Structure and Mechanism of CTP:Phosphocholine Cytidylyltransferase (LicC) from Streptococcus pneumoniae*

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Bo-Yeon Kwak‡, Yong-Mei Zhang§, Mikyung Yun‡, Richard J. Heath§, Charles O. Rock¶, Suzanne Jackowski§,§, and Hee-Won Park‡,¶

From the ‡Department of Structural Biology and the §Protein Science Division, Department of Infectious Diseases, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105 and the ¶Department of Molecular Biosciences, University of Tennessee Health Science Center, Memphis, Tennessee 38163

Pneumococcal LicC is a member of the nucleoside triphosphate transferase superfamily and catalyzes the transfer of a cytidine monophosphate from CTP to phosphocholine to form CDP-choline. The structures of apo-LicC and the LicC:CDP-choline-Mg\(^{2+}\) ternary complex were determined, and the comparison of these structures reveals a significant conformational change driven by the multivalent coordination of Mg\(^{2+}\). The key event is breaking the Glu\(^{146}\)-Arg\(^{228}\) salt bridge, which triggers the coalescence of four individual β-strands into two extended β-sheets. These movements reorient the side chains of Trp\(^{198}\) and Tyr\(^{199}\) for the optimal binding and alignment of the phosphocholine moiety. Consistent with these conformational changes, LicC operates via a compulsory ordered kinetic mechanism. The structures explain the substrate specificity of LicC for CTP and phosphocholine and implicate a direct role for Mg\(^{2+}\) in aligning phosphocholine for in-line nucleophilic attack and stabilizing the negative charge that develops in the pentacoordinate transition state. These results provide a structural basis for assigning a specific role for magnesium in the catalytic mechanism of pneumococcal LicC.

Choline metabolism plays a key role in cell separation, transformation, autolysis, and pathogenicity of Streptococcus pneumoniae. The only known metabolic fate of choline is to decorate the teichoic and lipoteichoic acids of the cell wall, and choline is an essential nutrient for S. pneumoniae (1). The cell surface P-Cho facilitates the interaction with the host surface and induces attachment and invasion (2, 3). The importance of choline in pathogenesis is not confined to S. pneumoniae but also plays a role in Hemophilus influenzae (4–7), Pseudomonas aeruginosa, and Neisseria gonorrhoeae (8, 9). In addition, cell wall P-Cho serves as the scaffold for a group of choline-binding proteins that are secreted from the cells and subsequently attached to the cell surface by their homologous choline-binding domains (see Ref. 10 and references therein). These choline-binding proteins are essential for many aspects of S. pneumoniae cell physiology including competence and stationary phase lysis.

The pathway for choline metabolism in S. pneumoniae and H. influenzae has been hypothesized to consist of a choline transport system, a choline kinase, CTP:phosphocholine cytidylyltransferase (CCT), and a choline phosphotransferase that transfers P-Cho from CDP-Cho to either lipoteichoic acid or lipopolysaccharide (5). The existence of this pathway is supported by the detection of choline kinase and CCT activity in crude extracts of S. pneumoniae (11, 12). Genetic elements required for choline incorporation into the lipopolysaccharide of H. influenzae are found in the licI locus, which contains four open reading frames. The hypothesis drawn from the bioinformatic analysis of the licI locus (4) is that licA corresponds to choline kinase based on a 31% identity to the choline kinase of Saccharomyces cerevisiae over the short span of 40 amino acids between residues 222 and 262. The licB gene has several predicted transmembrane domains and is thus postulated to be a choline transporter. The hydrophilic licC gene product is a candidate for the CCT due to the resemblance of its amino terminus to the amino-terminal 60 residues of NTP transferase family members, leaving the licD gene as a candidate for the choline phosphotransferase. A homologous licC gene exists in S. pneumoniae (13), and the predicted LicC proteins of H. influenzae and S. pneumoniae are 37% identical and 60% similar.

Recently, the LicC of S. pneumoniae was purified and demonstrated to catalyze the CCT reaction (14). The enzyme possesses a high degree of selectivity for P-Cho and CTP, although phosphoethanolamine and ATP are poor substrates. LicC lacks homology to either the prototypical metazoan CCTs (15, 16) or the CTP:glycerol-3-phosphate cytidylyltransferase from Bacillus subtilis (17). Rather, LicC is related to members of the NTP transferase superfamily that primarily activate sugars by transferring the NMP moiety to a phosphorylated acceptor substrate concomitant with the release of PP\(_1\) (see the NCBI Conserved Domain Database, protein family 00483) and contain the N-terminal signature sequence KA\(_N\)GXXGRX\(_S\)kG. The inclusion of LicC in this family is based on its similarity to the amino termini of Escherichia coli GalU and P. aeruginosa RmlA, two prototypical nucleotidyltransferases that form NDP-derivatives which are used in the biosynthesis of bacterial cell wall components (14). Members of this superfamily are characteristically dimers or tetramers; however, LicC is a monomer (14).

The crystal structures of three members of the NTP trans-
ferase superfamily are known. These are the dimeric GlmU (18) and methylenethiolyl-4-phosphate cytidylyltransferase from *E. coli* (19) and the tetrameric RmlA from both *P. aerugi- nosa* (20) and *Salmonella enterica* (21). The NTP transferase domains of these enzymes have a similar overall folding, and they share several key residues involved in substrate binding and/or catalysis, such as Gly<sup>14</sup>, Arg<sup>18</sup>, and Lys<sup>28</sup> (LicC numbering) in the signature sequence. Magnesium is required for activity. In *S. enterica* RmlA and *E. coli* methylenethiolyl-4-phosphate cytidylyltransfase, Mg<sup>2+</sup> is proposed to position the nucleotide substrate during the NTP transfer reaction and to stabilize the PP<sub>i</sub> leaving group after the reaction (19, 21); thus, Mg<sup>2+</sup> is not thought to play a role in the catalytic mechanism. In the other two structures, the magnesium binding site is undefined (18, 20). Several positively charged residues that are not conserved in all of the NTP transferase structures are proposed to be involved in catalysis of RmlA and methylenethiolyl-4-phosphate cytidylyltransferase by providing a favorable electrostatic environment, although their exact role is unclear (19–21). Exceptions are Arg<sup>18</sup> and Lys<sup>28</sup> (LicC numbering) in the signature sequence that bind the triphosphate group of the nucleotide substrate to stabilize the negative charge developed on the oxygens of the α-phosphate in the pentacoordinate transition state and the released PP<sub>i</sub> product. We have determined the kinetic mechanism of LicC from *S. pneumoniae* and the structures of LicC and the LicC-CDP-Chol-Mg<sup>2+</sup> ternary complex. The structures explain the specificity of LicC for CTP and identify the active site residues involved in binding the product, CDP-choline, with magnesium. We propose the mode of substrate binding and the direct role of the magnesium in catalysis based on the kinetic data and structures.

**Experimental Procedures**

Materials—Sources of supplies were Sigma (for CTP, CDP-Chol, P-Cho, and seleno-l-methionine) and Amersham Biosciences, Inc. (for phospho[methyl-<sup>14</sup>C]choline (specific activity 55 mCi/mmol)). Histagged native LicC was expressed and purified as described previously (14). For metabolic incorporation of selenomethionine into LicC, PET-15b containing the licC gene was transformed into BL21-Codon- Plus(DE3)-RIL-Y (Stratagene), and protein was expressed as previously (22) and purified in the same manner as the His-tagged native LicC. Protein concentration was determined by the method of Bradford (23). All other chemicals were reagent grade or better.

LicC Enzyme Assay—The enzymatic activity of LicC under different conditions was studied using the assay method described previously (14). The apparent *K<sub>m</sub>* for CTP or P-Cho in the presence of different concentrations of the second substrate was determined. In a final volume of 50 μl, the LicC assay contained the substrates, CTP and phospho[methyl-<sup>14</sup>C]choline (specific activity 3.68 mCi/mmol), at the appropriate concentrations for each study indicated in the figure legends, and 10 mM MgCl<sub>2</sub> to the protein to a final concentration of 10 mM each, and the reaction was stopped by the addition of 5 μl of 0.5 μM EDTA. A 40-μl aliquot of the reaction mixture was applied to a preabsorbent bed (Stratagene). The Crystallization condition was studied using the assay method described previously (14). For metabolic incorporation of selenomethionine into LicC, pET-Se-Met LicC was obtained from Hampton Research screen- ing) in the signature sequence. Magnesium is required for catalysis based on the kinetic data and structures.

| Data source | Apoenzyme | CDP-choline-bound enzyme |
|-------------|-----------|--------------------------|
| Wavelength (Å) | 0.9785 | 0.9793 |
| Resolution (Å) | 50–1.5 | 50–1.5 |
| Number of reflections | 910,139 | 1,187,180 |
| Measured | Unique | 95,290 |
| Completeness, % | Overall | 92.6 |
| | Last shell<sup>a</sup> | 45.5 |
| R<sub>sym</sub>(%)<sup>b</sup> | 0.039 | 0.048 |
| Average I/σ(I) | 19.1 | 13.8 |
| Anomalous scattering factors | r<sup>f</sup> values | −5.52 |
| | r<sup>f</sup> values | −5.52 |
| | Centric reflections | 2.2 |
| | Acentric reflections | 4.6 |
| Phasing statistics | Mean figure of merit | 0.42 |
| | r.m.s.(FH)/r.m.s.(E) | 0.78 |
| | Acentric reflections | 0.67 |

<sup>a</sup> Last shell includes the resolution between 1.55 and 1.5 Å in apoenzyme and between 2.44 and 2.4 Å in CDP-choline-bound enzyme.

<sup>b</sup> R<sub>sym</sub> = Σ<sub>hkl,i</sub>[Σ<sub|i</sub> I<sub>hkli</sub> − (I<sub>hkli</sub>)]/Σ<sub>i</sub> I<sub>hkli</sub>, where I<sub>hkli</sub> is the intensity of an individual measurement of the reflection with Miller indices h, k, and l, and (I<sub>hkli</sub>) is the mean intensity of that reflection.

<sup>c</sup> r.m.s. (FH) is the ratio of the root mean square value of the calculated heavy atom structure factor (FH) to the root mean square value of the difference between calculated and observed derivative structure factors (E), where it is averaged not only over all reflections but over all phases for each reflection, weighted by the phase probability.

**Data Collection and Structure Determination—**Multiwavelength anomalous dispersion data were measured from a single crystal of Se-Met apo-LicC at beam line X8-C at the National Synchrotron Light Source, equipped with an ADSC Quantum 4R detector operating at 100 K. Two wavelengths near the selenium absorption edge, 0.9789 Å (the peak) and 0.9795 Å (the inflection point), were chosen by measuring the x-ray absorption spectrum of the protein crystal. The data sets from both wavelengths were collected, reduced, and scaled by the program HKL 2000 (24). The crystals of apo-LicC belong to space group P2_<sub>1</sub> 2_<sub>1</sub> 2_<sub>1</sub> with unit cell dimensions of a = 42.6 Å, b = 63.6 Å, c = 122.4 Å. The program SOLVE was used to scale the two wavelength data sets together, to locate selenium positions, and to calculate the protein phases (25). The data for the four selenium atoms in the LicC enzyme were confirmed. The program RESOLVE was used for reciprocal space solvent flattening (26). The statistics of data collection and phasing are summarized in Table I. The atomic model was built using the programs WARP (27) and XTALVIEW (28), followed by crystallographic refinement in the program XPLOR (29). The refinement steps included simulated annealing, conjugated gradient minimization, and individual B-factor refinement. The refinement statistics are shown in Table II.

Complete diffraction data from a single crystal of the LicC-CDP-Chol-Mg<sup>2+</sup> complex were measured at beamline 19-BM at the Advanced Photon Source, equipped with a SBC-2 CCD x-ray detector operating at 100 K. The program HKL 2000 (24) was used to integrate and scale the data set. The crystals of the ternary complex belonged to space group P1 with unit cell dimensions of a = 48.2 Å, b = 69.0 Å, c = 81.6 Å, α = 93.4°, β = 92.8°, γ = 97.1°. The data collection statistics are summarized in Table I. The structure of the CDP-choline-bound LicC was determined by the molecular replacement method using the refined apo-enzyme as a search model. The cross-rotation and translation functions and Patterson correlation refinement were calculated using the program XPLOR (29). Using the data between 15.0 and 4.0 Å, the rotation function, followed by Patterson correlation refinement, gave four outstanding solutions that correspond to four molecules of LicC. Because any point can be taken as an origin in space group P1, we rotated the search model according to the highest solution of rotation function and considered this rotated molecule as the first molecule. With fixing the first molecule, the orientations of the other three molecules were determined by applying the three independent noncrystal-
Overview of the LicC Structure—The apo-LicC structure reveals a mixed seven-stranded β-sheet flanked by three α-helices on one side (α1, α2, and α6) and another three α-helices on the other (α3, α4, and α5) (Fig. 1). The seven-stranded sheet has six parallel strands (strands β1, β2, β3, β4, β5, and β7), and strand β6 is antiparallel to others, representing the typical nucleotide-binding fold (31). Four additional strands (β5a, β5b, β5c, and β5d) located between strands β5 and β6, which are perpendicular to the seven-stranded β-sheet, and another four strands (β1a and β1b, β4a, and β7a) are not part of the main β-sheet. Comparison of the apo-LicC structure with known structures in the Protein Data Bank using the DALI server (32) revealed that the two best matches were with two members of the NTP transference superfamily: P. aeruginosa RmlA (Protein Data Bank code 1iin, r.m.s. deviation 1.9 Å, Cα atoms of 171 residues) and E. coli methylerythritol-4-phosphate cytidylyltransferase (Protein Data Bank code 1i52, r.m.s. deviation 1.7 Å, Cα atoms of 131 residues). The finding of these low r.m.s. deviation values indicates that LicC is structurally similar to the three members of the NTP transference superfamily. The structural similarity along with the conservation of the N-terminal signature sequence supports the classification of LicC as a member of the NTP transference superfamily (14). Among the three members of the NTP transference superfamily homologous to LicC, GlmU is an uridylyltransferase involved in the production of UDP-N-acetylgalacosamine, an essential precursor for the cell wall and membrane biosynthesis in bacteria (18). RmlA is a thymidylyltransferase and the first enzyme in the biosynthesis of deoxy-TDP-D-rihamnose that is a component of the cell wall in many bacteria (20). E. coli methylerythritol-4-phosphate cytidylyltransferase is a cytidylyltransferase involved in the mevalonate-independent pathway for isoprenoid biosynthesis (19).

RESULTS

Overview of the LicC Structure—Helices are shown in blue, strands in green, and other secondary structural elements in yellow. The core seven β-sheet strands and flanking α-helices on either sides are labeled as β1–7 and α1–6. Additional β-strands that are not part of the core sheet are labeled by alphabetizing after the preceding β-strand number. For example, the β-strand inserted between β4 and β6 is labeled as β6a. Figs. 1, 3, and 4 were produced by the program RIBBONS (40).

![Fig. 1. Overall stereoview of the LicC structure. Helices are shown in blue, strands in green, and other secondary structural elements in yellow. The core seven β-sheet strands and flanking α-helices on either sides are labeled as β1–7 and α1–6. Additional β-strands that are not part of the core sheet are labeled by alphabetizing after the preceding β-strand number. For example, the β-strand inserted between β4 and β6 is labeled as β6a. Figs. 1, 3, and 4 were produced by the program RIBBONS (40).](http://www.jbc.org/)

| Table II | Refinement statistics |
|----------|-----------------------|
| Apo-enzyme | CDP-choline-bound enzyme |
| Refinement | | |
| Resolution (Å) | 20–1.5 | 20–2.4 |
| Rwork | 0.241 | 0.239 |
| Rfree | 0.218 | 0.238 |
| Number of reflections (F > 2σ(F)) | 49170 | 35102 |
| Number of protein atoms | 1903 | 7580 |
| Number of ligand atoms | 128 | |
| Number of water molecules | 237 | 178 |
| Stereochemistry | | |
| r.m.s. deviation bond length (Å) | 0.007 | 0.010 |
| r.m.s. deviation bond angles (degrees) | 1.37 | 1.51 |
| Average B factors (Å²) | 20.8 | 35.8 |
| Protein main-chain/ side-chain | 19.5/22.0 | 34/47.1 |
| Ligand | | |
| Residues from Ramachandran plot | | |
| Most favored regions (%) | 89.4 | 84.4 |
| Additional allowed regions (%) | 10.6 | 14.6 |
| Generously allowed regions (%) | 0 | 0.9 |

* Rwork is equivalent to Rwork except that 5% of the total reflections were set aside for an unbiased test of the progress of refinement.
Fig. 2. Solvent-accessible surface and electrostatic surface potential. Red and blue represent negative and positive electrostatic surface potential, respectively, whereas white represents neutral electrostatic surface potential. Bound ligands are shown with a ball and stick representation. A, the electrostatic potential of LicC with bound CDP-Cho-Mg\(^{2+}\). B, the electrostatic potential of RmlA from E. coli with bound CTP (PDB code 1fwy). C, the electrostatic potential of GlmU with bound UDP-N-acetylglucosamine (PDB code 1fvy). D, the electrostatic potential of methylerythritol-4-phosphate cytidylyltransferase from E. coli with bound CTP (PDB code 1l52). We used the coordinates of the CTP-bound structure in D, because the product-bound structure is not available. This figure was produced with the program GRASP (41).

It is known that the trimethylammonium group of choline is recognized by electron-rich aromatic rings (34–36). Indeed, in the LicC-CDP-Cho-Mg\(^{2+}\) complex, the binding pocket of the trimethylammonium group is formed by the aromatic rings of Tyr\(^{190}\) and Trp\(^{196}\) and the carboxyl group of Asp\(^{192}\). The side chains compensate for the positive charge of the trimethylammonium group through a stabilizing interaction with their ε electrons (Fig. 3B). We hypothesize that the negative charge of Asp\(^{192}\) may also be a factor in binding of the trimethylammonium group, because an electrostatic bond is stronger than a cation–π bond (37).

Magnesium is required for the catalytic activity of LicC (14). In the LicC-CDP-Cho-Mg\(^{2+}\) structure, the magnesium atom is fixed in space by interactions with six oxygen atoms, two oxygens from each of the α- and β-phosphates of CDP-choline, the carboxyl groups of Asp\(^{107}\), Glu\(^{216}\), and Asp\(^{218}\), and a water molecule, forming an octahedral geometry (Fig. 3B). This bound water molecule is further hydrogen-bonded to the carboxyl group of Asp\(^{192}\). The α-phosphate oxygen of CDP-Cho and the carboxyl groups of Asp\(^{107}\) and Asp\(^{218}\), which bind the magnesium, form hydrogen bonds with the amino group of Lys\(^{28}\) in the signature sequence (Fig. 3B), suggesting that Lys\(^{28}\) participates in orienting the side chains of Asp\(^{107}\) and Asp\(^{218}\) for binding both magnesium and the nucleotide substrate. The interaction between the amino group of Lys\(^{28}\) and the α-phosphate oxygen can enhance the catalytic reaction rate by stabilizing the developing negative charge in the pentacoordinate transition state. When the corresponding Lys of NTP transferases from other organisms was mutated, the catalytic activity was decreased (18, 19), which is consistent with the proposed role of Lys\(^{28}\) of LicC in catalysis. A β-phosphate oxygen...
tion of Arg_{129} with the amide nitrogen of Gly_{211}. The displaced Glu_{216}, and Asp_{218}, the pyrophosphate of CDP-Cho, and a water molecule are octahedrally arranged about Mg^{2+} (magenta). The side chain pi electrons of Trp_{136}, Tyr_{190}, and Asp_{192} interact with the trimethylammonium group of CDP-Cho. This type of interaction is observed in other proteins that recognize the trimethylammonium group of choline (34–36). The closest distances between the side chains of three residues and the trimethylammonium groups are 3.5, 3.7, and 3.2 Å, respectively.

that does not bind magnesium is hydrogen-bonded to the hydroxyl group of Tyr_{190} that also interacts with the trimethylammonium group via its aromatic ring (Fig. 3B).

Structural Differences between LicC and LicC-CDP-Cho-Mg^{2+}.—The most significant change in the secondary structural elements induced by CDP-Cho-Mg^{2+} binding involves the four strands (β5a, β5b, β5c, and β5d) located between strands 5 and 6. In the LicC-CDP-Cho-Mg^{2+} structure, strands β5a and β5b with their intervening loop become the continuous, long strand β5a, and strands β5c and β5d with their intervening loop coalesce to form strand β5c (Fig. 4A). These new, longer strands β5a and β5c are interconnected by a hydrogen bond network to form a two-stranded β sheet.

CDP-Cho-Mg^{2+} binding also alters the side chain positions of several residues. Side chains of Glu_{107} and Asp_{118} move toward bound magnesium by 7.4 and 3.8 Å, respectively, to coordinate the magnesium (Fig. 4B). Since Glu_{107} in the apoenzyme structure is salt-bridged to Arg_{129}, the movement of Glu_{107} disrupts the salt bridge interaction with Arg_{129}, displacing the guanidinium group of Arg_{129}, breaking the hydrogen bond interaction of Arg_{129} with the amide nitrogen of Gly_{211}. The displaced guanidinium group of Arg_{129} is hydrogen-bonded to the side chain of Gly_{211}. Also, the side chains of Trp_{136} and Tyr_{190} move toward the trimethylammonium group of CDP-choline. Arg_{129}, Glu_{135}, and Trp_{136} are all located in the newly formed strand β5a.

Arg_{18} (LicC numbering) in the signature sequence of NTP transferase structures interacts with the nucleotide tripohosphate (19–21). In the apo-LicC structure, the guanidinium group of Arg_{18} points down to the active site pocket, forming a hydrogen bond network with the carboxyl group of Asp_{107} and the carbonyl oxygen of Ile_{217} (Fig. 4C). In contrast, the guanidinium group is flipped out and exposed to solvent in the LicC-CDP-Cho-Mg^{2+} complex (Fig. 4C). Since the LicC-CDP-Cho-Mg^{2+} complex is the structure of the enzyme intermediate immediately after the release of PPi (see below), the solvent-exposed guanidinium group of Arg_{18} may correspond to the conformational change that facilitates the release of PPi, to the
solvent. The side chain of the corresponding Arg in *E. coli* methylerythritol-4-phosphate cytidylyltransferase forms hydrogen bonds with the α- and γ-phosphate oxygens of CTP (19). In LicC, after adjusting the torsion angles of Arg18, similar interactions can be formed between the side chain of Arg18 and the α- and γ-phosphate oxygens of CTP modeled using the CTP coordinates of *E. coli* methylerythritol-4-phosphate cytidylyltransferase (Protein Data Bank code 1i52). This finding suggests that Arg18 may be involved in the release of PPi and offset the negative charge developed on the α-phosphate oxygen of CTP during the catalytic reaction.

**Kinetic Mechanism for LicC**—A series of kinetic experiments were performed to define the kinetic mechanism for LicC. Double reciprocal plots of 1/v versus 1/[CTP] at different concentrations of P-Cho (Fig. 5A) intersected far to the left of the 1/v axis and far below the 1/[CTP] axis, characteristic for an ordered bi bi mechanism. Kinetic data were obtained using the assay described under “Experimental Procedures,” and the error bars present the range of data.

Substrates. CDP-Cho is a competitive inhibitor with respect to CTP, whereas PPi is a mixed-type inhibitor with respect to CTP (Fig. 6). These data show that CTP and CDP-Cho bind to the same form of the enzyme. On the other hand, both PPi and CDP-Cho are mixed-type inhibitors with respect to P-Cho (Fig. 7). This pattern of the product inhibition is characteristic of a sequential bi bi reaction with CTP as the leading substrate and CDP-Cho as the last product to leave, which is the same as the catalytic mechanism of *E. coli* glucose-1-phosphate thymidylyltransferase, a homologue of *P. aeruginosa* RmlA (39).

**DISCUSSION**

We propose a model for the catalytic cycle of LicC that is consistent with the kinetic and structural data (Fig. 8). CTP-Mg$^{2+}$ is the leading substrate. The LicC nucleotide specificity for CTP is conferred by specific interactions that include the N-4 nitrogen of the cytosine base and the carbonyl oxygens of Tyr82 and Tyr85, the CTP O2 oxygen with the amide nitrogen of Ala13 and the hydroxyl group of Ser90. The α,γ-phosphate oxygens are proposed to form a salt bridge with Arg18, which points into the active site of LicC. The planar aromatic ring of CTP is sandwiched between the loop of strands β1 and β1a and the loop of strand β3 and helix α5. The side chains of Asp107, Glu216 Asp218 and the α-phosphate of CTP coordinate the Mg$^{2+}$. Movement of Glu216 breaks the salt bridge with Arg129...
that in turn forms hydrogen bond to Glu<sub>135</sub>. This induces four β-strands in LicC into coalesce into two long β-strands. This rearrangement causes the repositioning of Trp<sub>136</sub> and Tyr<sub>190</sub> to optimize the binding of the second substrate P-Chol and position the bound P-Chol for the CMP transfer reaction. The structure is consistent with the reaction occurring by in-line nucleophilic attack of the phosphate oxygen of P-Chol on the α-phosphate of CTP, similar to other members of the NTP transferase superfamily. The first product released from the active site is PP<sub>i</sub>, which may be facilitated by its interaction with Arg<sub>129</sub>, which points away from the active site in the active site is PP<sub>i</sub>, which may be facilitated by its interaction with the incoming Mg<sup>2+</sup> (see “Discussion”). The subsequent binding of P-Chol is facilitated by the movement of Trp<sub>136</sub> and Tyr<sub>190</sub> to optimize the interaction of the enzyme with the incoming trimethylammonium group of P-Chol. Catalysis occurs in the ternary complex, and PP<sub>i</sub> is released first, facilitated by the movement of Arg<sub>129</sub>. CDP-Chol-Mg<sup>2+</sup> is the last product released from the enzyme, which allows the Glu<sub>216</sub>-Arg<sub>129</sub> salt bridge to reform and regenerate the apo-enzyme structure.

Our structures expand the understanding of the catalytic mechanism of members of the NTP transferase superfamily by implicating Mg<sup>2+</sup> as an integral component in catalysis. The Mg<sup>2+</sup> in previously determined NTP transferase structures is postulated to stabilize the PP<sub>i</sub> leaving group, and thus plays an indirect role in catalysis (19, 21). However, the Mg<sup>2+</sup> bound at the active site of the LicC-CDP-Chol-Mg<sup>2+</sup> structure suggests the direct involvement of the Mg<sup>2+</sup> in the catalytic mechanism of LicC. We propose two direct roles for Mg<sup>2+</sup> in NTP transferases. First, the binding of Mg<sup>2+</sup> accelerates catalysis by positioning the phosphate oxygen of P-Chol adjacent to the α-phosphate of CTP to align the incoming second substrate of in-line nucleophilic attack. Second, the interactions of the positively charged Mg<sup>2+</sup> works in concert with the side chains of Arg<sub>129</sub> and Lys<sub>28</sub> to stabilize the developing negative charge in the pentacoordinate transition state during CMP transfer.

The role of Arg<sub>129</sub> and Lys<sub>28</sub> in the in-line nucleophilic substitution mechanism proposed for LicC is the same as proposed for the structurally known members of the NTP transferase family, GlmU, RmlA, and E. coli methylenecrithol-4-phosphate cytidylyltransferase. When the nucleotide and phosphosugar substrates bind to GlmU to form the ternary complex, the proximity of the substrates and the catalytic Arg<sub>129</sub> (also Arg<sub>18</sub> in LicC) aids the reaction by stabilizing a charged transition state. In RmlA, three residues, Arg<sub>194</sub>, Lys<sub>162</sub>, and Arg<sub>18</sub> appear important in the catalytic mechanism. Arg<sub>194</sub> (Arg<sub>18</sub> in LicC) also functions by stabilizing the charged transition state, 

**FIG. 7. Product inhibition of the LicC reaction with respect to P-Chol.** A, double reciprocal plots of 1/v versus 1/[P-Chol] in the presence of different concentrations of CDP-Chol revealed that CDP-Chol is a mixed-type inhibitor with respect to P-Chol. B, double reciprocal plots of 1/v versus 1/[P-Chol] in the presence of different concentrations of PP<sub>i</sub> revealed that PP<sub>i</sub> is a mixed-type inhibitor with respect to P-Chol. The concentration of CTP (160 μM) was held constant in the experiments. Kinetic data were obtained using the assay described under “Experimental Procedures,” and the error bars present the range of data.

**FIG. 8. The LicC catalytic cycle.** LicC operates by a sequential ordered mechanism that is interpreted based on the enzyme kinetics and the conformational differences between the structure of apo-LicC and the LicC-CDP-Chol-Mg<sup>2+</sup> ternary complex. LicC first binds CTP-Mg<sup>2+</sup>, which triggers a conformation change that breaks the salt bridge between Glu<sub>216</sub> and Arg<sub>129</sub>. This action reorganizes the β-sheet structure of the protein to allow Glu<sub>216</sub> to directly interact with the incoming Mg<sup>2+</sup> (see “Discussion”). The subsequent binding of P-Chol is facilitated by the movement of Trp<sub>136</sub> and Tyr<sub>190</sub> to optimize the interaction with the incoming trimethylammonium group of P-Chol. Catalysis occurs in the ternary complex, and PP<sub>i</sub> is released first, facilitated by the movement of Arg<sub>129</sub>. CDP-Chol-Mg<sup>2+</sup> is the last product released from the enzyme, which allows the Glu<sub>216</sub>-Arg<sub>129</sub> salt bridge to reform and regenerate the apo-enzyme structure.
and Lys$^{162}$ (not conserved in LicC) interacts with the phosphate of the phosphosugar substrate to increase its nucleophilicity. The function of Arg$^{194}$ (not conserved in LicC) is less clear because the side chain conformation is different in two RmlA structures such that the side chain of Arg194 in S. enterica RmlA interacts with the phosphate of the phosphosugar substrate, whereas its side chain in P. aeruginosa RmlA does not.

In E. coli methylenecyclitol-4-phosphate cytidylyltransferase, Arg$^{90}$ and Lys$^{77}$ (Arg$^{18}$ and Lys$^{28}$ in LicC, respectively) interact with the α-phosphate of CTP and are considered to function in transition state stabilization. Arg$^{157}$ from the second subunit of the dimer (not conserved in LicC) and Lys$^{213}$ (Glu$^{216}$ in LicC that coordinates Mg$^{2+}$) interact with the phosphate of the phosphoalcohol substrate, increasing the nucleophilicity of the phosphoalcohol during the catalytic reaction. Taken together, these results suggest that Arg$^{18}$ and Lys$^{28}$ (LicC numbering) in the signature sequence play a common role in transition state stabilization and substrate activation in catalytic mechanism for the NTP transferase family members.

The active sites of GlmU and RmlA have negative electrostatic potential that is unfavorable for binding of the negatively charged substrates. Mg$^{2+}$-coordinating Asp$^{107}$ and Glu$^{216}$ in LicC correspond to Asp$^{110}$ and Asp$^{225}$ in RmlA and Asp$^{105}$ and Gly$^{225}$ in GlmU. In RmlA, these conserved Asp residues contribute a putative, LicC-like metal binding site, but metal binding was not observed in the structures of RmlA (19, 21). Mg$^{2+}$ may bind to Asp$^{110}$ and Asp$^{225}$ of RmlA to function as catalytic metal. The side chain of Asp$^{110}$ in P. aeruginosa RmlA is within hydrogen bond distance to the α-phosphate oxygen of the nucleotide substrate, and the charge repulsion between Asp$^{110}$ and the α-phosphate of the nucleotide substrate must be compensated during the catalytic reaction. In LicC, Mg$^{2+}$ bound by Asp$^{107}$ and Glu$^{216}$ neutralizes the negative charges of the substrates and increases the catalytic activity by stabilizing the transition state. Interestingly, Mg$^{2+}$-coordinating Asp$^{107}$ and Glu$^{216}$ in LicC are replaced by Ala$^{108}$ and Lys$^{213}$ in E. coli methylenecyclitol-4-phosphate cytidylyltransferase, respectively. These two replacements not only explain why the active site of E. coli methylenecyclitol-4-phosphate cytidylyltransferase has positive electrostatic potential but also suggest that this enzyme may not require the direct involvement of Mg$^{2+}$ for binding substrates and catalysis.

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Bo-Yeon Kwak, Yong-Mei Zhang, Minkyung Yun, Richard J. Heath, Charles O. Rock, Suzanne Jackowski and Hee-Won Park

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