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Mechanisms of Product Feedback Regulation and Drug Resistance in Cytidine Triphosphate Synthetases from the Structure of a CTP-Inhibited Complex†‡

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ABSTRACT: Cytidine triphosphate synthetases (CTPSs) synthesize CTP and regulate its intracellular concentration through direct interactions with the four ribonucleotide triphosphates. In particular, CTP product is a feedback inhibitor that competes with UTP substrate. Selected CTPS mutations that impart resistance to pyrimidine antimetabolite inhibitors also relieve CTP inhibition and cause a dramatic increase in intracellular CTP concentration, indicating that the drugs act by binding to the CTP inhibitory site. Resistance mutations map to a pocket that, although adjacent, does not coincide with the expected UTP binding site in apo Escherichia coli CTPS [EcCTPS; Endrizzi, J. A., et al. (2004) Biochemistry 43, 6447–6463], suggesting allosteric rather than competitive inhibition. Here, bound CTP and ADP were visualized in catalytically active EcCTPS crystals soaked in either ATP and UTP substrates or ADP and CTP products. The CTP cytosine ring resides in the pocket predicted by the resistance mutations, while the triphosphate moiety overlaps the putative UTP triphosphate binding site, explaining how CTP competes with UTP while CTP resistance mutations are acquired without loss of catalytic efficiency. Extensive complementarity and interaction networks at the interfacial binding sites provide the high specificity for pyrimidine triphosphates and mediate nucleotide-dependent tetramer formation. Overall, these results depict a novel product inhibition strategy in which shared substrate and product moieties bind to a single subsite while specificity is conferred by separate subsites. This arrangement allows for independent adaptation of UTP and CTP binding affinities while efficiently utilizing the enzyme surface.

Cytidine triphosphate (CTP) is synthesized de novo from uridine triphosphate (UTP), adenosine triphosphate (ATP), and glutamine by cytidine triphosphate synthetases (CTPSs), EC 6.4.3.2, 525–630 residues (Figure 1a) (1–3; reviewed in ref 4). CTPSs catalyze three discrete reactions: MgATP-dependent phosphorylation of the UTP uracil O4 atom, glutamine hydrolysis to generate ammonia, and ammonia displacement of the uracil O4 phosphate (5–7). CTPSs also regulate intracellular CTP levels in response to the four ribonucleotide triphosphate concentrations (3, 8–16) and to protein phosphorylation in yeast (17) and likely other eukaryotes. In particular, substrate UTP and ATP binding induce oligomerization of inactive dimers to active tetramers, leading to positive cooperative behavior at physiological enzyme concentrations (Figure 1b) (3, 4, 11–13). Further, the product CTP provides negative feedback by acting as a competitive inhibitor of substrate UTP (3, 8, 9, 14), with a comparable Kᵢ for CTP (110 μM) and Kᵢ for UTP (150 μM) (3) (Figure 1a). This particular interaction determines the upper limit for intracellular CTP, and Saccharomyces cerevisiae carrying CTPS mutants defective in product inhibition exhibit 16–20-fold increased levels (18).

Antimetabolite pyrimidine analogues cyclopentenylcytosine (CPEC) and 3-deazauridine (3-deazaU) are effective CTPS inhibitors that arrest cancer cell proliferation (18–24) and are toxic to yeast and bacteria (18, 25). Moreover, using these inhibitors to deplete the intracellular CTP concentration increases the efficacy of the anticancer/antiviral drugs cytosine arabinoside (26, 27), 2′,3′-dideoxy-3′-thiacytidine (28), and 2′,2′-difuoro-2′-deoxyctydine (29). Trypanosoma brucei CTPS is a validated African sleeping sickness drug target (30), and giardiosis (31), giardiosis (32), chlamydia (33), and hemorrhagic fevers (34) are also potentially treatable using anti-CTPS therapies. However, spontaneous resistance to these drugs arises frequently through clustered CTPS gene mutations that release CTP feedback inhibition and increase intracellular CTP levels (Figures 2 and 6) (18, 25, 35, 36). These results define the
CTPS role in regulating intracellular CTP as well as suggesting that the drugs act by binding to the CTP inhibitory site. Visualizing the structural mechanisms for CTP and CTP analogue inhibition will provide the basis for rational improvement of efficacy and resistance evasion of CTPS antagonists.

Recently, we determined a prototypical CTPS structure, apo *Escherichia coli* CTPS, at 2.3 Å resolution (apo-EcCTPS, Protein Data Bank entry 1S1M) (4). Apo-EcCTPS is a nearly 222-symmetric homotetramer. Each monomer consists of an N-terminal ALase domain, which provides the oligomeric interfaces, and a C-terminal GATase domain (Figure 1b). The four kinase/ligase active site clefts where CTP is produced are assembled by highly conserved ALase domain surfaces from three different monomers, while GTP-regulated glutamine hydrolysis is carried out in the GATase domain glutaminase active site. In this *P*2,2,2 crystal form, a crystallographic 2-fold axis defines the tight “dimer interface” (A′–A’ or B–B’ interfaces²), while substrates bound in the kinase/ligase active sites mediate tetramer formation across the noncrystallographic dissociable “tetramer interface” (A–B’ and A’–B interfaces) (4).

Conservation of functionally and structurally important residues, which are ~20% identical with relatively few insertion or deletion differences between all members, suggests that EcCTPS is a viable model for understanding many aspects of CTPS function. This idea finds support in the structural similarity of EcCTPS and CTPS from *Thermus thermophilus* (37).

Previously, we used bioinformatic analysis to identify potential nucleotide binding sites (4). Structural relatedness of the ALase domain to the functionally related dethiobiotin synthetase (DTBS) provided predictions for the catalytic and ATP binding sites. The UTP site was deduced by modeling the uracil ring O4 position overlapping the analogous substrate oxygen position in the DTBS–DAPA–AlF₃ complex (PDB entry 1BS1) (38), and inferring the UTP γ-phosphate position from that of a sulfate or iodine ion ligated by a conserved P-loop-like structure located at the tetramer interface (Figure 2).

Parsimonious interpretation of existing data suggested that the CTP inhibitory site and the UTP catalytic site were the same. CTP competitively inhibits UTP binding (3), is isosteric to UTP, and similarly induces tetramer formation (13). However, some selected drug resistance mutations that abolish CTP binding do not interfere with UTP binding, actually increase the *k*_cat/*K*_m (18), and are located away from the predicted uracil ring catalytic site (4). This behavior raises questions about how a single active site can both simultaneously recognize and discriminate between two highly similar ligands, and how mutations could severely affect the binding of one ligand but not the other.

To address the structural mechanisms of CTP inhibition, infer the binding sites of pyrimidine analogue anti-CTPS drugs, and understand the molecular basis for drug resistance, we determined the structures of two ADP/CTP CTPS complexes, obtained by soaking apo crystals in either ammonium sulfate and Tris-HCl at pH 8.5 as previously

**FIGURE 1: CTP synthesis and regulation by CTP synthetases.** (a) CTPSs catalyze the amination of UTP to form CTP. The uracil O4 position is activated via ATP-dependent phosphorylation, and the resulting phosphate is displaced by ammonia generated by glutamine hydrolysis. CTPSs are regulated by all four nucleotide triphosphates: ATP and UTP promote oligomerization of inactive dimers to active tetramers (see panel b), GTP increases the *k*_cat/*K*_m of rate-limiting glutamine hydrolysis, and CTP is a negative feedback inhibitor that is competitive with UTP. (b) Space-filling representation of the EcCTPS tetramer of nearly identical subunits. Tetramerization of A–A’ (red and blue) and B–B’ (yellow and green) tightly associated dimers is mediated by the kinase ammonia ligase (ALase) domain (saturated yellow and red). GTP-activated glutamine hydrolysis is catalyzed by the glutamine amidotransferase (GATase) domain (light yellow and red). Tetramer formation is promoted by ATP and UTP substrates, as well as CTP product, binding in the CTP synthesis active site at the tetramer interfaces (black arrows). Bound ADP and CTP ligands at the B–A interface are also indicated (black).

EXPERIMENTAL PROCEDURES

CTPS crystals in space group *P*2₁2₁2 were grown in ammonium sulfate and Tris-HCl at pH 8.5 as previously.

² The asymmetric unit of the EcCTPS *P*2₁2₁2 crystal contains subunits A and B. Symmetry chains in the functional tetramer are denoted by a prime; for example, the dyad-related subunit of chain B is B’.
in the CTP and ADP positions were unambiguously visualized in the EcCTPS 1S1M model lacking solvent or ligand molecules. 41

Anisotropic temperature factor scaling (40) and processed using MOSFLM/SCALA (41, Table 1). This structure was deposited in the Protein Data Bank as entry 2AD5. The identities of the ADP and CTP ligands were confirmed by similar omit Fn - Fc difference density from ADP- and CTP-soaked crystals (2.8 Å resolution, 4.0σ, orange netting). Ribbons indicate secondary structure for the A (red), B (yellow), and B′ (green) EcCTPS subunits. The CTP and ADP positions from the refined 2AD5 model are enclosed by the electron density (black carbons). The hypothetical UTP positioning is also shown (4). Side chains for conserved catalytic residues K18, D72, and E140 identify the ALase catalytic site and the likely location of the UTP O4 atom for phosphorylation and/or amination, in analogy to the DTBS structure (38). Side chains at positions of drug resistance mutations (25, 35) that inhibit or abolish CTP feedback inhibition are also indicated (gray sticks; see Figure 6 for details).

Refinement was carried out using TNT Release 5F with anisotropic temperature factor scaling (41, 42). The apo-EcCTPS 1S1M model lacking solvent or ligand molecules was used as a starting model. After one round of refinement, the CTP and ADP positions were unambiguously visualized in Fn - Fc and 2Fc - Fc maps (Figure 2). However, no GTP density was discernible in preliminary or refined maps. Close inspection indicated a rearrangement in residues 227–233, and the formation of a new disulfide bond between Cys261 and Cys268 which are adjacent but reduced in the apo structure. The structure converged after several more rounds of model building with O (43) and TNT refinement. The final models were generally similar to the 1S1M structure (main chain rmsd = 0.62 Å). The structure described here is that of the ATP/UTP-soaked crystals which had generally more well-defined CTP density. It contains 1050 of 1090 residues, two CTP and two ADP molecules, two Mg ions, and 252 solvent molecules, and has R-factor and R-free values of 0.201 and 0.277, respectively, with good geometry (Table 1). This structure was deposited in the Protein Data Bank as entry 2AD5.

Structural comparisons and accompanying model calculations were performed utilizing EDPDB (44). Figures were generated using MOLSCRIPT (45) or BOBSCRIPT (46) rendered with RASTER3D (47). Although the active site structures of the two unique monomers in the asymmetric unit are quite similar (rmsd = 0.83 Å for 372 atoms of residues within 8 Å of the bound CTP), the ligand densities were somewhat clearer for monomer B and all figures depict this active site (Figure 2).

The kinetic constants for ATP and 2′-deoxyATP (dATP) in glutamine-dependent CTP synthesis were determined using uncleaved N-terminally His6-tagged EcCTPS protein. The PCR-amplified gene was cloned into Ndel- and Xhol-cleaved pET28b (+), and the protein was overexpressed in B21(DE3) cells and purified to homogeneity using a single metal chelate chromatographic step. Assay for CTP formation was carried out in triplicate as previously described (12) with 100 nM recombinant His6-EcCTPS, following the change in absorbance at 291 nm in 60 mM Na-HEPES (pH 8.0), 10 mM MgCl2, 1 mM UTP, 0.2 mM GTP, 5 mM Gln, and 30, 50, 100, 200, 400, 600, and 1000 μM ATP or dATP at 37 °C. Apparent S0.5, Vmax, and Hill coefficient values were obtained by manual fitting to the Hill equation using Excel. For His6-tagged EcCTPS, the specific activity and kinetic constants for ATP were comparable to those of the native enzyme purified from E. coli (12) (H. Kim, unpublished results).

RESULTS

Data from the product- and substrate-soaked crystals yielded identical electron density maps in the vicinity of the CTP synthesis active sites, suggesting that they both depict the product complexes (Figure 2). Thus, the crystals are catalytically active and competent to carry out both the phosphorylation and ammonia ligase reactions. The crystallization mother liquor containing ~0.8 M ammonium sulfate at pH 8.5 likely provided ammonia for the reaction in lieu of Gln hydrolysis, which is readily utilized by CTPSs [Km(NH3) ~ 2 mM (2, 48)].

Difference electron density features in both substrate- and product-soaked crystals indicated that ADP was bound in
The ADP ribose ring is sandwiched between the Ile20 side chain and Ala182(A) from the adjacent noncrystallographically-related “dissociable” subunit (Figure 3). This direct contact combined with the extensive interactions of loop residues 176–187 of the adjacent subunit with ATP binding site residues provides the structural basis for ATP-induced tetramer formation. In addition, the ribose O4’ atom is bound via a water-mediated hydrogen bond to Ser15 carbonyl and Arg211 guanidinium moieties. The ribose 2’- and 3’-hydroxyls have no direct protein contacts, although they are proximal to Asp303 and Lys306. However, 2’-hydroxyl recognition is apparently unimportant since ATP and dATP were equally effective cosubstrates with UTP. The apparent S0.5, Vmax, and Hill coefficient values for ATP (140 ± 30 μM, 2.9 ± 0.1 s−1, and 1.4 ± 0.3, respectively) were essentially identical to those for dATP (120 ± 20 μM, 4.2 ± 0.4 s−1, 1.4 ± 0.2, respectively). However, ddATP was not an efficient substrate (data not shown).

The diphosphate moiety is bound by the P-loop via a network of main chain and side chain hydrogen bonds (Figure 3). The β-phosphate is ligated by amide hydrogen bonds with residues 15, 17, and 18, while the α-phosphate is ligated by residue 19 and 20 amides. A Mg2+ ion also bridges the β-phosphate and putative catalytic residues Glu140 and Asp72 in the ATP/UTP-soaked crystals.

The CTP binding site location was defined by unambiguous pyrimidine nucleotide triphosphate density at the interface between three subunits (Figure 2), and binding was accompanied by only minor structural changes. Compared to apo-EcCTPS, the main chain rmsd for the entire tetramer is 0.62 Å, with individual domain main chain rmsd values ranging from 0.4 to 0.5 Å. Relative to the B subunit, the ALase domains of the A and B’ subunits rotated 1.7° and 1.1°, respectively, and shifted their centers of mass 0.6 and 0.9 Å, respectively, bringing the B and B’ subunits 0.31 Å closer together. A 1.1° hinging of the B subunit ALase and GATase domains was also observed.

The adenine ribose ring packs into a pit created by the loops of residues 17–21 and 238–247 and the side chains of Ile20 and Arg211 (Figure 3). The specificity for adenine ring N1 and N6 atoms is provided by main chain hydrogen bonds from the amide of Val241 and carbonyl oxygen of Lys239, respectively. As in other similar kinases, such as APS kinase [PDB entry 1M7G (49)], a conserved arginine, Arg211, stacks against the adenine, and is stabilized by a salt bridge with Asp240.

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Table 1: Data Collection and Refinement Statistics

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* Data were collected at SSRL beamline 7-1 using a MAR imaging plate. The data were integrated by MOSFLM and merged with SCALA (39) assisted by Wedger Elves (40). The merging R values were based on intensities for all of the data calculated by SCALA. R-free and R-factor values were calculated by TNT release 5F (41) using the following scaling parameters: Ksd = 0.75, Bsd = 121, K = 1.33. Anisotropic thermal corrections were also used: Bsd = −10.17, Bsd = 19.57, and B33 = −9.40. Model deviations from ideal geometry were calculated by TNT using Engh and Huber parameters (59) and the BCORRELS library (42). Average model B-factors were calculated with EDPDB (44). * From PDB entry 1S1M (4).
N4. The cytosine C5 and C6 atoms are proximal to Phe227-(A); however, they do not form a tight interaction, and the side chain electron density is not clearly defined (data not shown).

The CTP ribose packs against residues 114–116 (B′) and Glu149. Gln114(B′) vacates the space occupied by ribose, simultaneously capping the main chain carbonyl of Ile116-(B′) (Figure 4a,b). The Glu149 side chain switches from capping its own main chain amide in apo-EcCTPS to accept hydrogen bonds from both the ribose 2′- and 3′-hydroxyl groups. Val115(B′) and Ile116(B′) main chain amide donate additional hydrogen bonds to the 2′-hydroxyl, with main chain atoms 114–117(B′) shifting 0.9–1.0 Å toward the ribose (Figures 4a,b and 5). This trivalent 2′-hydroxyl recognition explains the preference for CTP over dCTP as an inhibitor (50).

The triphosphate moiety overlaps the predicted UTP triphosphate binding site (4), providing the structural basis for CTP binding competition with UTP and the absolute triphosphate requirement for cytosine ribonucleotide inhibition (50) (Figure 2). The CTP triphosphate makes extensive protein contacts via a network of polar interactions, provided primarily by the noncrystallographically-related “dissociable” subunit (Figures 4c and 5). The γ-phosphate is effectively pinned by six hydrogen bonds from the Lys187(A) and Thr188(A) side chains and the Thr188(A) and Lys189(A) main chain amides, as well as water-mediated interactions with Ser14 and Glu220(A). Lys189(A), Gln192(A), and Lys223(A) donate side chain hydrogen bonds to the β-phosphate. Within the nucleoside-binding reference subunit, the α-phosphate is recognized directly by the Ser14 side chain and via water by the Thr144 side chain. The critical role of the Lys187 side chain–γ-phosphate interaction in UTP binding is underscored by the inability of Lys187Ala EcCTPS to perform Gln- or NH3-dependent CTP synthesis (51).

In addition to the individual interactions, a number of interdependent protein–protein contacts cooperatively increase the specificity for CTP. Prominently, Thr188(A), Gln192(A), Lys189(A), and Asp147 co-align for phosphate recognition (Figures 4c and 5). This interlocking recognition network also provides an energetic basis for CTP-dependent tetramerization (13) in several ways (Figure 1b). First, the parsing of phosphate and nucleoside contacts to different subunits allows CTP, and presumably UTP, to act as “mortar” to cement the tetramer contacts. Second, phosphate-directed ordering of Lys189(A) and cytosine ring-directed repositioning of Asp147 generate an intersubunit salt bridge network that includes His193(A), while Lys187(A) is repositioned to hydrogen bond with Ser14 (Figures 4c and 5). These interactions are absent in the apo structure. Interestingly, Lys187Ala EcCTPS is not defective in ATP/UTP-dependent tetramerization (51), perhaps because oligomerization is still driven by ATP binding (11, 12). Neutralization of clustered positive charges at the interface by bound triphosphate may also contribute to oligomerization, since Lys187Ala EcCTPS tends to form trimers in the absence of nucleotides (51).

**DISCUSSION**

Although feedback inhibition is a common theme in unbranched biosynthetic pathways, relatively few enzymes appear to be primarily product-regulated. The simplest mode entails product binding in a substrate-like pose, with product dissociation limiting the maximum flux through the enzyme. Indeed, E. coli chorismate lyase (52), adenylosuccinate synthase (53), brain hexokinase I (54), and Thermoaerobacter tencongensis HGPRT (55) are inhibited in this manner. Alternatively, second allosteric product binding sites may be present, as in diguanylate cyclase (56), but if substrates are structurally similar to products, substrate inhibition may ensue.

CTPSs have evolved a hybrid strategy for distinguishing between UTP and CTP. The overlapping regions of the product feedback inhibitory and substrate sites recognize a common feature in both substrates, the triphosphate moiety. The feasibility of UTP sharing the CTP triphosphate binding subsite is supported by the lack of obvious alternative phosphate binding sites and by the ease with which the uracil...
ring can be placed in the putative catalytic site (Figure 2), by rotating the nucleoside \(120^\circ\) about the O5'-P'-O3'-P' torsion angle, combined with further adjustments about the R, \(\beta\), and \(\gamma\) torsion angles, while maintaining the entire set of protein—triphosphate interactions. In pantothenate kinase [PDB entries 1ESM and 1ESN (57)], the adenine rings of the ATP substrate and coenzyme A downstream product inhibitor also occupy widely separated pockets while their phosphate positions overlap. The novel aspect for CTPS is utilization of alternative binding modes to distinguish its remarkably similar substrate and product in contiguous enzyme surfaces.

The observed CTP positioning, along with the expected overlapping UTP substrate pose, explains at once how CTP competes for UTP binding and promotes tetramer formation, while mutations can abolish the CTP binding without reducing UTP affinity. The physical spanning of the dissociable tetramer interface by the ligand binding sites provides the basis for substrate- and product-induced tetramer formation. The extensive interdependent contacts, particularly with the \(\gamma\)-phosphate, provide an explanation for the absolute requirement for triphosphate substrate and regulator, as well as the requirement for conversion of noninhibitory CPEC and 3deazaU in vivo to the inhibitory nucleoside triphosphates (22, 50, 58). The thermodynamic linkage between substrate binding and the assembly of competent CTP synthesis sites clearly explains the apparent positive cooperativity of ATP and UTP at subsaturating substrate concentrations, and supports the idea that CTPSs are regulated in part by an association—dissociation mechanism (3, 11, 12). In addition, the product-inhibited enzyme is still in the “active” tetrameric form, and is conceivably poised to receive...
substrates, potentially allowing for a faster response to changing metabolic needs for CTP. Indeed, at physiological enzyme concentrations, a significant lag in activity is observed if EcCTPS is not preincubated with UTP or ATP (12), and at low enzyme concentrations, CTP stimulates activity, presumably by promoting tetramerization (3, 13).

The ability to exchange the pyrimidine ring between sites without shifting the triphosphate suggests the possibility that CTP might bind the inhibitory site immediately after synthesis and prior to dissociating from the enzyme.

To differentiate isosteric substrate and product pyrimidine rings, an additional pocket, lined by residues 115, 116, and 146–148 and far from the expected kinase/ligase catalytic site, specifically recognizes the cytosine and ribose portions of the inhibitor. The bidentate hydrogen bonds donated by the Ile148 and Glu149 main chain amides distinguish the $\text{B}^{-}\text{B}^{-}\text{prime}$-acceptor interface. Potential hydrogen bonds were suggested from the $\text{A}^{-}\text{B}^{-}\text{prime}$-acceptor interface and potentially provide a direct interaction path between dimer interface-related CTP sites, suggesting both a multiplying effect of position 148 and 151 substitutions on CTP binding and the potential for allosteric communication of CTP sites with each other or with the interfacial UTP or ATP sites. Indeed, given the proximity of the segments of each of the four protein chains, binding CTP at even a single site could conceivably influence interactions of all of the subunits.

While the CTP complex structure presented here provides straightforward rationales for some of the resistance mutations, positions 155, 158, and 229 do not directly contact the bound CTP and potential for allosteric interactions between bound CTPs. Ribbon diagram of the B–B′ crystallographic 2-fold interface, showing bound CTP (ball and stick with black carbon atoms), and side chains of residues that are replaced in CTP feedback inhibition-resistant CTPS mutants are shown as gray sticks. The associated subunits and residue labels are indicated by color (red for A, blue for A′, yellow for B, and green for B′). The black oval indicates the location of the 2-fold crystallographic symmetry axis, which relates subunits of the tightly bound dimer. The previously characterized CTP resistance substitutions are D147E [Chlamydia trachomatis (25)], V*116F, G146E, I148T, M*151I, R158H, and H*229K [hamster (35)], and E155K [hamster (35) and yeast (18)] (an asterisk denotes residues different from those of EcCTPS). Note the potential for substitutions at residues 148 and 151 to disrupt binding at both sites, and the potential for binding at one CTP site to influence binding at the two-fold-related site. Residues 155, 158, and 229 are not in direct contact with the bound CTP but could potentially interact with each other if the B–A′ and A–B′ interface distances were reduced by 1 Å.

bound CTP (Figure 4a,b). Interestingly, Ile148 and Leu151 participate in the B′–B′ interface and potentially provide a direct interaction path between dimer interface-related CTP sites, suggesting both a multiplying effect of position 148 and 151 substitutions on CTP binding and the potential for allosteric communication of CTP sites with each other or with the interfacial UTP or ATP sites. Indeed, given the proximity of the segments of each of the four protein chains, binding CTP at even a single site could conceivably influence interactions of all of the subunits.

While the CTP complex structure presented here provides straightforward rationales for some of the resistance mutations, positions 155, 158, and 229 do not directly contact the bound CTP and it is not apparent how the associated resistance substitutions exert their effects (Figure 6). These sites line a loosely associated and sequence-conserved interface formed by the B, B′, and A subunits. The lack of intimate contacts and the disorder of residues near the CTP site, such as Phe227 in the apo or CTP-inhibited forms described above, may indicate a yet-unidentified conforma-
tional change that tightens the interface upon CTP binding in solution but is not allowed in the crystals.

The hybrid strategy used by CTPSs for differentiation of substrate and product allows for greater evolutionary adaptability without requiring a separate product regulatory site. Affinity and specificity for substrates and products can be fine-tuned by adaptive mutation or protein modification independently, allowing responses to intracellular CTP concentrations without compromising catalytic efficiency. For CTPS, this property also makes cytidine analogues less attractive as anti-CTPS drugs since resistance to them can be acquired while maintaining CTP synthesis activity. A further limitation, in agreement with inhibitor studies (50), is the suggestion from the CTP complex structure that modifications to improve cytidine triphosphate analogue binding would be limited to cytosine N4, with size limits to C5 and C6. Finally, selected resistances to some pyrimidine compounds, such as 5-fluorouracil (36) which is not a CTP substrate (50), may not involve altered drug–enzyme interactions but rather selection for increased intracellular CTP levels that inhibit their uptake and incorporation.

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