

Kinetic and Structural Analysis of α -D-Glucose-1-phosphate Cytidylyltransferase from *Salmonella typhi**[§]

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Tyvelose is a 3,6-dideoxyhexose found in the *O*-antigen of the surface lipopolysaccharides of some pathogenic bacteria. It is synthesized via a complex biochemical pathway that is initiated by the formation of CDP-D-glucose. The production of this ligand is catalyzed by the enzyme glucose-1-phosphate cytidylyltransferase, which utilizes α -D-glucose 1-phosphate and MgCTP as substrates. Previous x-ray crystallographic investigations have demonstrated that the *Salmonella typhi* enzyme complexed with the product CDP-glucose is a fully integrated hexamer displaying 32 point group symmetry. The binding pocket for CDP-glucose is shared between two subunits. Here we describe both a detailed kinetic analysis of the cytidylyltransferase and a structural investigation of the enzyme complexed with MgCTP. These data demonstrate that the reaction catalyzed by the cytidylyltransferase proceeds via a sequential rather than a Bi Bi ping-pong mechanism as was previously reported. Additionally, the enzyme utilizes both CTP and UTP equally well as substrates. The structure of the enzyme with bound MgCTP reveals that the binding pocket for the nucleotide is contained within one subunit rather than shared between two. Key side chains involved in nucleotide binding include Thr¹⁴, Arg¹⁵, Lys²⁵, and Arg¹¹¹. In the previous structure of the enzyme complexed with CDP-glucose, those residues defined by Thr¹⁴ to Ile²¹ were disordered. The kinetic and x-ray crystallographic data presented here support a mechanism for this enzyme that is similar to that reported for the glucose-1-phosphate thymidylyltransferases.

O-Antigens are glycan side chains of bacterial surface lipopolysaccharides that play a role in pathogenicity and are recognized by the immunological defense system of the host (1). Tyvelose, as shown in Scheme 1, is a 3,6-dideoxyhexose that occurs in the *O*-antigens of some types of Gram-negative bacteria such as *Yersinia pseudotuberculosis* IVA and *Salmonella typhi* (2). It is produced via a complex biochemical pathway

that employs α -D-glucose 1-phosphate as the starting ligand. The first committed step in the pathway, as shown in Scheme 1, is the transfer of a CMP moiety from CTP to glucose 1-phosphate. This reaction is catalyzed by α -D-glucose-1-phosphate cytidylyltransferase, which was first purified in the early 1960s from various bacterial species (3–7). These initial reports established the kinetic parameters of the enzyme and were in general agreement despite the varying sources of protein. All of the studies demonstrated that the cytidylyltransferase has a strict preference for CTP, with only trace activity in the presence of dCTP and UTP, and no measurable activity with any other NTP or dNTP (4, 6, 7). The enzyme was shown to have maximal activity at pH ~8 and to display feedback inhibition by CDP-paratose, CDP-tyvelose, and other intermediates in the production of CDP-tyvelose (4–7). Additionally, the type of inhibition observed was consistent with multiple binding sites for the inhibitor. The overall reaction was shown to be readily reversible with an equilibrium constant in the direction of CDP-glucose formation of 0.57 to 1.0 (4, 6). These initial reports also indicated that the quaternary structure of the enzyme was monomeric with an overall molecular weight of 110,000 (7).

Following these earlier studies, highly purified samples of α -D-glucose-1-phosphate cytidylyltransferase were subsequently obtained from *Salmonella enterica* strain LT2, *Y. pseudotuberculosis*, and *Streptomyces glaucescens* GLA.0 (8–10). In contrast, these investigations indicated the quaternary structure of the enzyme to be tetrameric rather than monomeric with a subunit molecular weight of ~30,000 (9). A detailed kinetic analysis of the cytidylyltransferase from *S. enterica* suggested that its catalytic mechanism proceeds via a Bi Bi ping-pong reaction (8). In such a mechanism, CTP binds first to the enzyme followed by an attack of an enzymatic base on the α -phosphorus of the nucleotide, resulting in the formation of an enzyme-CMP covalent intermediate and the release of pyrophosphate. Glucose 1-phosphate subsequently binds and conducts a nucleophilic attack on the phosphorus of the enzyme-CMP intermediate resulting in CDP-glucose formation. Strikingly, this mode of catalysis is in sharp contrast to the sequential mechanism reported for the related glucose-1-phosphate thymidylyltransferases (11, 12).

Recently, the x-ray crystallographic structure of the *S. typhi* glucose-1-phosphate cytidylyltransferase complexed with CDP-glucose was determined in this laboratory (13). The enzyme crystallized in the space group P6₃22 with one subunit in the asymmetric unit. Contrary to previous reports, the crystallographic data clearly demonstrated the enzyme to be a fully integrated hexamer displaying 32 point group symmetry. Subsequent analytical ultracentrifugation experiments confirmed the hexameric nature of the cytidylyltransferase (13). A view of one-half of the hexamer is shown in Fig. 1. Note that each active site is formed by residues contributed from two subunits, specifically those related by the crystallographic dyads. Each subunit displays a “bird-like” appearance with the “body” dom-

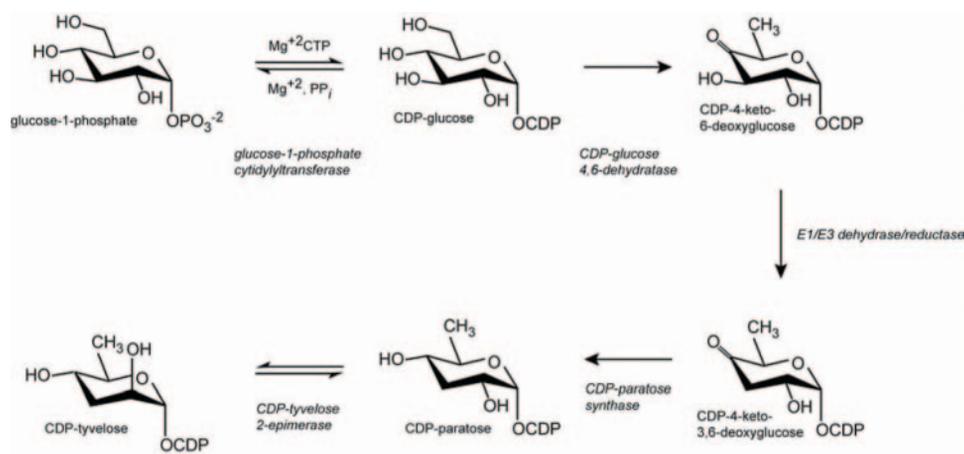
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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Fig. S1.

The atomic coordinates and structure factors (code 1WVC) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

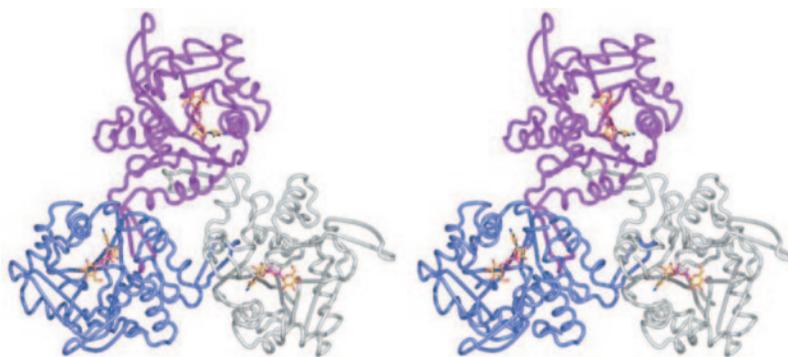
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SCHEME 1

FIG. 1. Ribbon representation of glucose-1-phosphate cytidylyltransferase from *S. typhi*. The cytidylyltransferase from *S. typhi* is a hexamer displaying 32 symmetry. For the sake of clarity, only half of the hexamer is shown. The active sites are indicated by the positions of the CDP-glucose ligands, which are displayed in ball-and-stick representations.



initiated by a seven-stranded mixed β -sheet and the two “wings” formed by β -hairpin motifs. Here we present both a detailed kinetic analysis of the enzyme and the three-dimensional structure of the protein complexed with CTP. These biochemical and x-ray crystallographic data indicate that the reaction proceeds via a sequential mechanism, that the enzyme can utilize both UTP and CTP, that the binding site for CTP is contained within a single subunit of the hexamer, and that the wings of each subunit adopt different orientations when CTP *versus* CDP-glucose is bound in the active site.

EXPERIMENTAL PROCEDURES

Protein Purification—The *S. typhi* glucose-1-phosphate cytidylyltransferase was purified according to previously published procedures (13). All chemicals and enzymes were purchased from Sigma. The concentrations of CTP, CDP-glucose, UTP, and UDP-glucose were determined by UV spectrophotometry assuming extinction coefficients at 271 nm of $9.3 \text{ mM}^{-1} \text{ cm}^{-1}$ for CTP and CDP-glucose and $10.3 \text{ mM}^{-1} \text{ cm}^{-1}$ for UTP and UDP-glucose.

Enzymatic Assays for the Forward and Reverse Reactions—The activity of glucose-1-phosphate cytidylyltransferase in the forward reaction was followed by two spectrophotometric methods. Absorbance readings were taken with a Beckman DU 640B spectrophotometer equipped with a Peltier temperature-controlled 6-cell autosampler. The substrate specificity of cytidylyltransferase was assessed in a coupled assay in which the production of pyrophosphate was linked to the oxidation of NADH. These assays were performed in a discontinuous manner utilizing the pyrophosphate detection kit from Sigma (product P-7275). The pyrophosphate detection kit contained the coupling enzymes pyrophosphate-dependent phosphofructokinase, aldolase, triose-phosphate isomerase, and glycerophosphate dehydrogenase, as well as the appropriate substrates. For this set of experiments, the pyrophosphate reagent was prepared by resuspending one vial of the lyophilized reagent mixture in 2 ml of sterile water, rather than the recommended 4 ml. The assay was initiated by the addition of cytidylyltransferase (18 μl , 0.25 $\mu\text{g}/\mu\text{l}$) to 3 ml of solution containing 1–2 mM NTP, 3–4 mM MgCl_2 , 1–2 mM of various sugar 1-phosphates, and 25 mM HEPPS (pH 8.0). At various times (0 min to 24 h), 800 μl were removed and quenched by the addition of 40 μl of 2 M HCl and 20 μl of CCl_4 followed by 30 s of

vortexing. These aliquots were subsequently centrifuged for 1 min and 800 μl were withdrawn and transferred to a Microfuge tube containing 35 μl of 2 M NaOH to neutralize the pH. To this solution, 165 μl of the pyrophosphate reagent were added and the reaction was incubated at 30 $^\circ\text{C}$ for 20 min. The amount of pyrophosphate produced over time was assessed by the decrease in absorbance at 340 nm according to the manufacturer's instructions. Negative controls were performed in which cytidylyltransferase, CTP, or glucose 1-phosphate were omitted from the reaction.

The kinetic constants for the cytidylyltransferase forward reaction were assessed via a continuous coupled assay for the detection of pyrophosphate. Unfortunately, an unidentified contaminant in the Sigma pyrophosphate detection kit acted as a substrate for the cytidylyltransferase reaction when only CTP or UTP were added to the reaction. Therefore, this kit could not be used to perform a continuous assay. Instead, the individual reagents of the kit were purchased from Sigma and the pyrophosphate reagent was reconstituted from the individual components. Each 350- μl reaction contained 14.4 mM imidazole (pH 7.4), 1.6 mM citrate, 32 μM EDTA, 64 μM MnCl_2 , 6.4 μM CoCl_2 , 256 μM β -NADH, 3.8 mM fructose 6-phosphate, 0.06 units of pyrophosphate-dependent phosphofructokinase, 0.6 units of glycerophosphate dehydrogenase, 5.6 units of triose-phosphate isomerase, 0.84 units of aldolase, 0.5 mg of bovine serum albumin, and 0.35 μg of cytidylyltransferase, and varying amounts of CTP with MgCl_2 and glucose 1-phosphate. CTP was varied from 50 μM to 1 mM at fixed levels of glucose 1-phosphate in the range of 25 μM to 1 mM. The MgCl_2 concentration was fixed at 2.5 mM above the concentration of CTP in each reaction. 40 μl of the variable substrate, glucose 1-phosphate or CTP and MgCl_2 , were transferred to a quartz cuvette and the reaction was initiated by the addition of 310 μl containing the remaining reaction components. The reaction was quickly mixed and the absorbance at 340 nm was monitored every 15 s over 7 min. During this time, the reactions were kept at a constant temperature of 26 $^\circ\text{C}$. The continuous assay was also performed varying α -D-xylose 1-phosphate while keeping the concentration of CTP fixed at 1 mM. Similarly, the concentrations of dCTP or UTP were varied while maintaining the concentration of glucose 1-phosphate at 1 mM. In the assays involving dCTP and xylose 1-phosphate, 20 times the concentration of cytidylyltransferase was required to achieve an observable reaction velocity. To compare reaction velocities, the rate of the reaction with CTP and glucose 1-phosphate at this higher enzyme concentration

was also measured. Control experiments for the continuous assay were performed in which cytidylyltransferase, glucose 1-phosphate, or NTP were omitted from the assay to ensure that pyrophosphate production did not occur unless both of the substrates and cytidylyltransferase were present.

The products of the reaction with CTP, UTP, glucose 1-phosphate, and xylose 1-phosphate were identified via HPLC¹ analysis. HPLC analysis of the reaction products was performed on an ÄKTA HPLC (Amersham Biosciences) equipped with a 1-ml Resource Q column using two different sets of conditions. The reaction products were analyzed at pH 4.0 with a gradient of 20 to 500 mM ammonium acetate over 20 ml and at pH 8.5 with a gradient of 20 to 500 mM ammonium carbonate over 20 ml. The products of the reaction were identified by comparing the chromatograms to those of authentic samples of CMP, UMP, CDP, UDP, CDP-glucose, UDP-glucose, CTP, or UTP.

The kinetic constants for the reverse (NTP synthesis) reaction were determined via a discontinuous HPLC assay. Each 1–5-ml reaction contained 1 µg/ml cytidylyltransferase and 25 mM HEPPS (pH 8.0), whereas the concentrations of CDP-glucose and sodium pyrophosphate or UDP-glucose and sodium pyrophosphate were varied over the range of 20 µM to 10 mM. The concentration of MgCl₂ was fixed at 2 mM above the concentration of pyrophosphate. Reaction aliquots were removed at various times and quenched with HCl and carbon tetrachloride as described for the discontinuous coupled assay. One-ml samples of the reaction were injected onto a 1-ml Resource Q column pre-equilibrated with 20 mM ammonium acetate (pH 4.0). The NTP product was separated from the nucleotide 5'-diphosphate-glucose substrate with a linear gradient from 0 to 300 mM LiCl over 20 ml. The elution profile was monitored by UV absorbance at 271 nm. The retention volumes for CDP-glucose, CTP, UDP-glucose, and UTP were 9.2, 13.4, 9.7, and 15.3 ml, respectively. The area under the elution peak for UTP and CTP was calculated and compared with a standard curve relating the area under the elution peak to the micromoles of NTP.

Measurement of the Equilibrium Constant and Divalent Cation Dependence—The equilibrium constant and Mg²⁺ dependence for the cytidylyltransferase reaction were assessed via an HPLC-based assay to detect the relative proportion of CDP-glucose to CTP and vice versa. For the measurement of the equilibrium constant, the standard reaction contained 1 mM each of sodium pyrophosphate and CDP-glucose or 1 mM each of CTP and glucose 1-phosphate, 3 mM MgCl₂, and 25 mM HEPPS (pH 8.0). Each 1-ml reaction contained 2.5 µg of cytidylyltransferase. To determine the dependence of the cytidylyltransferase reaction on the concentration of Mg²⁺, CTP and glucose 1-phosphate were fixed at 0.2 and 1 mM, respectively, whereas the concentration of MgCl₂ was varied from 0.7 to 5.2 mM. These 1-ml reactions contained 1.0 µg of cytidylyltransferase. The reaction products were separated on a 1-ml Resource Q column with a linear gradient of 20 to 250 mM ammonium carbonate (pH 8.5) over 20 ml. The elution profile was monitored by UV absorbance at 271 nm and the area under the peaks was used to determine the proportion of CDP-glucose to CTP.

Kinetic Data Analysis—The kinetic constants for the forward reaction were assessed using the data obtained from the continuous spectrophotometric assay. Before the final data analysis, the initial velocity pattern was determined by plotting each data set to the equation.

$$\frac{1}{v} = \left(\frac{K}{V}\right)\frac{1}{A} + \left(\frac{1}{V}\right) \quad (\text{Eq. 1})$$

The double reciprocal plot clearly showed a series of intersecting lines suggesting a sequential mechanism (Fig. 2). Therefore, all 60 data points from the continuous assay with CTP and glucose 1-phosphate were fitted to Equation 2.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (\text{Eq. 2})$$

The data were fitted with the program SEQUENO to calculate V , K_a , K_b , K_{ia} , and K_{ib} from $K_{ib} = (K_{ia}K_b)/K_a$, where K_{ia} and K_{ib} are the dissociation constants for binary EA and EB complexes, K_a and K_b are Michaelis constants for A and B, and V is the maximum velocity (14). In this equation, A is the concentration of CTP, whereas B is the concentration of glucose 1-phosphate.

The kinetic parameters for the back reaction and for the alternate substrates for the forward reaction were obtained by fitting the data to the Michaelis-Menten equation (Equation 3) of the form.

¹ The abbreviations used are: HPLC, high performance liquid chromatography; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.

TABLE I
X-ray data collection statistics

Wavelength (Å)	0.95730
Resolution limits (Å)	50–2.5
	2.59–2.5 ^a
No. independent reflections	11,780
Completeness (%)	1,064
	97.4
Redundancy	90.3
	9.5
	5.3
Average I/average σ (I)	26.4
	3.1
R_{sym} (%)	6.2
	27.4

^a Statistics for the highest resolution bin.

^b $R_{\text{sym}} = (\sum |I - \bar{I}| / \sum I) \times 100$.

$$v = \frac{VA}{K + A} \quad (\text{Eq. 3})$$

Crystallization of Glucose-1-phosphate Cytidylyltransferase—Prior to crystallization, the protein was concentrated to 9.5 mg/ml. CTP and MgCl₂ were added to final concentrations of 4 and 8 mM, respectively. Crystals were grown by the hanging drop method of vapor diffusion against a precipitant solution containing 24–28% poly(ethylene glycol) 400, 100 mM MgCl₂, 100 mM HEPES (pH 7.0). The crystals belonged to the space group P6₃22 with unit cell dimensions of $a = b = 84.2$, $c = 157.4$, and one subunit per asymmetric unit.

X-ray Data Collection and Structural Analysis—Prior to x-ray data collection, the crystals were serially transferred from the hanging drop experiments to a cryoprotectant solution containing 30% poly(ethylene glycol) 400, 10% ethylene glycol, 400 mM NaCl, 100 mM MgCl₂, 4 mM CTP, and 100 mM HEPES (pH 7.0), flash-cooled to –150 °C in a stream of nitrogen vapor, and stored under liquid nitrogen until synchrotron beam time became available. X-ray data from a single crystal were collected on a CCD detector at the COM-CAT 32-ID beamline (Advanced Photon Source, Argonne National Laboratory, Argonne, IL). All x-ray data were processed with HKL2000 and scaled with SCALEPACK (15). Relevant x-ray data collection statistics are presented in Table I.

The structure of *S. typhi* glucose-1-phosphate cytidylyltransferase complexed with CTP was solved via molecular replacement with the program AMoRe and employing as a search model the previously determined structure of the enzyme with bound CDP-glucose (Protein Data Bank 1TZF) (16). Alternate cycles of manual model building with the graphics program TURBO (17) and least-squares refinement with TNT (18) reduced the R-factor to 20.2% for all measured x-ray data from 25 to 2.5 Å. The polypeptide chain extends from Ala²-Ala¹⁶², Gly¹⁶⁴, Gln¹⁷⁷, and Lys¹⁸¹-Glu²⁵⁹. There is a metal coordinated to His⁷⁹, which is most likely a nickel ion resulting from the nickel-nitriloacetic acid column chromatography purification. Note that the His₈ tag at the N terminus was removed from the protein prior to the kinetic and structural analyses. Relevant least-squares refinement statistics are presented in Table II.

RESULTS AND DISCUSSION

To determine the substrate specificity for cytidylyltransferase, pyrophosphate production was assessed utilizing the pyrophosphate detection kit marketed by Sigma. The control experiments, however, indicated that pyrophosphate was produced even when only CTP and cytidylyltransferase were added to the coupling enzymes. The products from the reaction containing CTP, coupling reagent, and cytidylyltransferase were verified by HPLC and indicated that in the absence of glucose 1-phosphate, a compound consistent with the HPLC profile of CDP-glucose was produced. In addition, electrospray ionization mass spectrometry on the product of this reaction showed that it had the same molecular weight as CDP-glucose. It should be noted that CMP was not detected as a product of this reaction nor was it detected in prolonged incubations of cytidylyltransferase, CTP, and MgCl₂. Because the pyrophosphate reagent in the detection kit appeared contaminated with a substrate for the cytidylyltransferase reaction, this reagent

TABLE II
Least-squares refinement statistics

Resolution limits (Å)	25.0–2.5
R-factor ^a (overall) %/No. of reflections	20.2 (11746)
R-factor (working) %/No. of reflections	19.9 (10623)
R-factor (free) %/No. of reflections	27.2 (1123)
No. of protein atoms	1996
No. of hetero-atoms	123
Average B values (Å ²)	
Protein atoms	48.7
CTP	42.8
Solvents	52.2
Weighted root mean square deviations from ideality	
Bond lengths (Å)	0.010
Bond angles (deg)	2.2
Trigonal planes (Å)	0.004
General planes (Å)	0.006
Torsional angles ^b (deg)	20.0

^a R-factor = $(\sum |F_o - F_c| / \sum |F_o|) \times 100$, where F_o is the observed structure-factor amplitude and F_c is the calculated structure-factor amplitude.

^b The torsional angles were not restrained during the refinement.

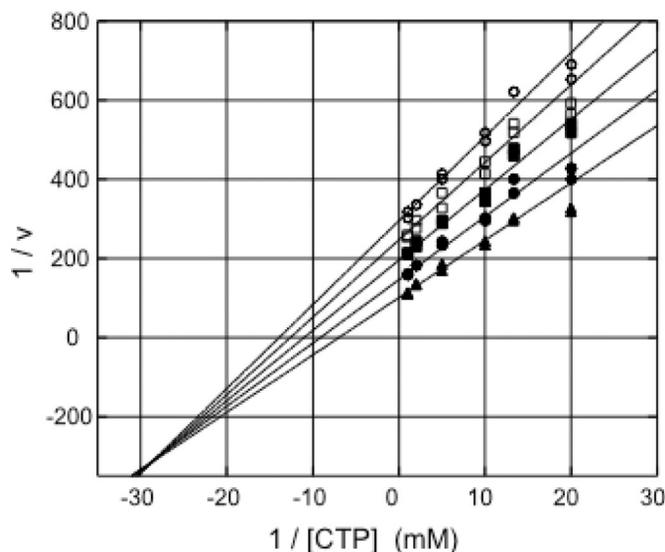


FIG. 2. Double reciprocal plots of initial rates of formation of pyrophosphate. In this plot, the concentration of CTP is varied at several fixed concentrations of glucose 1-phosphate equal to 25 μM (open circles), 33 μM (open squares), 50 μM (filled circles), 0.1 mM (filled circles), and 1 mM (filled triangles). Initial velocities (v) are expressed as millimolar/min of pyrophosphate.

could only be utilized to perform a discontinuous assay as described under "Experimental Procedures." In this set of assays, CTP, dCTP, UTP, dUTP, ATP, and GTP were tested for activity with glucose 1-phosphate. Similarly, D-glucose 1-phosphate, D-xylose 1-phosphate, D-mannose 1-phosphate, D-galactose 1-phosphate, D-fructose 6-phosphate, and D-glucose 6-phosphate were tested as substrates with CTP. Only reactions containing CTP, dCTP, UTP, glucose 1-phosphate, and xylose 1-phosphate produced detectable levels of pyrophosphate. The kinetic parameters of the cytidylyltransferase reaction with these substrates were further assessed in a continuous assay system.

To confirm the identity of the products from the cytidylyltransferase reaction in the forward direction, samples of the reaction with CTP, dCTP, UTP, glucose 1-phosphate, and xylose 1-phosphate were analyzed by HPLC. The substrate and product peaks from the reaction were identified by comparing the retention volumes to those of known samples of CTP, CDP,

TABLE III
Kinetic parameters for the forward reaction with CTP and glucose 1-phosphate

These parameters were generated from a least-squares fit of the data to the rate equation for a sequential mechanism, assuming A = CTP and B = glucose 1-phosphate. The fit gave $\sigma = 0.18 \mu\text{M}/\text{min}$.

V_{\max}	110.4 (± 0.2) $\mu\text{M}/\text{min}$
K_A	148 (± 7) μM
K_B	52 (± 2) μM
K_i^a	35 (± 8) μM
K_{ib}	12 (± 3) μM

TABLE IV
Kinetic parameters of the reverse reaction (NTP synthesis)
These parameters were calculated by fitting the data to the Michaelis-Menten equation.

Substrate	K_m (μM)	V_{\max} ($\mu\text{M}/\text{min}$)
CDP-glucose	11 (± 2)	8.0 (± 0.2)
Pyrophosphate	67 (± 8)	8.3 (± 0.3)
UDP-glucose	2535 (± 240)	7.6 (± 0.3)

CMP, CDP-glucose, UTP, UDP, UMP, and UDP-glucose at both pH 8.5 and 4.0. In all reactions, the only product identified was the nucleotide 5'-diphosphate-sugar, with no concomitant formation of nucleotide 5'-monophosphate. In addition, prolonged incubation of cytidylyltransferase with CTP or UTP did not result in CMP or UMP formation, confirming that the enzyme does not perform a pyrophorylysis side reaction. Thus it can be concluded that the measurement of pyrophosphate is an appropriate method to determine the kinetics of product formation.

The kinetic constants for the forward cytidylyltransferase reaction were determined using a continuous assay coupling the production of pyrophosphate to the oxidation of NADH. To establish whether the cytidylyltransferase reaction follows a sequential or ping-pong mechanism, the glucose 1-phosphate concentration was varied at several fixed concentrations of CTP while measuring the initial velocity of the reaction. Double reciprocal plots of the initial velocity with respect to substrate concentration resulted in a series of lines that intersected in the lower left quadrant as shown in Fig. 2. The initial velocity pattern is consistent with a sequential mechanism for cytidylyltransferase. This is in sharp contrast to that previously reported for the *Salmonella* glucose-1-phosphate cytidylyltransferase that suggested a ping-pong mechanism on the basis of double reciprocal plots of the initial velocity pattern (8). The initial velocity data in the present investigation were fitted to the rate equation for a sequential mechanism using the least-squares fitting program SEQUENO (14). The data fit the rate equation for a sequential mechanism well with $\sigma = 0.18 \mu\text{M}/\text{min}$ for the initial velocity values. The results from this analysis are reported in Table III. The K_m for CTP (K_A) is $148 \pm 7 \mu\text{M}$ and the K_m for glucose 1-phosphate (K_B) is $52 \pm 2 \mu\text{M}$. The V_{\max} for the forward reaction with CTP and glucose 1-phosphate is $10.4 \pm 0.2 \mu\text{M}/\text{min}$. The dissociation constants (K_i) of CTP and glucose 1-phosphate are 35 ± 8 and $12 \pm 3 \mu\text{M}$, respectively. The fact that the dissociation constants are lower than the K_m values for CTP and glucose 1-phosphate argues that the substrates bind in an anti-synergistic fashion in the forward reaction.

The continuous assay was also utilized to determine the kinetic parameters from the reaction with UTP and glucose 1-phosphate, dCTP and glucose 1-phosphate, and CTP and xylose 1-phosphate. The K_m for UTP is $159 \pm 8 \mu\text{M}$ and the V_{\max} of the reaction is $15.4 \pm 0.3 \mu\text{M}/\text{min}$. The ratio of the V_{\max} of the reaction with UTP versus CTP is 1.5 ± 0.04 , suggesting that the rates of the reaction with UTP or CTP are essentially the same. These data indicate that the enzyme appears to utilize

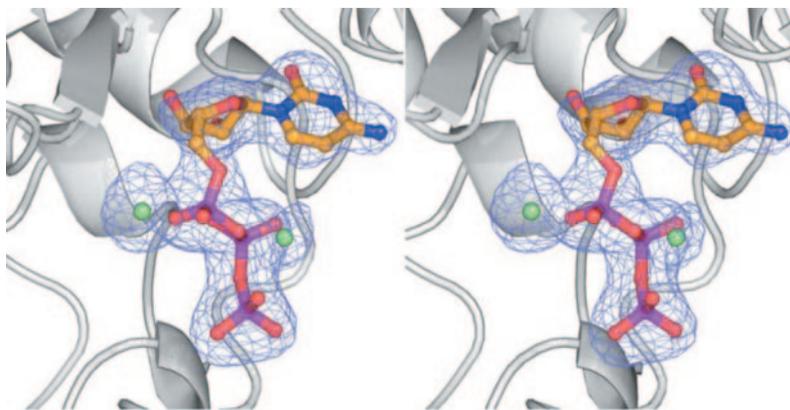


FIG. 3. **Representative electron density.** Shown is the electron density corresponding to the nucleotide. The map was calculated with coefficients of the form $(F_o - F_c)$, where F_o was the native structure factor amplitude and F_c was the calculated structure factor amplitude from the model lacking coordinates for the MgCTP ligand. The map was contoured at 3σ . There were two magnesium ions modeled into the electron density and they are represented by the *green spheres*.

CTP and UTP equally well, a finding that has not been reported for any other hexose 1-phosphate cytidylyltransferase or uridylyltransferase. However, the rate of the reaction is severely decreased when CTP is replaced with dCTP. The K_m for dCTP is 3.0 ± 0.5 mM, which is 20 times greater than the K_m for CTP and the rate of the reaction is 30% that of the reaction with CTP. Of the hexose 1-phosphates tested, only xylose 1-phosphate acted as a substrate in the forward reaction. The K_m for xylose 1-phosphate is 3.6 ± 0.3 mM, which is 72 times greater than the K_m for glucose 1-phosphate. The rate of the reaction with xylose 1-phosphate, however, is $\sim 40\%$ faster than the reaction with glucose 1-phosphate. This rate increase could be explained if the product CDP-xylose has a low affinity for the enzyme in which case the product release step for CDP-xylose would be much faster than that for CDP-glucose. This would give a higher apparent velocity for the reaction of CTP and xylose 1-phosphate.

The equilibrium constant for cytidylyltransferase was analyzed by measuring the proportion of substrate *versus* product via HPLC analysis, obtained from both the forward and reverse reactions. These data indicate a K_{eq} of 0.27 with the reaction having a slight preference for CTP synthesis. There is some indication in the kinetic data that a second Mg^{2+} ion is required in addition to that forming the Mg^{2+} -CTP complex. Thus a 2 mM excess of Mg^{2+} over Mg^{2+} -CTP was needed for full activity although a 0.5 mM excess should have been sufficient to keep MgCTP in that form. The dissociation constant of the second metal ion is presumably less than 1 mM.

Kinetic parameters for the back reaction (NTP synthesis) indicate that the enzyme has a clear preference for CDP-glucose over UDP-glucose as shown in Table IV. The K_m for CDP-glucose is 11 ± 2 μ M, whereas the K_m for UDP-glucose is 2535 ± 240 μ M. However, the V_{max} for both substrates is nearly the same at ~ 8 μ M/min. The K_m for pyrophosphate is 67 ± 8 μ M.

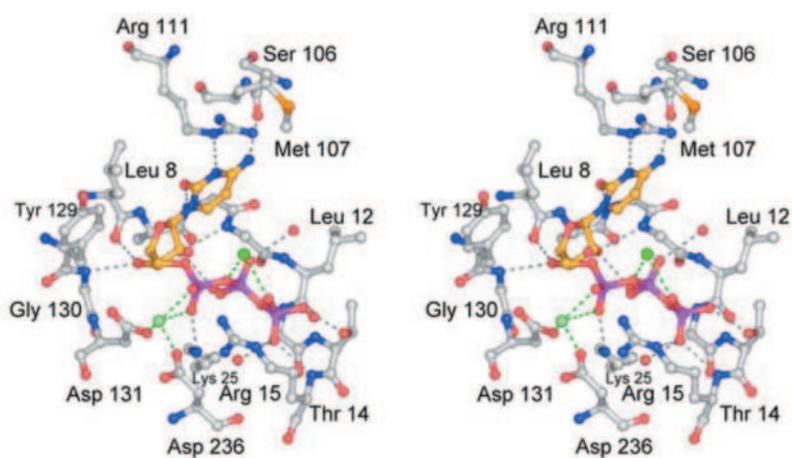
The dramatic difference in the K_m for CDP-glucose *versus* UDP-glucose but a nearly identical K_m for CTP and UTP may be explained if the binding of the triphosphate nucleotide is driven by the affinity of the enzyme for pyrophosphate. Indeed, the K_m for pyrophosphate is less than the K_m for CTP or UTP (158 and 149 μ M, respectively). The relatively low affinity of the enzyme for UDP-glucose suggests that the rate of dissociation of the UDP-glucose product is likely not rate-limiting and thus explains the slightly elevated V_{max} for the forward reaction with UTP *versus* CTP. The results from the back reaction are somewhat more difficult to explain. Additional x-ray crystallographic and kinetic data, including the determination of the K_m

for glucose 1-phosphate and pyrophosphate in the presence of UTP and UDP-glucose, respectively, will be required to fully explain the current observations.

Electron density corresponding to the bound nucleotide, MgCTP, is shown in Fig. 3. As can be seen, the cytosine ring is in the *anti*-conformation, whereas the ribose adopts the C_2' -*endo* pucker. At this resolution it is not possible to completely define the coordination geometry about the Mg^{2+} ion. However, it is apparent from this structure that the Mg^{2+} ion sits within 2.5 and 3.1 Å of the α -phosphoryl oxygens (Fig. 4a). Additionally, the side chains of Asp¹³¹ and Asp²³⁶ lie within coordination distance of the magnesium (2.0 and 2.1 Å, respectively). There is a second peak of electron density positioned near the α,β -bridging oxygen (2.4 Å) and a γ -phosphoryl oxygen (2.8 Å) of the nucleotide. This has been tentatively modeled as a magnesium ion.

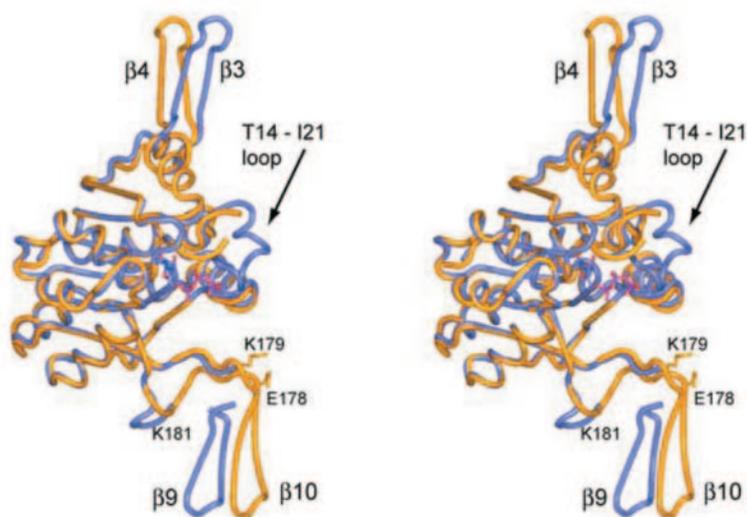
A close-up view of the active site with bound MgCTP is shown in Fig. 4a. The backbone amide nitrogen of Gly¹⁰, the carbonyl oxygen of Ser¹⁰⁶, and the guanidinium group of Arg¹¹¹ all lie within hydrogen bonding distance to the carbonyl oxygen, the amino group, and the ring nitrogen, respectively, of the cytosine base. The 2-hydroxyl group of the nucleotide ribose sits within ~ 2.9 Å of the backbone amide nitrogen of Gly¹¹ and a water molecule, whereas the 3-hydroxyl group lies within 2.9 and 2.7 Å of the backbone nitrogen of Gly¹³⁰ and the carbonyl oxygen of Leu⁸, respectively. In the previously determined structure of the enzyme complexed with its product, CDP-glucose, the loop defined by Thr¹⁴ to Ile²¹ was disordered (13). Strikingly, in the presence of CTP, the loop becomes ordered and both O γ of Thr¹⁴ and N ϵ of Arg¹⁵ participate in hydrogen bonding interactions with γ -phosphoryl oxygens (Fig. 4a). Note that the side chain of Lys²⁵ projects toward the α -phosphoryl group of CTP with its ϵ -nitrogen sitting at 2.4 Å from a phosphoryl oxygen.

Shown in Fig. 4b is a superposition of the subunits with either bound CDP-glucose or CTP. The polypeptide chains for these two models correspond with a root mean square deviation of 0.45 Å for 203 structurally equivalent α -carbons. The most dramatic structural changes that occur upon binding either CTP or CDP-glucose are limited primarily to the β -hairpin regions labeled $\beta 3$, $\beta 4$, and $\beta 9$, $\beta 10$ in Fig. 4b. All of the residues involved in anchoring CTP to the protein are contained within the same subunit of the hexamer. This is in marked contrast to that observed in the model of the enzyme with bound product, CDP-glucose, whereby part of the binding pocket is provided by a second subunit in the hexamer. Specifically, Glu¹⁷⁸ and Lys¹⁷⁹ from a neighboring molecule form

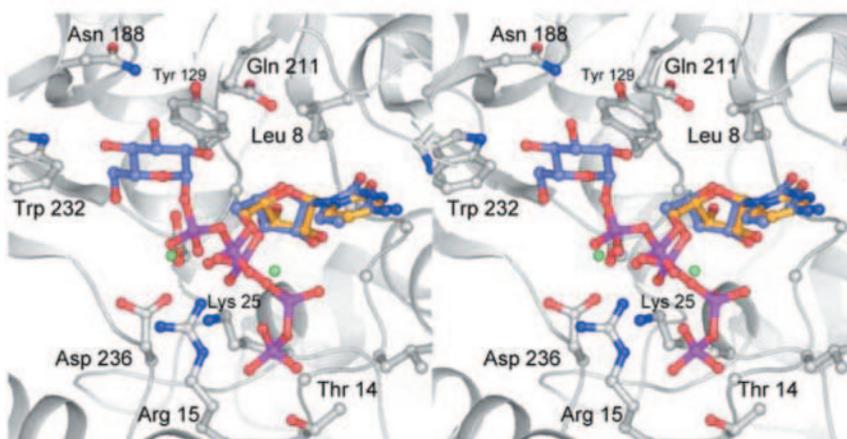


(a)

FIG. 4. Structure of the cytidylyltransferase with bound CTP. A close-up view of the active site, within ~ 3.2 Å, is shown in *a*. The green spheres represent bound magnesium ions. The dashed green lines indicate possible ligand coordination to these cations. At a resolution of 2.5 Å it is not possible to completely define the coordination spheres of the magnesiums. Ordered water molecules are depicted as red spheres. Possible hydrogen bonds (within 3.2 Å) between CTP and the protein are indicated by the dashed lines. A superposition of the α -carbon traces for the models of the cytidylyltransferase with either bound CDP-glucose (yellow) or CTP (blue) is depicted in *b*. Note that the β -hairpin motif labeled $\beta 9$, $\beta 10$ in *b* forms part of the active site in a neighboring molecule in the hexamer. The manner in which the enzyme accommodates either CTP or the product, CDP-glucose, is depicted in *c*.



(b)



(c)

electrostatic interactions with CDP-glucose (13). These residues are disordered in the absence of CDP-glucose (Fig. 4*b*).

An overlay of the CTP and CDP-glucose ligands, when bound

in the active site of the cytidylyltransferase, is given in Fig. 4*c*. As can be seen, the two ligands adopt similar positions with respect to their base and ribosyl moieties. Quite remarkable

conformational differences, however, occur beginning at their α -phosphorus atoms. Specifically, the torsional angle defined by the bond between O-5 of the ribosyl group and the α -phosphorus atom differs by $\sim 115^\circ$ in these two models. As a result the glucosyl group in CDP-glucose abuts the side chain of Tyr¹²⁹, whereas the γ -phosphoryl group of CTP sits near Thr¹⁴ and Arg¹⁵ (Fig. 4c).

These two structures further argue against a Bi Bi ping-pong mechanism in that the only potential base near the α -phosphorus is Lys²⁵. Strikingly, the β -phosphorus of the CDP-glucose ligand is located at ~ 3.4 Å from the α -phosphorus of CTP in the overlay presented in Fig. 4c. Assuming that the binding of the glucose moiety in CDP-glucose mimics what would occur for the substrate, glucose 1-phosphate, it is easy to envisage a catalytic mechanism for the cytidylyltransferase that proceeds via direct nucleophilic attack of the glucose 1-phosphate phosphoryl oxygen on the α -phosphorus of CTP. In conclusion, from both the structural and kinetic data presented here it can be concluded that the cytidylyltransferase reaction is sequential and similar to that reported for the glucose-1-phosphate thymidylyltransferases (11, 12).

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