Glycerol-3-phosphate Cytidylyltransferase

Structural Changes Induced by Binding of CDP-Glycerol and the Role of Lysine Residues in Catalysis*

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The bacterial enzyme, glycerol-3-phosphate cytidylyltransferase (GCT), is a model for mammalian cytidylyltransferases and is a member of a large superfamily of nucleotidyltransferases. Dimeric GCT from Bacillus subtilis displays unusual negative cooperativity in substrate binding and appears to form products only when both active sites are occupied by substrates. Here we describe a complex of GCT with the product, CDP-glycerol, in a crystal structure in which bound sulfate serves as a partial mimic of the second product, pyrophosphate. Binding of sulfate to form a pseudo-ternary complex is observed in three of the four chains constituting the asymmetric unit and is accompanied by a backbone rearrangement at Asp131 and ordering of the C-terminal helix. Comparison with the CTP complex of GCT, determined previously, reveals that in the product complex the active site closes around the glycerol phosphate moiety with a concerted motion of the segment 37-47 that includes helix B. This rearrangement allows lysines 44 and 46 to interact with the glycerol and cytosine phosphates of CDP-glycerol. Binding of CDP-glycerol also induces smaller movements of residues 92-100. Roles of lysines 44 and 46 in catalysis have been confirmed by mutagenesis of these residues to alanine, which decreases $V_{\text{max(app)}}$ and has profound effects on the $K_m(app)$ for glycerol-3-phosphate.

Cytidylyltransferases function predominantly in biosynthetic pathways where they are responsible for activation of metabolic intermediates. They catalyze reversible reactions in which CTP and an alcohol are substrates, and a cytidylyl ester and pyrophosphate are the products, as shown in Equation 1 (below) for glycerol-3-phosphate cytidylyltransferase (GCT),1 a bacterial enzyme from Bacillus subtilis. GCT, which functions in the biosynthesis of teichoic acid, a component of certain bacterial cell walls, is a member of a family of cytidyltransferases that we have been investigating. The other two principal members of this family are phosphocholine and phosphoethanolamine cytidyltransferases, which function in eukaryotic phospholipid biosynthesis. This family ranges in complexity from multidomain eukaryotic enzymes to simple bacterial prototypes in which the entire protein corresponds to a minimal catalytic domain. The complex cytidyltransferases have attracted considerable interest, because they are frequently regulatory in biosynthetic pathways, and the activities of some of these enzymes are modulated by membrane lipids (1).

$\text{CTP} + \text{glycerol-3-phosphate} \rightleftharpoons \text{CDP-glycerol} + \text{pyrophosphate}$ (Eq. 1)

We have previously determined the structure of GCT, in complex with its substrate CTP, as a model for the catalytic domain of this family of cytidyltransferases (2). GCT is a homodimer in which each monomer adopts an $\alpha/\beta$ fold with a central five-stranded parallel $\beta$ sheet. In this structure, a large pocket, termed the substrate-binding “bowl,” is formed by helices and loops that extend from the central $\alpha/\beta$ core (see Fig. 5 in Ref. 2). CTP is bound at one side of the bowl, primarily by interactions with two prominent conserved motifs. The histidine side chains of the $^{14}\text{HWGH}$ motif (3) interact with the $\alpha$ and $\beta$ phosphates of the CTP. Both the side chains and backbone atoms of the $^{113}\text{REEGISTT}$ motif that characterizes this family of cytidyltransferases (3) interact with CTP (see below).

The GCT structure is closely related to the structures of phosphopantetheine adenylyltransferase (PPAT) (4, 5) and nicotinamide mononucleotide adenylyltransferase (NMNAT) (6-8), key enzymes in cofactor biosyntheses. The GCT, PPAT, and NMNAT structures are representatives of a superfamily of nucleotidyltransferases, all of which are presumed to share a common catalytic fold (9, 10). They typically incorporate Rossman-like $\alpha/\beta$ domains that are topped by a substrate binding region, and they contain the signature $\text{HXGH}$ sequence, which forms part of the nucleotide phosphate binding site. The $\text{HXGH}$ sequence was first noted in class I aminoaeryl-tRNA synthetases (11), which are also members of this nucleotidyltransferase superfamily. The GCT family of cytidyltransferases is distin-

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1 The abbreviations used are: GCT, glycerol-3-phosphate cytidylyltransferase; ECT, ethanolamine-phosphate cytidylyltransferase; CCT, choline-phosphate cytidylyltransferase; PPAT, phosphopantetheine adenylyltransferase; NMNAT, nicotinamide mononucleotide adenylyltransferase; NCS, non-crystallographic symmetry.
guished from the other nucleotidyltransferases in the superfamily by the presence of the RTEGISTT motif, which is unique to the GCT family. The structure of the CTP complex (2) showed interactions of the Arg115 side chain of this motif with the substrate but also revealed that specificity for the cytosine base was conferred primarily by hydrogen bonds with backbone atoms of the motif. Despite its broad distribution in nature, the GCT-like fold is not universally found in cytidylyltransferases. For example, a recent x-ray analysis of phosphocholine cytidylyltransferase from *Streptococcus pneumoniae* (12) demonstrates that the structure of this enzyme, which lacks the HXGH and RTEGISTT signatures, assigns it to a different family of nucleotidyltransferases, many of which catalyze the activation of sugars rather than lipid phosphates.

To define the binding site for glycerol-3-phosphate and to examine the conformational changes that accompany product formation (13, 14), we have determined the structure of GCT complexed with its product, CDP-glycerol (see Fig. 1). The structure reported here is a pseudo-ternary complex, with sulfaate as a partial mimic for the second product, pyrophosphate, and suggests roles for Lys44 and Lys46 in substrate binding and catalysis. These lysines are part of a substructure termed the “40s flap,” which closes around the glycerol phosphate end of the active site in the product complex.

### EXPERIMENTAL PROCEDURES

**Materials**—CTP, glycerol-3-phosphate, protease inhibitors, and imidazole were from Sigma. Nickel-nitrioltriocetic acid-agarose was from Qiagen. Vent DNA polymerase and restriction enzymes were from New England Biolabs. [44°C]Glycerol-3-phosphate was from Amersham Biosciences. Oligonucleotides were synthesized by the University of Michigan Biomedical Research Core Facility.

**Site-directed mutagenesis**—Site-directed mutagenesis employed a two-step PCR protocol and mutagenic oligonucleotides with pET11a-GCT (His-tag) as a template. Briefly, PCR reaction #1 amplified 5′ to the Lys44 or Lys46 codon. PCR reaction #2 amplified 3′ to the Lys44 or Lys46 codon. PCR reaction #3 mixed the overlapping products of PCR #1 and PCR #2 and the 5′ and 3′ flanking primers. The mutagenic oligonucleotides for K44A were 5′-CCGATGAAATTTAATACAGCG-3′ (forward) and 5′-GCTTTGTTATGAAACC-3′ (reverse) and for K46A were 5′-CCGATGAAATTTAATACAGCG-3′ (forward) and 5′-GCTTTGTTATGAAACC-3′ (reverse). Thermocycle parameters were 94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min. The final PCR product was cloned into pET11a using the NdeI and BamHI restriction sites. pET11a constructs were sequenced by the University of Michigan sequencing core.

**Protein Expression and Purification**—Histagged mutant constructs were expressed in BL21(DE3)pLysS *Escherichia coli* in a 4-h induction with 1 mM isopropylthiogalactoside. Cells were centrifuged at 5,000 g. Cells were expressed in BL21(DE3)pLysS

### Table 1 Data collection

| Parameter | Value |
|-----------|-------|
| Resolution (Å) | 1.87–1.80 |
| Highest resolution shell (Å) | 6.59–6.00 |
| Wavelength (Å) | 1.5416 |
| Number of reflections | 121437 |
| Number of unique reflections | 40610 |
| Completeness (%) | 87.5 (82.1) |
| Rmerge<sub>s</sub> | 0.051 (1.91) |
| I/σ<sub>obs</sub> | 20.6 (5.7) |
| Redundancy | 3.0 |

* Values in parentheses refer to the statistics in the highest resolution shell.

### Table 2 Model refinement statistics

| Parameter | Value |
|-----------|-------|
| Resolution (Å) | 15.0–1.8 |
| Reflections used in refinement | 40610/20322 |
| Protein atoms | 4166 |
| Waters | 518 |
| Product atoms | 120 |
| Sulfate | 15 |
| Average B-factor<sub>o</sub> for protein atoms (Å<sup>2</sup>) | 11.13, 10.57, 12.38, 10.26 |
| R<sub>work</sub>/R<sub>free</sub> | 0.2180/0.2541 |
| Root mean square deviation bonds (Å) | 0.007 |
| Root mean square deviation angles (°) | 1.30 |

* Total reflections and number used in calculation of R<sub>work</sub>.

### APPENDIX

#### APPENDIX A

**Data collection**

| Space group | P1 | a = 37.50 Å, b = 55.93 Å, c = 63.70 Å, α = 88.99°, β = 75.03°, γ = 82.54° |
|-------------|----|------------------|
| Resolution (Å) | 1.87–1.80 |
| Highest resolution shell (Å) | 6.59–6.00 |
| Wavelength (Å) | 1.5416 |
| Number of reflections | 121437 |
| Number of unique reflections | 40610 |
| Completeness (%) | 87.5 (82.1) |
| Rmerge<sub>s</sub> | 0.051 (1.91) |
| I/σ<sub>obs</sub> | 20.6 (5.7) |
| Redundancy | 3.0 |

#### APPENDIX B

**Model refinement statistics**

| Parameter | Value |
|-----------|-------|
| Resolution (Å) | 15.0–1.8 |
| Reflections used in refinement | 40610/20322 |
| Protein atoms | 4166 |
| Waters | 518 |
| Product atoms | 120 |
| Sulfate | 15 |
| Average B-factor<sub>o</sub> for protein atoms (Å<sup>2</sup>) | 11.13, 10.57, 12.38, 10.26 |
| R<sub>work</sub>/R<sub>free</sub> | 0.2180/0.2541 |
| Root mean square deviation bonds (Å) | 0.007 |
| Root mean square deviation angles (°) | 1.30 |

* Values in parentheses refer to the statistics in the highest resolution shell.

**Binding of CDP-glycerol to Cytidylyltransferase**

**Structure**

The crystal was flash-cooled to 140 K. Images were processed and scaled using DENZO and SCALEPACK (17). Data collection statistics are reported in Table I. The outermost data between 1.80 and 1.87 Å were 82% complete, with a missing wedge of reflections, but the observed reflections in this annulus were included in the final refinements.

The structure was solved by molecular replacement using the GCT-CTP complex (Protein Data Bank code 1COZ) as a model. Rotation searches with the coordinates for the GCT dimer (2), but omitting CTP, were carried out with X-PLOR (18). The highest peak determined the orientation of the first dimer and fixed the origin. The second dimer, whose orientation was derived from the rotation search, was positioned in a phased translation function computation (18).

Refinements were conducted in CNS (19) with cross-validation and maximum likelihood targets (20). Initial rigid body refinement with data from 15.0 to 2.0 Å used first the dimer model and then single chains as rigid groups and was followed by minimization with non-crystallographic symmetry (NCS) restraints applied to all atoms. At this stage R<sub>work</sub> was 0.404. R values of minimization and simulated annealing from 2000 K in torsional space were conducted using NCS restraints. After adding bound product and sulfate to the model, R<sub>work</sub>
RESULTS

Overview of the Product Complex—The structure of the triclinic crystal form of GCT with bound CDP-glycerol was solved by molecular replacement using the structure of the CTP-bound complex as a model. Because the asymmetric unit of the triclinic crystal includes two dimers, designated I and II, there are four independent views of the fold and of the enzyme-product interactions. Each of the monomers binds CDP-glycerol with thermal factors similar to the B-factors for the surrounding protein, consistent with high occupancy of the binding sites. Both chains of dimer I (Fig. 1A) contain strong, isolated electron density with protruding lobes characteristic of phosphate or sulfate, located close to the position corresponding to the β phosphate of CTP in the substrate complex (Fig. 1B). This bound ligand was assigned as sulfate because the crystals were grown in lithium sulfate with no added phosphate. The location of the sulfate suggests that it is a mimic of the other product, pyrophosphate. In dimer II this sulfate is slightly displaced in one monomer (chain D) and absent in the other (chain C).

Measurements of the Binding of CDP-glycerol to GCT—The affinities of the enzyme for CDP-glycerol were measured to compare the enzyme-CTP complex with the enzyme-CDP-glycerol complex. Binding to GCT was monitored by the quenching of the intrinsic fluorescence of tryptophan as described for the substrates of the forward reaction (13). Changes in fluorescence upon binding of CDP-glycerol (data not shown) were

backbones of the product complex and the CTP complex. The product complex is shown in blue, and the CTP complex is shown in silver. Binding of product induces structural changes in the positions of residues 37–47, the 40s flap, as can be seen at the upper left. CDP-glycerol and sulfate (colored) and CTP (silver) are drawn in ball-and-stick mode.
biphasic as has been observed for CTP and glycerol-3-phosphate. Dissociation constants of 0.4 ± 0.1 and 16 ± 9 μM were obtained for the first and second steps of product binding to the GCT dimer. These affinities are similar to those determined for CTP or glycerol-3-phosphate (13, 23).

**Interactions of GCT with CDP-glycerol**—The bound product adopts a zig-zag shape, with the α-(cytidine) phosphate positioned above the ribose ring and the glycerol moiety extended across the substrate binding bowl toward helix D (Fig. 1B). Interactions of the CMP moiety of the product with the fingerprint sequences 14HWGH and 113RTGISFF (see Table III and Fig. 2A) are similar to those found with CTP (2). The side chains of His44 and His46 interact with the sulfate in the monomers that contain sulfate. As shown in the CTP complex (2), hydrogen bonding of the cytosine moiety to backbone atoms from Thr414 and Ile117 establishes the selectivity for the cytosine base (Fig. 2A). However, in the CDP-glycerol complex the ribose 2' and 3' hydroxyls interact with the Asp94 carboxylate and with the amide of Gly92, respectively, whereas these ribose hydroxyls have no close protein partners in the CTP complex. These differences in product and substrate interactions result from a small movement of residues 92–99 toward the product.

The structure of the complex with CDP-glycerol reveals the binding determinants for glycerol-3-phosphate (see Table III and Fig. 2B). The oxygen that bridges the α- and β-phosphates of the product hydrogen bonds with the amide of Thr9 (not shown), and an exo-oxygen of the α-phosphate interacts with Lys46 (Fig. 2B). The indole nitrogen of Trp95 and the e-amino groups of Lys44 and Lys46 form hydrogen bonds to the β-phosphate, Lys77 binds the terminal 1' hydroxyl of glycerol, and Glu14' binds both of the hydroxyl groups of the glycerol moiety (see Table III and Fig. 2B). Closure of the rims of the substrate bowl (see below) brings Lys44, Lys46, and Trp95 within hydrogen bonding distance of the product.

The orientations of the substrate and product molecules in the respective complexes of GCT are compared in Fig. 3. The β- and γ-phosphates from CTP and the glycerol phosphate from CDP-glycerol extend on opposite sides of the CMP phosphate. The α-phosphate configuration in CDP-glycerol is inverted from that in CTP. These two states represent the configurations of this phosphate prior to and after formation of the transition state in which the α-phosphate assumes a trigonal bipyramidal configuration.

**Flap Closure and Other Structural Changes**—The overall folds of the product and CTP complexes are very similar (Fig. 1B). However, the two structures differ significantly at residues 37–47 in the vicinity of the glycerol phosphate moiety and more subtly at residues 92–99. The difference between the CTP and the product complexes at residues 37–47 is striking (Fig. 3). This piece of the structure, which includes a short helix at residues 37–43, acts as a flap or lid that closes over the glycerol phosphate. The concerted rearrangement can be approximated as a rigid body motion and has been analyzed using the program DynDom (24, 25). The motion of the flap is best described as a rotation of ~14° about an axis passing through the hinge residues 36 and 48. The backbone of residue 45 moves about 3.0 Å in this rearrangement. The smaller displacements at residues 92–99 and the C-terminal helix (residues 119–126) are not concerted and cannot be treated as rigid body motions.

In the triclinic crystal form, the two dimers of the asymmetric unit contact one another at an interface that includes residues 44–48 from each chain. This raises the possibility that crystal packing might influence the position of the flap in the product complex. The chains at this interface are related by an improper rotation of about 160° and a translation of 4.2 Å, and the interchain interactions are asymmetric. For example, Glu45 from the C chain intrudes into the substrate bowl of its neighboring A chain, approaching the sulfate ion, but the opposing Glu45 wraps back against its own chain. Despite this asymmetry, the motion of the flap is essentially the same in all four chains. Similarity of the flap motions and the studies of mutants that establish the functional importance of lysines 44 and 46 (see below) argue that flap closure is functional and not simply an artifact of crystal packing.

**Role of Lysines 44 and 46 in GCT: Analysis of Mutations**—The movement of the 40s flap toward the glycerol-3-phosphate portion of the product suggested that residues in this region were important for binding the substrate, glycerol-3-phosphate, and possibly important for catalysis. The two lysines in this segment, Lys44 and Lys46, are conserved in other putative bacterial glycerol-3-phosphate cytidylyltransferases (Fig. 4). Moreover, a conserved lysine is found in a similar segment of

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**TABLE III**

| Ligand interactions | Donor/acceptor | Distance (Å) | Donor/acceptor | Distance (Å) |
|---------------------|----------------|--------------|----------------|--------------|
| Cytosine 4-NH₂       | Thr114 O       | 2.77         | Sulfate O1     | 17N μ2       |
|                     | Ile117 O       | 2.94         | α-Phosphate O1 | 3.37         |
| Cytosine N3         | Thr114 NH      | 3.09         | α-Phosphate O2 | 3.03         |
| Cytosine O2         | Wat 211/201    | 2.71         | Wat 424/215    | 2.25         |
| Ribose 2’ OH        | Asp23 O62      | 2.97         |                 |              |
| Ribose 3’ OH        | Asp25 NH       | 2.93         | Sulfate O2     | 14 N μ2      |
| α-Phosphate O1      | Wat 232/248    | 2.83         | 119 NH         | 2.96         |
| α-Phosphate O2      | Phe46 NH       | 2.92         |                 |              |
|                      | Sulfate O1     | 3.37         | Sulfate O3     | 119 O μ1     |
|                      | Wat 424/215    | 2.53         |                 | 3.06         |
| α-Phosphate O3      | Sulfate O1     | 3.03         | Sulfate O4     | α-Phosphate O2 |
| β-Phosphate O1      | Sulfate O4     | 3.23         | Solvent “W1”   | 3.32         |
| β-Phosphate O2      | Lys46 N        | 3.13         |                 |              |
|                      | Solvent “W1”   | 2.94         |                 |              |
| Glycerol O2’        | Lys46 N        | 3.00         | α-Phosphate O2 | 2.94         |
|                      | Lys46 N        | 2.83         | β-Phosphate O2 | 2.67         |
| Glycerol O1’        | Lys44 N        | 2.98         | Sulfate O4     | 3.31         |
|                      | Glu44 O        | 2.64         |                 |              |
|                      | Lys77 N        | 3.01         |                 |              |

*Distances are averages of the distances observed in the two chains of dimer I.*
S0.5, the substrate concentration at half-maximum, were fit to a Hill equation. This allowed a calculation of the Hill coefficient, which represents the degree of cooperativity. Because of this cooperativity, the kinetic parameters for glycerol-3-phosphate, but K44A was negatively cooperative toward glycero-1-phosphate. Each mutant displayed a small degree of positive cooperativity (ECT) cytidylyltransferases (Fig. 4).

Selected hydrogen bonds between the glycerol phosphate and protein residues are shown as red dashed lines. Selected hydrogen bonds between the glycerol phosphate and protein residues are shown as dashed lines. See the text for descriptions of the tightly bound solvents and Table III for a complete list of the interactions of CDP-glycerol and sulfuric acid and solvents.

eukaryotic phosphocholine (CCT) and phosphoethanolamine (ECT) cytidylyltransferases (Fig. 4).

To assess the functional contributions of Lys44 and Lys46, each residue was mutated to alanine in the context of Histagged GCT (3). The mutant proteins were purified and assayed as a function of substrate concentration to determine whether Km and Vmax were altered toward either substrate (Table IV). Both the K44A and K46A mutants were considerably less active than the wild-type enzyme, with the apparent Vmax for each mutant enzyme reduced by a factor of about 10. Each mutant displayed a small degree of positive cooperativity toward CTP; K46A was positively cooperative toward glycerol-3-phosphate, but K44A was negatively cooperative toward glycerol-1-phosphate. Because of this cooperativity, the kinetic data were fit to a Hill equation. This allowed a calculation of S0.5, the substrate concentration at half Vmax.

The S0.5 for glycerol-3-phosphate was altered dramatically, increasing about 125-fold for each of the mutants (Table IV). This change is consistent with the conclusion that each lysine plays a role in the reaction. In addition, the S0.5 for CTP for the mutants was about 5-fold higher than for the wild-type, a result that is not surprising, because all previous mutations that increase Km for one substrate also increase Km for the other substrate (3).

The Ternary Complex and Comparisons with the Sulfate-free Chain—As already noted, sulfate from the crystallization medium appears in three of the four chains. In both chains of dimer I it occupies a site very close to the β-phosphate of CTP, a likely position for one of the phosphates of pyrophosphate (see Table III and Fig. 1B and Fig. 3); in the D chain of dimer II the sulfate is bound at a site between the positions of the β- and γ-phosphates of CTP.

Chain C of dimer II is a binary complex of enzyme and product without bound sulfate (Fig. 5). Comparisons of this chain with the chains of dimer I indicate that binding of sulfate to form a pseudo-ternary complex is accompanied by significant changes in the nucleotide end of the substrate-binding bowl (Fig. 5B). One of the structural differences between chains with and without sulfate is the orientation of the peptide connecting Asp11 and Leu12, with the carbonyl oxygen of Asp11 pointing toward the substrate bowl in the ternary complex with sulfate bound and away from the bowl in the binary complex. This oxygen also points away from the bowl in the CTP complex (Fig. 5B). The orientation of this peptide seems to depend on the presence and nature of the ligands that interact with His14, His17, and Thr119. In addition, the C-terminal region of the unique sulfate-free chain is much less ordered than the corresponding regions of the remaining chains in the structure and cannot be modeled beyond residue 115. We believe this apparent mobility of the sulfate-free chain has functional significance (see “Discussion”).

Structure of the Dimer Interfaces: Comparisons with the CTP Complex—Negative cooperativity (13) leads one to expect that substrate binding will induce structural changes that propagate to the dimer interface. The interfaces were examined to look for differential effects of CTP and CDP-glycerol. Comparisons of dimer I from the product complex with the CTP complex reveal small differences in the overall orientations of the monomer chains relative to one another; one chain rotates 4.2° and translates by 0.6 Å. Many of the direct interactions between chains, such as the stacking of Trp15 of chain A with...
His34 of chain B (Fig. 6), are retained in both structures, but several local differences between CTP and CDP-glycerol complexes are observed. Hydrogen bonding of the interface residue, His36, to its own chain is disrupted by the peptide flip at Asp11 that occurs in chains binding product and sulfate (Fig. 6). Small shifts of this side chain are altered by CTP binding (14). Invariant residues up- and downstream of the conserved lysines are included to establish the register of the alignments. CCTs are from B. subtilis (Bsu), Listeria innocua (Lmo), Staphylococcus aureus (Sau), Lactococcus lactis (Lac), Aquifex aeolicus (Aae), and S. pneumoniae (Spu). ECTs are from human (Hsa), Arabidopsis thaliana (Ath), and Saccharomyces cerevisiae.(Sce). CCTs are from Plasmodium falciparum (Pfa), Caenorhabditis elegans (Cee), rat (Rno), A. thaliana (Ath), and S. cerevisiae (Sce).

Table IV

| V_{max,app} | S_{0.5} | V_{max} | K_{m} | S_{0.5} | V_{max} |
|-------------|---------|---------|------|---------|---------|
| Wild-type   | 5.5 ± 0.3 | 1.4 ± 0.1 | 1.1 ± 0.1 | 0.74 ± 0.07 | 1.1 ± 0.02 |
| K4A         | 7.3 ± 0.5 | 7.4 ± 0.4 | 1.8 ± 0.1 | 97 ± 22 | 0.88 ± 0.04 |
| K46A        | 5.3 ± 0.5 | 6.8 ± 0.6 | 1.6 ± 0.1 | 89 ± 5 | 1.4 ± 0.1 |

The substrate concentration at half V_max,app, where S_{0.5} is the n-th root of K_{m} (14).

The role of the 40s flap in binding and reactivity: Comparisons with other nucleotidylytransferases—For the large family of transferases bearing the HXGH signature, including the aminoaacyl-tRNA synthetases, studies of mechanism converge on a scheme in which catalysis results from stabilization of the highly charged pentavalent transition state (27) by countercharges provided by basic residues and/or metal ions.3 The role of basic residues has been thoroughly documented for the aminoaacyl-tRNA synthetases by single and multiple mutations (28). Substitutions of alanine for the lysine residues in the KMSKS signature sequences of aminoaacyl-tRNA synthetases reduce k_{cat}/K_{m} by factors ranging from 100 to 5000 (29–33). The KMSKS signature of aminoaacyl-tRNA synthetases corresponds approximately to ^116GISTT, located near the start of the E helix in the structure of GCT (2). The Ile and Thr residues of this sequence interact with CTP and CDP-glycerol (Fig. 2A), but the lysines that are important for stabilization of the transition state in the synthetases are missing from the GCT signature sequence (Fig. 1C). Two downstream lysines, 121 and 123, are found in GCT but are not conserved and do not interact.

3 In an intriguing variation, the catalytic function of the second lysine of the KMSKS sequence in human tyrosyl-tRNA synthetase is replaced by potassium (37).
with phosphates of the substrate or product. The functional roles of the lysines from the KMSKS motif in aminoacyl-tRNA synthetases seem to be assumed by lysines 44 and 46 from the flap in GCT. Mutation of GCT residues Lys44 or Lys46 to alanine reduces $V_{\text{max}}/K_m$ for glycerol-3-phosphate by factors of 1100, an effect of the same order of magnitude as observed for the critical lysines in aminoacyl-tRNA synthetases. The present study thus reveals surprising variability in the positions of the basic residues that are presumed to stabilize the transition state. Another variation is found in some of the NMN adenylyltransferase enzymes (see below), which appear to utilize arginine residues from yet a third sequence for transition state stabilization. As a result of these variations in the positions of critical basic residues, a signature for essential basic groups is difficult to trace through the entire nucleotidyltransferase superfamily. Nevertheless the conservation of basic residues in the 40s can be detected within the GCT subfamily of cytidylyltransferase sequences as shown in Fig. 4. Indeed, substitution of alanine for the single lysine in this segment of rat choline phosphate cytidylyltransferase reduces $V_{\text{max}}/K_m$ by a factor of 240,000 (34), indicating an important functional role for the conserved lysine from the 40s region in this member of the GCT family.

**Fig. 5.** Binding of CDP-glycerol and sulfate to form a pseudo-ternary complex. *A,* an omit map from the B chain, computed after simulated annealing, showing the density corresponding to CDP-glycerol, sulfate, and one adjoining solvent. The contours are drawn at 3 $\sigma$. In chain C there is no density at the sulfate position. *B,* a view in the same orientation as Fig. 2A, showing the differences between the chain that binds only CDP-glycerol (silver) and the chains that also bind sulfate. In the sulfate-free chain there is no significant density corresponding to W1, and the structure cannot be modeled beyond residue 114.

**Fig. 6.** Dimer interfaces in the CTP complex and in the product (ternary) complex. *A,* a view of the dimer interface in the vicinity of His50 showing the backbone rearrangement at Asp11. The structure of the CTP complex is in silver; the blue and gold chains are from dimer I of the product complex, in which both chains bind sulfate. Reorientation of Asp11 is the major difference that is evident in this superposition of the two structures. Side chains of Leu42, Leu43, Ser41, and Glu48 have been omitted for clarity.

**Fig. 7.** Structural changes induced by binding of deamido-NAD$^+$ to NMNAT from *E. coli.* As in GCT the 40s segment of this enzyme has closed over the product analog, positioning basic residues near the dinucleotide phosphates. The 40s loop adopts several positions in the free enzyme; this drawing compares coordinates from the A chains.
Structures have been determined for four nucleotidyltransferases with folds similar to GCT, PPAT from *E. coli* (4, 5) and NMNATs from *E. coli* and two Archaea (6–8). As in GCT, the 40s regions of NMNAT and PPAT from *E. coli* incorporate basic residues that bind product. The effects of mutations in GCT suggest that these residues in NMNAT and PPAT are likely to play a catalytic role. As shown in Fig. 7, the 40s region of the *E. coli* NMNAT enzyme moves significantly relative to apoenzyme when NAD⁺ binds, bringing His⁶⁵ and Arg⁶⁶ close to the NAD phosphates (8). A smaller displacement of the loop carrying Lys⁴⁵ is observed in comparisons of free and product-bound PPAT (4). In contrast, in the archaeal NMNAT enzymes the segment equivalent to the flap of GCT is shortened, and primary interactions with the product phosphates are provided by arginine side chains at positions corresponding to Thr⁹ and near Lys¹⁸² of GCT, rather than by residues in the 40s region.

Cooperativity—Dimeric GCT binds its substrates CTP and glycerol-3-phosphate in random fashion (15), but it displays an intriguing form of negative cooperativity in substrate binding (13). At the first step in binding of either substrate, the apparent *Kₚ* is about 0.2 μM; at the second step *Kₚ* increases to ~30 μM. As reported here, the substrate for the reverse reaction, CDP-glycerol, similarly displays negative cooperativity in binding. Kinetic analyses and simulations have suggested that the enzyme turns over only when both active sites are occupied by substrates (13). Cooperativity has also been inferred for PPAT, where structures provide evidence for half-of-the-sites reactivity (4, 5). However, cooperativity may not be a universal feature of the GCT-like enzymes. A very recent study of the properties of a tetrameric GCT from *Staphylococcus aureus* has failed to find any evidence for cooperative behavior in that species of GCT (35).

The structural bases for negative cooperativity in *B. subtilis* GCT and for the requirement that the enzyme be fully loaded to turn over are incompletely understood. NMR studies of CTP binding to GCT support a sequential model for negative cooperativity (14). Measurements of Arg¹⁵N and backbone ¹⁵NH resonances as a function of increasing CTP concentration revealed changes in chemical shifts that were linked to binding of the second CTP and consistent with conformational changes near the dimer interface. These studies identified residues that might be important for cooperativity. Residues displaying chemical shift changes during the titration included Arg⁵⁵ and Arg⁶⁵; there were also changes in the C-terminal helix and the conserved region following Arg¹¹¹. The NMR experiments compared free enzyme with CTP-bound species and were therefore expected to detect conformational changes associated with cooperativity in binding of CTP, whereas the available x-ray structures only permit comparison of the saturated CTP- and CDP-glycerol complexes. Nevertheless the x-ray structures of these latter two complexes display some structural differences in the same regions that are associated with the chemical changes observed in the NMR experiments. Arg⁶⁵ appears flexible and adopts multiple conformations. Rearrangements are seen near His⁵⁵, a neighbor of the Arg⁶⁵ that was tracked in the NMR experiments. Evidence from the x-ray structures suggests that Asp¹¹ and His⁵⁵ sense the presence of ligands in the pyrophosphate/sulfate site. However, comparisons of the x-ray structures of substrate and product complexes do not reveal how the changes at one active site are communicated to the other chain of the dimer.

Both the x-ray experiments and NMR data indicate disorder and flexibility in GCT. In particular, the apparent disorder of the C terminus of the sulfate-free chain, where there is no ligand in the pyrophosphate subsite, strongly correlates with the flexibility of the C terminus of substrate-free GCT discerned by NMR (14). Measurements of relaxation times by NMR imply that substrate binding in the CTP subsite is associated with an increase in the ordering of the chains and the subunit interface and suggest a possible role for mobility in the cooperative behavior of this enzyme. The loss of entropy resulting from the reduced flexibility associated with substrate binding may contribute to the free energy of negative cooperativity in GCT (14).

Changes Accompanying Formation of a Ternary Complex—An intriguing property of GCT is that rapid turnover occurs only when both substrates are bound to both chains (13). Comparisons of the sulfate-free chain with the chains of dimer I, described under “Results,” reveal some structural changes that are associated with formation of the pseudo-ternary complex, CTP-CDP-glycerol-sulfate, from the binary complex with CDP-glycerol (Fig. 5B).

However, the structural changes that elicit full activity in true ternary complexes of GCT (13) are probably incompletely expressed in the complexes with CDP-glycerol and sulfate. Attempts to obtain fully occupied ternary complexes by cocRYSTALizing GCT with glycerol-3-phosphate and the nucleotide analog CPNPP, in which the α-β bridge oxygen is replaced by an amide (36), have met with limited success. Occupancies of the glycerol-3-phosphate site are low, and crystals are disordered by further soaking with very high concentrations of the ligands. Similarly, despite considerable effort, it has been not been possible to obtain crystals of ternary complexes of PPAT (5). These observations suggest that binding of Mg²⁺ and the highly charged pyrophosphate to form the ternary complex for the reverse reaction may be associated with much larger structural rearrangements than we see in comparisons of the sulfate-bound and sulfate-free chains. From the many studies of reactivity in the enzymes related to GCT it is evident that electrostatic interactions play a dominant role in both substrate binding and activity of nucleotidyltransferases (27, 28).

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