Standard Free Energies for Uridylyl Group Transfer by Hexose-1-P Uridylyltransferase and UDP-Hexose Synthase and for the Hydrolysis of Uridine 5′-Phosphoimidazolate†

A. Arabshahi, F. J. Ruzicka, S. Geeganage, and P. A. Frey*

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

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ABSTRACT: The reversible reaction of UDP-glucose with imidazole (Im) to produce uridine 5′-phosphoimidazolate (UMPIm) and glucose-1-P is catalyzed by UDP-hexose synthase, which is the mutant H166G of hexose-1-P uridylyltransferase (EC 2.7.7.12) [Kim, J., Ruzicka, F. J., & Frey, P. A. (1990) *Biochemistry 29,* 10590−10593]. The availability of UDP-hexose synthase allows the equilibrium constant for the reaction UDP-glucose + Im = UMPIm + glucose-1-P to be measured, and it is found to be 2.2×10^{-2} at pH 8.5 and 27 °C. At pH 7.0 and 27 °C the equilibrium constant is 6.4×10^{-4}. The equilibrium constant for the formation of the covalent uridylyl-enzyme intermediate of hexose-1-P uridylyltransferase (**E**-His^{166} + UDP-glucose = **E**-His^{166}-UMP + glucose-1-P) is found to be 1.8×10^{-4} at pH 7.0 and 25 °C, which is slightly less favorable than the formation of UMPIm from UDP-glucose and Im. These equilibrium constants, when considered in the light of other data in the literature, allow the standard free energy changes for the hydrolysis of UMPIm and the analogous covalent uridylyl-enzyme intermediate to be calculated. The results show that $\Delta G'^{\circ}$ ($\Delta G^{\circ}_{pH7.0}$) for the hydrolyses of UMPIm and **E**-His^{166}-UMP are −14.7 and −15.4 kcal mol^{-1}, respectively at pH 7.0. At pH 8.5, the corresponding values of $\Delta G^{\circ}_{pH8.5}$ are −12.6 and −9.9 kcal mol^{-1}, respectively. It is concluded that noncovalent binding interactions between the active site and the UMP group of **E**-His^{166}-UMP provide little or no stabilization in the formation of this species as an intermediate in the reaction of hexose-1-P uridylyltransferase.

Hexose-1-P uridylyltransferase (EC 2.7.7.12) catalyzes the transformation of galactose-1-P[1] and UDP-glucose into UDP-galactose and glucose-1-P by a ping pong mechanism. The mechanism can be described by eqs 1 and 2 in the case of the *Escherichia coli* enzyme, in which the active-site nucleophilic catalyst is His^{166} (Frey et al., 1982; Kim et al., 1990):

$$\text{UDP-glucose} + \textbf{E}\text{-His}^{166} = \textbf{E}\text{-His}^{166}\text{-UMP} + \text{glucose-1-P} \quad (1)$$

$$\text{galactose-1-P} + \textbf{E}\text{-His}^{166}\text{-UMP} = \text{UDP-galactose} + \textbf{E}\text{-His}^{166} \quad (2)$$

An active-site mutant of the *E. coli* hexose-1-P uridylyltransferase catalyzes the reaction of UDP-glucose or UDP-galactose with imidazole to produce UMPIm and the corresponding hexose-1-phosphates according to eqs 3 and 4 (Kim et al., 1990):

$$\text{UDP-glucose} + \text{Im} = \text{UMPIm} + \text{glucose-1-P} \quad (3)$$

$$\text{UDP-galactose} + \text{Im} = \text{UMPIm} + \text{galactose-1-P} \quad (4)$$

The mutant enzyme lacks the active-site nucleophile His^{166}, which is replaced by Gly^{166}, and is designated H166G. Imidazole acts in place of the nucleophilic His^{166} in the wild-type enzyme; therefore, eqs 3 and 4 are analogous to eq 1, with the substitution of Im for **E**-His^{166}. No other enzyme catalyzes either eq 3 or eq 4, nor does the wild-type uridylyltransferase, so that H166G is an enzyme in its own right and has been named UDP-hexose synthase (Kim et al., 1990).

The availability of UDP-hexose synthase allows the standard free energy for eq 3 to be measured, and that for eq 1 can be measured independently. Comparison of these values will allow the effect of binding interactions between the active site and the UMP-group of **E**-His^{166}-UMP on the stability of the covalent intermediate to be assessed. The standard free energy for eq 3, when considered in the light of the standard free energy for the hydrolysis of UDP-glucose to UMP and glucose-1-P, also allows the free energy for the hydrolysis of UMPIm to be calculated. In the present paper, we report these free energies and show that the formation of the covalent intermediate in the wild-type uridylyltransferase is energized largely by the cleavage of the phosphoanhydride bond in UDP-glucose rather than by enzymatic binding interactions.

MATERIALS AND METHODS

Materials. Glucose-1-P, UDP-glucose, UDP-galactose, MOPS, NAD$^+$, NADP$^+$, glucose-6-P dehydrogenase, phosphoglucomutase, UDP-glucose dehydrogenase, Tris, EDTA, DTT, and Sephadex G-25 were purchased from Sigma. The following were obtained from the vendors indicated: Q-Sepharose (fast flow) and DEAE-Sephadex from Pharmacia;

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[1] Abbreviations: UDP-glucose, uridine 5′-diphosphate glucose; UMPIm, uridine 5′-phosphoimidazolate; glucose-1-P, α-D-glucose 1-phosphate; galactose-1-P, α-D-galactose 1-phosphate; Im, imidazole; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NAD$^+$, nicotinamide adenine dinucleotide; NADP$^+$, nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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Affi-Gel Blue from Bio-Rad; PM 30 ultrafilter membranes and Microcon 10 microconcentrators from Amicon, and Ultrafree-MC 10K NMWL filter unit from Millipore. Hexose-1-P uridylyltransferase was purified from *E. coli* BL21-(DE3)pLysS cells transformed with plasmid pTZ18ROT (Wedekind, 1995) by a modification of the procedure of Arabshahi et al. (1986). All buffers contained 10 mM 2-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride. The pooled fractions from the Affi-Gel Blue column were desalted by ultrafiltration using an Amicon system equipped with a PM30 membrane instead of by gel-permeation chromatography, and the DEAE-Sephadex column was replaced with a Q-Sepharose column. The purified enzyme was concentrated in the Amicon ultrafiltration system and frozen as pellets in liquid nitrogen. The mutant H166G of this enzyme, UDP-hexose synthase, was purified by the same procedure from *E. coli* CA 13 transformed with the expression vector containing the mutant gene (Kim et al., 1990). UMPIm was synthesized as described (Ruzicka & Frey, 1993).

Assays. Hexose-1-P uridylyltransferase was assayed in the direction of glucose-1-P formation as described (Wong & Frey, 1974). Glucose-1-P was assayed by an enzymatic−fluorometric method (Passoneau & Lowry, 1993) and UDP-glucose by an enzymatic−spectrophotometric method (Keppler & Decker, 1974). UMPIm and UDP-glucose were assayed by HPLC using a Waters Nova-Pak column for the stationary phase and 5 mM NaCl as the mobile phase. Retention times for UMPIm and UDP-glucose were 10 and 1 min, respectively.

Equilibrium Constant for Uridylylation of Hexose-1-P Uridylyltransferase. The equilibrium constant for the reaction of the uridylyltransferase with UDP-glucose to produce the covalent UMP-enzyme according to eq 1 was measured at pH 7.0 by measuring the amount of glucose-1-P produced. The reaction was carried out at 0, 21, and 42 μM uridylyltransferase and 0.98 or 1.96 mM UDP-glucose to verify whether the equilibrium constant is independent of these concentrations, as it proved to be. The reaction mixtures (255 μL) consisted initially of 0−42 μM enzyme (120 units mg^{-1}), 0.98 or 1.96 mM UDP-glucose, and 95 mM MOPS buffer at pH 7.0 and 25 °C. The reactions proceeded for 15 min, except for two that were allowed to proceed for 30 or 60 min to confirm that the reaction had reached equilibrium in 15 min. The reactions were stopped by addition of 2 μL of 0.25 M EDTA and heating in boiling water for 2 min. The coagulated enzyme was removed by centrifugation, and the supernatant fluids were filtered through an Ultrafree-MC from Millipore (cutoff 10K). The filtrates were assayed for glucose-1-P by the enzymatic−fluorometric method. The concentrations of glucose-1-P produced ranged from zero in the absence of enzyme to 2.9 μM at the highest enzyme and UDP-glucose concentrations. Glucose-1-P was found in control experiments to be stable under the conditions of the reaction and sample preparation, and additional control experiments verified that ultrafiltration to remove the enzyme did not alter the concentration of glucose-1-P.

Equilibrium Constant for Reaction of UDP-Hexose with Im. The equilibrium constant was measured by observing the reaction in both forward and reverse directions at pH 7.0 and 8.5 at 27 °C. The reaction mixtures in the forward direction consisted at pH 7.0 of 2.0 mM UDP-glucose, 20 mM Im, 95 mM MOPS buffer, and sufficient UDP-hexose synthase to bring the reaction to equilibrium within a few minutes (0.1 mg). After 180 min at pH 7.0, samples of the solution were passed through a Microcon 10 filter to remove the enzyme, and the filtrate was adjusted to pH 8.3 by use of sodium bicinate. Aliquots were analyzed for UMPIm and UDP-glucose by HPLC. At pH 8.5 the initial solution contained 1.87 mM UDP-glucose, 20 mM Im, 95 mM sodium bicinate buffer, and sufficient UDP-hexose synthase (mutant H166G of uridylyltransferase) to bring the reaction to equilibrium within a few hours (0.04 mg). After 30 h at pH 8.5, the sample was heated for 3 min at 100 °C and aliquots were subjected to analysis by HPLC for UMPIm and UDP-glucose. The results of control experiments showed UMPIm and UDP-glucose to be stable under the conditions of the reaction and sample preparation, and the enzyme remained active throughout the approach to equilibrium. In the reverse direction, the reaction mixtures consisted initially of 2.0 mM UMPIm, 2 mM glucose-1-P, 20 mM Im, 95 mM sodium bicinate at pH 8.5 or 95 mM MOPS buffer at pH 7.0, and sufficient enzyme to bring the reaction to equilibrium within a few minutes at pH 7.0 or a few hours at pH 8.5. After 180 min at pH 7.0, samples were passed through a Microcon 10 filter to remove the enzyme, and the filtrate was adjusted to pH 8.3 and analyzed for UMPIm and UDP-glucose by HPLC. After 30 h at pH 8.5, the sample was heated for 3 min at 100 °C and aliquots were subjected to analysis by HPLC for UMPIm and UDP-glucose.

RESULTS AND DISCUSSION

Equilibrium Constant in the Formation of UMPIm. The reaction of UDP-glucose with Im to produce UMPIm and glucose-1-P (eq 3) is catalyzed by UDP-hexose synthase (mutant H166G of hexose-1-P uridylyltransferase). The equilibrium constant can be measured by HPLC analysis for the ratio UMPIm/UDP-glucose at equilibrium, and the equilibrium is readily observable in both directions through the judicious selection of initial reactant and product concentrations. We find the value of $K_{eq}8.5$ to be 2.20×10^{-2}, when measured in the forward direction, and 2.17×10^{-2} when measured by approach to equilibrium from the reverse direction. Our values for K_{eq}' are 6.4×10^{-4} when measured in the forward direction and 5.3×10^{-4} when measured by approach to equilibrium from the reverse direction. Taking the mean values, the ratio $K_{eq}8.5/K_{eq}'$ is 38, which is in accord with the effects of pH and the values of pK_a for Im, 6.99; glucose-1-P, 6.13; and UMPIm, 5.68 (Ruzicka & Frey, 1993).

Equilibrium Constant in the Formation of ***E****-His^{166}-UMP.* The covalent intermediate of hexose-1-P uridylyltransferase is produced in its reaction with UDP-glucose according to eq 1. The equilibrium constant at pH 8.5 ($K_{eq}8.5$) is 2.1 (Wong & Frey, 1974). This value shows that the covalent intermediate is practically isoenergetic with UDP-glucose at the active site at pH 8.5. The value of the equilibrium constant at pH 7.0, as calculated from the amount of glucose-1-P formed at several enzyme and UDP-glucose concentrations in five reactions, is $(1.8 \pm 0.2) \times 10^{-4}$. The lower value of K_{eq}' compared with $K_{eq}8.5$ can be attributed only in part to the consequences of lower pH and the pK_a of His^{166}. As shown above, $K_{eq}8.5$ for the reaction of UDP-glucose with Im to produce UMPIm and glucose-1-P (eq 3) is more favorable than K_{eq}' by a factor of 38, whereas $K_{eq}8.5$ is more

Table 1: Standard Free Energy Changes for Reactions of UDP-Glucose, **E**-His^{166}-UMP, and UMPIm

reaction	$\Delta G'^{\circ}$ (kcal mol^{-1})	$\Delta G^{\circ}_{pH8.5}$ (kcal mol^{-1})
UDP-glucose + H_2O = UMP + glucose-1-P	−10.3[a]	−10.3[a]
UMPIm + glucose-1-P = UDP-glucose + Im	−4.4	−2.3
UMPIm + H_2O = UMP + Im	−14.7	−12.6
E-His166-UMP + glucose-1-P = **E**-His166 + UDP-glucose	−5.1	0.44[b]
E-His166-UMP + H_2O = **E**-His166 + UMP	−15.4	−9.9

[a] Frey & Arabshahi, 1995. [b] Wong & Frey, 1974.

favorable by 12 000-fold in the formation of the covalent intermediate.

*Standard Free Energy Changes for Reactions of **E**-His^{166}-UMP and UMPIm.* UMPIm is a donor of the uridine 5′-phosphoryl group and is expected to be a high-energy compound. The standard free energy change for the hydrolysis of UMPIm can be calculated from the free energies of the reactions studied in this paper and that for the hydrolysis of UDP-glucose to UMP and glucose-1-P (Frey & Arabshahi, 1995). The standard free energy change for the hydrolysis of the covalent intermediate **E**-His^{166}-UMP can be calculated in the same way, and the values for $\Delta G'^{\circ}$ and $\Delta G^{\circ}_{pH8.5}$ are given in Table 1.[2]

At pH 7, the standard free energies for hydrolysis of UMPIm and the uridylyl-enzyme form of hexose-1-P uridylyltransferase are similar and about 5 kcal mol^{-1} more negative than that for the hydrolysis of UDP-glucose to UMP and glucose-1-P. UMPIm and **E**-His^{166}-UMP are, therefore, very high-energy species. The standard free energies for their hydrolysis are more negative at pH 7 than at pH 8.5. It is interesting that uridylyl group transfer to the enzyme by UDP-glucose is favored by about −0.4 kcal mol^{-1} at pH 8.5 but is disfavored by about 5 kcal mol^{-1} at pH 7.0. The molecular basis for this difference is under investigation in this laboratory.

Inasmuch as the standard free energy changes for the hydrolysis of UMPIm and **E**-His^{166}-UMP are comparable at pH 7.0, noncovalent binding interactions between the active site and the uridylyl group of the covalent intermediate cannot be enhancing the stability of the intermediate. Therefore, the driving force for the formation of the uridylyl-enzyme is derived from the free energy of the phosphoanhydride bond in UDP-glucose. At pH 8.5, the covalent intermediate is 2.7 kcal mol^{-1} more stable to hydrolysis than UMPIm, which represents only a moderate stabilization of the enzymatic intermediate through noncovalent interactions within the active site. The absence of noncovalent stabilization of the uridylyl group in **E**-His^{166}-UMP at pH 7.0 stands in contrast to the substantial noncovalent stabilization of tyrosyl adenylate at the active site of tyrosyl-tRNA synthetase (Fersht, 1987).

In addition to being intermediates in uridylyltransferase reactions, nucleoside 5′-phosphoimidazolates are intermediates in nonenzymatic nucleotide synthesis (Eckstein & Gindl, 1970). They are also used as models for activated nucleotides in template-directed nonenzymatic synthesis of oligonucleotides (Inoue & Orgel, 1983; Orgel, 1986). Nucleoside 5′-phosphoimidazolates have been regarded as high-energy species, but the standard free energy for hydrolysis to the nucleoside 5′-monophosphates and Im has not been known. The standard free energy for the hydrolysis of uridine 5′-phosphoimidazolate reported in this paper should be similar to that for other nucleoside 5′-phosphoimidazolates.

[2] The recommendation of the International Union of Pure and Applied Chemistry is that $\Delta G'^{\circ}$ refers to total concentrations of the ionic species of each component of the reaction at pH 7.0, 25 °C, and 1 mM free Mg^{2+} (Alberty, 1994). In this paper, most standard free energy changes follow this recommendation except for the absence of Mg^{2+}, which does not participate in the reactions described in this paper. In cases in which free energy changes are reported at pH 8.5, they will be specified as $\Delta G^{\circ}_{pH8.5}$.

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