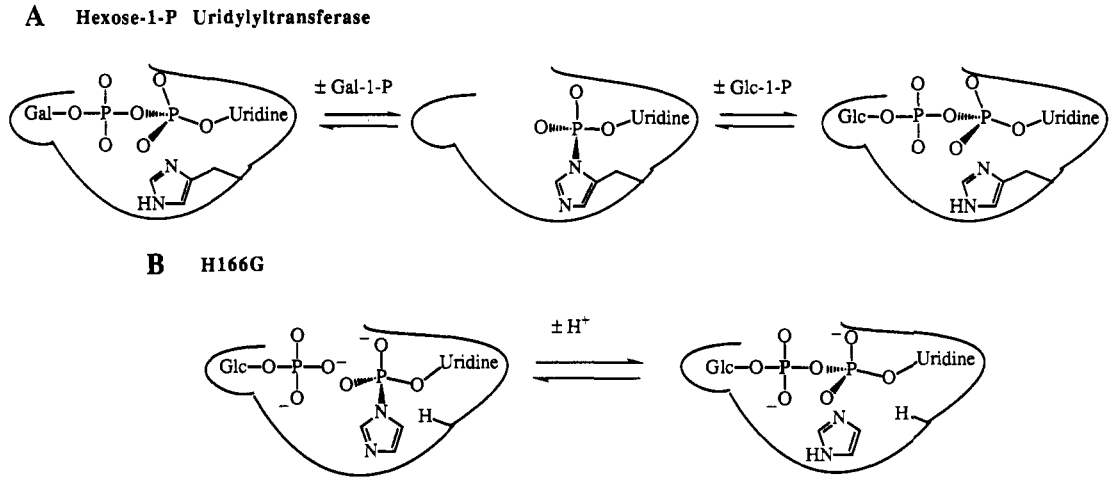
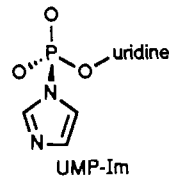


FIGURE 1: Active site conceptual models for hexose-1-P uridylyltransferase and the mutant H166G. Part A shows how the active site of hexose-1-P uridylyltransferase is subdivided by the nucleophilic histidine into subsites for hexose 1-phosphates and the uridylyl group, respectively. This illustration also shows how the active site histidine acts as a nucleophilic catalyst to cleave UDP-galactose into the covalent uridydyl enzyme and galactose-1-P, which vacates the hexose-1-P subsite. In the second step, glucose-1-P binds to this subsite and accepts the uridylyl group. Part B shows how the mechanism in part A is chemically reconstructed in a mutant protein, in which the active site histidine has been mutated to a glycine. This mutant is H166G, and it catalyzes eq 4, the reaction of UMP-Im with glucose-1-P to form UDP-glucose and imidazole.

catalyze group transfer by a double-displacement mechanism (Sheu et al., 1979; Frey, 1982, 1989). According to this model, the active site of such an enzyme consists of a single binding site for a phosphoryl or nucleotidyl donor substrate, and this site is subdivided by a nucleophilic amino acid that undergoes transient phosphorylation or nucleotidylation to form the covalent intermediates. Part A in Figure 1 illustrates this conceptualization of active site structure and function for hexose-1-P uridylyltransferase. Since glucose-1-P and galactose-1-P are sterically similar and isoelectrostatic, they have the capacity to bind at the same site in the active center, and the principle of economy in the evolution of binding sites led to the evolution of a mechanism that allows them to share this binding site. This mechanism is the ping pong kinetic mechanism, in which the UMP group is covalently bonded to an active site nucleophile during the interchange of glucose-1-P and galactose-1-P in their common binding site.

We undertook to assign nucleophilic function to H164 or H166 by site-directed mutagenesis and chemical reconstruction of catalytic function. To do this, we synthesized a chemical analogue of the uridylyl enzyme in Figure 1, uridine 5′-(phosphoimidazolate) (UMP-Im), and inquired whether it



UMP-Im

would react as a substrate of one or the other of two specific mutants, H164G or H166G, of hexose-1-P uridylyltransferase. We sought to determine whether one or the other mutant enzyme would catalyze the reaction of UMP-Im with glucose-1-P to form UDP-glucose according to part B of Figure 1.

In this paper we show that the mutant protein H166G catalyzes the reaction of uridine 5′-(phosphoimidazolate) (UMP-Im) with glucose-1-P to form UDP-glucose and imidazole. This reaction is a chemical model for the second step, eq 2, in which UMP-Im is a model for the uridylyl enzyme. The mutant protein H166G is a new enzyme, UDP-hexose synthase, because the reaction of UMP-Im with hexose 1-phosphates is not catalyzed by any other enzyme.

Materials and Methods

UMP-Im was synthesized by the method of Cramer et al. (1961). UMP-Im has been used as an intermediate in synthesis by Kozarich, et al. (1973) and by Lohrmann and Orgel (1978).

The mutant genes for H164G and H166G were prepared by the methods used earlier to prepare the genes for the mutants H164N and H166N (Field et al., 1989). These genes and the wild-type gene gal T were excised by *Eco*RI and *Hin*dIII digestion from the corresponding replicative forms of M13 used for sequence analysis and in the mutagenesis. They were cloned into a double-stranded expression vector designated pKF and constructed as follows. The plasmid pKK223-3 (Pharmacia) was linearized by *Eco*RI and *Hin*dIII digestion, the 5′-ends were dephosphorylated, and a 24-bp polylinker that had been synthesized to contain the *Hin*dIII and *Eco*RI sites in reverse orientation was ligated into the plasmid. The new plasmid pKF, which confers ampicillin resistance and contains the strongly inducible tac promoter, was purified from cells of *E. coli* CA13(galT118, relA1, 1–, spoT1). The WT and mutant genes were cloned into the *Hin*dIII- and *Eco*RI-digested pKF plasmid and designated pKFt, pKF4, and pKF6 for WT, H164G, and H166G, respectively. The plasmids were transformed into strain CA13 of *E. coli*, containing an ochre mutation in gal T of the chromosome, and the cells were induced through the tac promoter to overproduce the proteins. The cells were grown up, initially from single colonies, in 40 mL of 2× YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter of distilled, deionized water) under the pressure of ampicillin (0.1 mg/mL). The initial cultures were used to inoculate 4 L of the same medium; the cultures were shaken at 37 °C for 9 h, with addition of ampicillin (0.1 mg/mL) every 3 h, harvested, and frozen in liquid N_2. Hexose-1-P uridylyltransferase and the mutant proteins were purified as described (Field et al., 1989), with the exception that the DEAE-Sephadex column was changed to QAE-Sephadex.

Hexose-1-P uridylyltransferase was assayed as previously described (Wong & Frey, 1974b). The mutant enzyme H166G was assayed as a catalyst for the reaction of eq 4 by coupling with UDP-glucose dehydrogenase to the production of NADH; in the reverse direction it was coupled with phosphoglucomutase and glucose-6-P dehydrogenase to the

Table I: Kinetic Parameters for H166G and Hexose-1-P Uridylyltransferase[a]

enzyme	reaction	kinetics	K_m (mM) UMP donor	K_m (mM) Hex-1-P acceptor	k_{cat} (s^{-1})
H166G	4; forward	sequential	0.085 ± 0.011 (UMP-Im)	0.22 ± 0.02 (Glc-1-P)	1.31 ± 0.01
H166G	4; reverse	sequential	0.12 ± 0.05 (UDP-Glc)	290 ± 51 (imidazole)	4.5 ± 0.5
WT[b]	1 + 2; forward	ping pong	0.12 ± 0.04 (UDP-Gal)	0.16 ± 0.01 (Glc-1-P)	350
WT[b]	1 + 2; reverse	ping pong	0.20 ± 0.02 (UDP-Glc)	0.30 ± 0.03 (Gal-1-P)	960

[a] All parameters were measured at pH 8.5 in 0.1 M sodium *N*,*N*-bis(2-hydroxyethyl)glycinate with 8 mM cysteine at 27 °C. [b] Data from Wong and Frey (1974b).

production of NADPH. The reaction of eq 5, also catalyzed by H166G, was assayed by coupling with UDP-galactose 4-epimerase and UDP-glucose dehydrogenase to the production of NADH.

Results

Catalytic Properties of the Mutant H166G. The mutant H166G of hexose-1-P uridylyltransferase exhibits no catalytic activity in reactions 1 and 2, as was expected from the inactivity of the mutant H166N reported earlier (Field et al., 1989). However, H166G catalyzes reactions 4 and 5, the reactions of glucose-1-P or galactose-1-P with UMP-Im to form UDP-glucose or UDP-galactose, respectively.

$$\text{UMP-Im} + \text{glucose-1-P} \rightleftharpoons \text{UDP-glucose} + \text{imidazole} \quad (4)$$

$$\text{UMP-Im} + \text{galactose-1-P} \rightleftharpoons \text{UDP-galactose} + \text{imidazole} \quad (5)$$

We detected reaction 4 and measured its rate by coupling UDP-glucose formation to the production of NADH, which could be observed spectrophotometrically by its absorption at 340 nm, using the coupling enzyme UDP-glucose dehydrogenase. We detected reaction 5 by using both UDP-galactose 4-epimerase and UDP-glucose dehydrogenase as the assay enzymes coupling UDP-galactose formation to the production of NADH. We detected and measured the *reverse* of reaction 4 by coupling the production of glucose-1-P from UDP-glucose to NADPH formation using phosphoglucomutase and glucose-6-P dehydrogenase as the coupling enzymes.

Equation 6 is the rate equation for reaction 4 catalyzed by the mutant H166G, and the kinetic parameters are given in

$$v = \frac{V[A][B]}{[A][B] + K_{mA}[B] + K_{mB}[A] + K_{iA}K_{mB}} \quad (6)$$

forward reaction: A = UMP-Im; B = glucose-1-P

reverse reaction: A = UDP-glucose; B = imidazole

Table I. Equation 6 is the rate law for the rapid equilibrium random binding pathway and for the ordered steady-state binding pathway for bisubstrate enzymatic reactions (Cleland, 1970). These are sequential kinetic pathways, in which both substrates must bind to the enzyme to form a ternary complex prior to any product being released (eq 7). The kinetics,

$$\text{E} + \text{UMP-Im} + \text{glucose-1-P} \rightleftharpoons \text{E·UMP-Im·glucose-1-P} \rightleftharpoons \text{E} + \text{UDP-glucose} + \text{Im} \quad (7)$$

therefore, differ from the ping pong kinetics required for eqs 1 and 2, the kinetic mechanism for the wild-type enzyme, which does not involve ternary complex formation.

The kinetic parameters for H166G, catalyzing reaction 4 in the forward direction, are compared in Table I with the corresponding values for the wild-type enzyme catalyzing reactions 1 and 2 under the same experimental conditions. The K_m values for glucose-1-P are similar for the wild-type enzyme and H166G, and the K_m for UMP-Im is slightly smaller than that for UDP-glucose. In the reverse direction the K_m for UDP-glucose is comparable to the value for the wild-type enzyme. The K_m for imidazole, which is not a substrate for the wild-type enzyme, is 0.29 M.

The reaction of the wild-type uridylyl enzyme with glucose-1-P is compared in Figure 1 with the reaction of H166G with UMP-Im and glucose-1-P. The two reactions differ by the absence of a covalent bond and a methylene bridge between the active site and the imidazole ring in Figure 1B. The rates may be compared to a first approximation by considering the rates at substrate saturation. The values of k_{cat} differ by a factor of 267 in the direction of glucose-1-P formation and by a factor of 213 in the direction of UDP-glucose formation. Therefore, deletion of the methylene group and the covalent bonds linking the imidazole ring of H166 to the enzyme backbone reduces the catalytic efficiency at substrate saturation about 250-fold. The comparable values of k_{cat}/K_m for the mutant and wild-type enzymes differ by similar factors of 128 for the reaction of UDP-glucose and 367 for the reaction of glucose-1-P.

Properties of the Mutant H164G. Overexpression of the mutant H164G in *E. coli*, with the same vector used for the expression of H166G, leads to the appearance of the expected band on polyacrylamide gel electrophoretograms, and the protein emerges as a focused band from the QAE-Sephadex anion-exchange column used to purify the wild-type enzyme and the mutant H166G. However, the protein H164G exhibits no detectable enzymatic activities; moreover, it is much more labile to proteolysis than the native enzyme or the mutant H166G. It appears that H164 in hexose-1-P uridylyltransferase is important for maintaining the tertiary structure of the protein.

Discussion

Active Site Nucleophile. The simplest and most obvious interpretation of our results is that the active site nucleophile of hexose-1-P uridylyltransferase is the imidazole ring provided by H166. In the mutant H166G, the function of H166 in the wild-type enzyme is assumed by the imidazolate substituent of UMP-Im in the forward direction of reaction 4 and by imidazole itself in the reverse direction. H164 is also an essential component of the active site, but the available evidence does not suggest that it is the nucleophilic catalyst. It seems to be involved in maintaining the tertiary structure of the enzyme, but it may have additional essential functions in catalysis. The role of H164 is under further investigation in this laboratory.

Mutant H166G Is a New Enzyme. The mutant H166G efficiently catalyzes reactions that are not catalyzed by any other known enzyme, and it is not a hexose-1-P uridylyltransferase. It is not simply a new species of a known enzyme; therefore, it is a new enzyme. We base this claim on the following: (a) H166G catalyzes reactions 4 and 5 and does not catalyze the wild-type reaction. (b) Hexose-1-P uridylyltransferase does not catalyze reactions 4 and 5. (c) Hexose-1-P uridylyltransferase catalyzes its reaction by a ping pong kinetic pathway via a covalent uridylyl enzyme and reactions

1 and 2, whereas the mutant H166G catalyzes reaction 4 by a sequential kinetic pathway via a ternary complex. (d) No other enzyme is known to catalyze reaction 4. (e) Reactions 4 and 5 are analogous to the reactions catalyzed by nucleotide sugar pyrophosphorylases, such as UDP-glucose pyrophosphorylase, which also catalyze uridylyl transfer by single-displacement mechanisms and sequential kinetic pathways involving ternary complexes. We suggest the name *UDP-hexose synthase* for the mutant H166G of hexose-1-P uridylyltransferase. This name describes the new functional capacities of the enzyme and has not been used for any other enzyme.

Importance of the Covalent Uridylyl Enzyme. The significance and mechanistic role of covalent enzyme–substrate intermediates in enzymatic reactions have long been a subject of interest and controversy. Page and Jencks (1971) showed that the binding of a substrate to an active site can enhance a reaction rate by a factor of up to 10^8, relative to the corresponding second-order reaction in solution, owing to losses of translational and rotational entropy in the bound state. Jencks has analyzed the various contributions to catalysis that are associated with the binding of a substrate to an active site, and he has pointed out that one means by which maximal losses in translational and rotational entropy can be realized is through the formation of a covalent bond between the enzyme and substrate (Jencks, 1975, 1987). Thus, in a reaction such as that catalyzed by hexose-1-P uridylyltransferase, the histidine imidazole ring is highly immobilized by being covalently bonded to the enzyme, and the uridylyl group of the UMP–enzyme is similarly immobilized; entropy loss through covalent bonding might be a major mechanism of rate enhancement.

One of us has advanced the hypothesis that the major advantage conferred by covalent intermediates in simple enzymatic group transfer reactions is that the covalent bond conserves the bond energy of the transferred group during some physical process that is an essential step for the reaction (Frey, 1982, 1989). For example, in the simple bisubstrate group transferring enzymes that follow ping pong kinetics, the bonding potential of the group is maintained in the intermediate through a covalent bond to the enzyme during the dissociation of one product and the binding of the group acceptor. Thus, for hexose-1-P uridylyltransferase, the bonding potential of the uridylyl group (UMP) is maintained by covalent bonding to H166 in reactions 1 and 2 in the interval of time during which glucose-1-P dissociates and galactose-1-P binds.

The basic difference between the complex E-His–UMP·Glc-1-P in eq 2 and the complex E·UMP-Im·Glc-1-P in eq 5, both of which react to form UDP-glucose, is the absence in the latter complex of the β-methylene carbon and covalent bond to the phosphoimidazolate. The rate difference is about 250-fold, which is a modest factor compared with the overall rate enhancement factors of 10^{10}–10^{14} typically associated with enzymatic catalysis. The covalent bond clearly does not confer a major rate enhancement in the hexose-1-P uridylyltransferase reaction. However, the covalent phosphoimidazolate bond remains a decisively important factor as a means to maintain group transfer potential for both hexose-1-P uridylyltransferase and UDP-hexose synthase (H166G). Part of the rate loss might be attributed to the cavity created by the absence of the methylene group in H166G, which may allow nonproductive binding modes. Further protein engineering can examine the questions of whether this cavity can be filled by the methyl group of alanine in the mutant H166A and whether this mutation can restore catalytic efficiency lost to nonproductive binding in H166G.

We emphasize that the foregoing interpretation in no way brings into question the importance of substrate binding by hexose-1-P uridylyltransferase and UDP-hexose synthase (H166G). Both enzymes must bind and immobilize their substrates, and binding must lead to major losses in rotational and translational entropy of substrates. However, it appears that noncovalent enzyme–substrate binding interactions are the major means by which the rotational and translational entropies of substrates are reduced.

Surrogates of Catalytic Groups in Enzymes. The method here used to identify the active site nucleophile of hexose-1-P uridylyltransferase, the three-dimensional structure of which is unknown, is related to one that has been used to study general acid–base catalysts in enzymes for which three-dimensional structures are known. The general base function of the active site histidine in subtilisin can be partially restored in the subtilisin mutant H64A by a properly oriented histidine residue in a substrate (Carter & Wells, 1987). And base catalysis by K258 of aspartate aminotransferase can be partially restored for the mutant K258A by primary amines in solution (Toney & Kirsch, 1989). In the present work the catalytic group is a nucleophilic catalyst rather than a general acid–base catalyst, and it is carried into the active site by UMP-Im and cleaved from the substrate by the action of the mutant enzyme. Thus, the mutant catalyzes a new reaction by a different kinetic mechanism than that utilized by the wild-type enzyme. This new enzyme, UDP-hexose synthase, is a remarkably efficient enzyme in its own right.

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