# Identification of Lysine 122 and Arginine 196 as Important Functional Residues of Rat CTP:Phosphocholine Cytidylyltransferase Alpha†

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ABSTRACT: CTP:phosphocholine cytidylyltransferase α (CCTα) contains a central region that functions as a catalytic domain, converting phosphocholine and cytidine 5′-triphosphate (CTP) to CDP-choline for the subsequent synthesis of phosphatidylcholine. We have investigated the catalytic role of lysine 122 and arginine 196 of rat CCTα using site-directed mutagenesis and a baculovirus expression system. Arginine 196 is part of the highly conserved RTEGIST motif, while lysine 122 has not previously been identified by protein sequence alignment as a candidate catalytic amino acid. Removing the side chain of lysine 122 compromises the catalytic ability of CCTα, decreasing the apparent $V_{max}$ value in mutant enzymes Lys122Ala and Lys122Arg to 0.30 and 0.09% of the wild-type value, respectively. The decrease in $V_{max}$ is accompanied by dramatic 471- and 80-fold increases in the apparent $K_m$ value for phosphocholine but no greater than 3-fold increases in the apparent Hill constant ($K^*$) value for CTP. Mutation of arginine 196 to lysine results in an enzyme that retains 24% of the wild-type $V_{max}$ value with a modest 5-fold increase in the $K_m$ value for phosphocholine. However, the Arg196Lys mutant enzyme exhibits a 23-fold increase in the $K^*$ value for CTP. These data suggest lysine 122 and arginine 196 of rat CTP:phosphocholine cytidylyltransferase are functionally important amino acids, perhaps at or near the active site involved in forming contacts with the substrates phosphocholine and CTP, respectively.

The enzyme CTP:phosphocholine cytidylyltransferase (CCT)[1] is a member of the CDP-choline pathway for the biosynthesis of phosphatidylcholine, the major bilayer-forming lipid of eukaryotic cell membranes. CCT, the rate-limiting enzyme for the CDP-choline pathway (*1*), is present intracellularly as both a soluble and a membrane-associated form (*2*). The enzyme is activated simultaneously with the translocation of the enzyme from a soluble form to a membrane-associated form, and activation results in an increase in the rate of PC synthesis (*3*). Mammals have been found to possess two isoforms of CCT, denoted CCTα (*4*) and CCT*β* (*5*). Mammalian CCTα comprises several functional regions: the extreme N-terminus contains a nuclear localization signal, and the central portion constitutes a highly conserved catalytic domain, followed by C-terminal lipid binding and phosphorylation regions (*6*). The catalytic domain is conserved among the members of the cytidylyltransferase family, including CCT, CTP:phosphoethanolamine cytidylyltransferase (ECT) (*7*), and glycerol 3-phosphate cytidylyltransferase (GCT) (*8*). Mutagenesis studies utilizing GCT, the bacterial cytidylyltransferase, have identified amino acids critical for catalysis, including an arginine and two histidines (*9*). Similar studies have also implicated a histidine as critical for catalysis by rat CCTα (*10*).

Multiple protein sequence alignments have been employed as an initial strategy to identify conserved amino acids likely to serve a catalytic function in CCTα. This approach has resulted in the identification of several conserved motifs, including the HxGH and RTEGIST motifs (Figure 1). There is no three-dimensional structure of a mammalian CCT currently available, but the structure of the bacterial cytidylyltransferase GCT has been solved in complex with either the substrate CTP (*11*) or the product CDP-choline.[2] Therefore, an effective second strategy to aid in identification of catalytic amino acids of CCT is to utilize the known three-dimensional structure of bacterial GCT, previously postulated to be a model three-dimensional structure for the cytidylyltransferase family (*11*). GCT, like CCT, utilizes CTP as substrate, making it likely that amino acids at the active site of GCT within contact distance of CTP will be conserved in CCT. In the GCT:CTP binary complex multiple contacts are made with CTP by arginine 113, which corresponds to arginine 196 of CCT, the arginine of the conserved RTEGIST motif (Figure 1). However, since GCT utilizes glycerol 3-phosphate as substrate while CCTα utilizes choline phosphate as substrate, critical amino acids in contact with the phosphorylated alcohol substrate may not be revealed by sequence alignment of GCT with CCT. In the recently solved crystal structure of the enzyme:product structure of

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[1] Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; PC, phosphatidylcholine; ECT, CTP:phosphoethanolamine cytidylyltransferase; GCT, glycerol-3-phosphate cytidylyltransferase; CTP, cytidine 5′-triphosphate; PoC, phosphocholine.

[2] Pattridge, K. A., Weber, C. H., Friesen, J. A., Sanker, S., Kent, C., and Ludwig, M. L., submitted.

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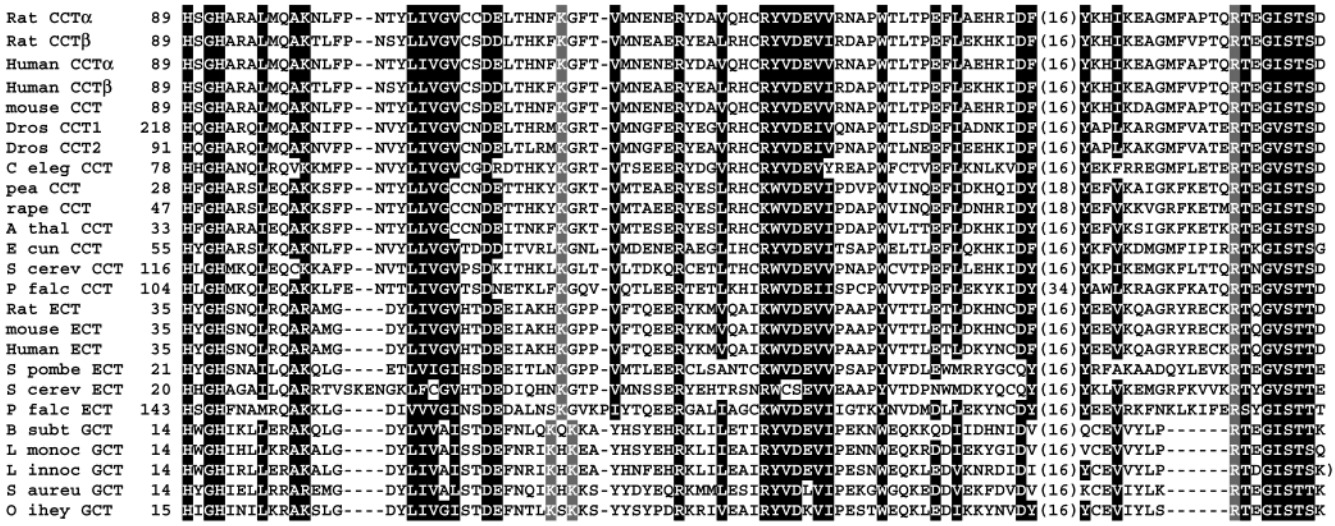

FIGURE 1: Comparison of the amino acid sequence of rat CCTα to 14 other CTP:phosphocholine cytidylyltransferases (CCT), six CTP:phosphoethanolamine cytidylyltransferases (ECT), and five glycerol-3-phosphate cytidylyltransferases (GCT). Boxed in black are amino acids identical or similar in at least 80% of the sequences. For consideration of similarity S = T, Y = W = F, L = I = V, R = K, and D = E. In gray are the positions corresponding to lysine 122 and arginine 196 of rat CCTα as well as lysine 44 and lysine 46 of GCT. Shown are positions corresponding to amino acids 89−204 of the catalytic domain of CCTα. Although not shown, there is an insertion with low conservation corresponding to amino acids 166−180 of rat CCTα, with the number of amino acids present in each sequence indicated in parentheses. Database accession numbers are as follows: rat CCTα, P19836; rat CCT*β*, Q9QZC4; human CCTα, P49585; human CCT*β*, Q9Y5K3; mouse CCTα, P49587; *Drosophila melanogaster* CCT1, NP_647621; *Drosophila melanogaster* CCT2, NP_647622; *Caenorhabditis elegans* CCT, P49583; *Pisum sativum* (pea) CCT, T06558; rapeseed CCT, T07982; *Arabidopsis thaliana* CCT, BAC01276; *Encephalitozoon cuniculi* CCT, CAD25880; *Saccharomyces cerevisiae* CCT, P13259; *Plasmodium falciparum* CCT, P49587; rat ECT, NP_446020; mouse ECT, AAH03473; human ECT, Q99447; *Saccharomyces pombe* ECT, T37720; *Saccharomyes cerevisiae* ECT, P33412; *Plasmodium flaciparum* ECT, CAD52599; *B. subtilis* GCT, NP_391455; *Listeria monocytogenes* GCT, NP_46614; *Listeria innocua* GCT, NP_470413; *Staphylococcuc aureus* GCT, BAB94468; *Oceanobacillus iheyensis* GCT, NP_693830.

GCT (GCT:CDP-glycerol) two lysines, at positions 44 and 46, were in position to interact with the glycerol moiety of the product.[2] However, these lysines were not aligned with lysines in CCT. There was only one lysine, at position 122, present in CCTα in that region of primary sequence, which aligned directly between lysine 44 and lysine 46 of GCT (Figure 1).

The question arose, then, what is the role of lysine 122 of CCTα in catalysis? In addition, is arginine 196 of the RTEGIST motif of CCTα also an active site amino acid, as is the case in GCT? We have investigated the catalytic role of lysine 122 and arginine 196 of rat CCTα using site-directed mutagenesis. Enzymes were expressed in Sf9 insect cells using a baculovirus expression system, purified to electrophoretic homogeneity, and kinetically characterized. From our data, we conclude that lysine 122 and arginine 196 each play an important role in CCTα function, perhaps involved in forming active site contacts with phosphocholine and CTP, respectively.

## MATERIALS AND METHODS

*Chemicals and Reagents.* Bac-to-Bac baculovirus expression system, serum-free *Spodoptera frugiperda* (Sf9) cells, Sf900II media, and trypan blue were from Invitrogen. Oligonucleotides were synthesized by the University of Michigan core facility. Restriction endonucleases were from New England Biolabs. [$^{14}$C]Phosphocholine was from Pharmacia. Cytidine 5′-triphosphate (CTP), DEAE Sepharose, CM-sepharose, phosphocholine, oleate, phosphatidylcholine, and protease inhibitors were from Sigma. Protein Assay kit was from Bio-Rad.

*Site-Directed Mutagenesis.* The full-length rat liver CCTα cDNA (*12*) was cloned into pFASTBAC1, the Bac-to-Bac baculovirus expression system donor plasmid, using the BamHI and SphI restriction sites of the multiple cloning site. Mutant CCTα cDNA constructs were produced by PCR-mediated overlap extension mutagenesis (*13*) employing mutagenic oligonucleotides encoding the desired codon change (Table 1). The resulting mutant cDNA fragments were placed in pFASTBAC1 using the BamHI and HindIII sites. The sequence of all cDNA constructs, including wild-type CCTα, was verified using automated DNA sequencing with fluorescent ddNTP dye terminators.

*Maintenance of Sf9 Cells.* Sf9 cells were grown in Sf900II Sf9 cell medium in suspension culture at 28 °C while shaking at 125 rpm. Cells were maintained at densities between $5 \times 10^5$ and $3 \times 10^6$ cells/mL. Cell viability was determined by trypan blue staining.

*Generation of Recombinant Baculovirus.* Production of recombinant Bacmid DNA and transfection of Sf9 cells was carried out as described in the Bac-to-Bac baculovirus expression manual. Viral supernatant from the initial transfection (0.2 mL) was used to infect 10 mL of Sf9 cells at $1 \times 10^6$ cells/mL. Viral supernatant liquid harvested 6 days post-infection (0.5 mL) was then used to infect 50 mL of Sf9 cells at $1 \times 10^6$ cells/mL. The viral supernatant harvested 6 days post-infection was used for protein expression. Viral titer was determined by plaque assay as described in the Bac-to-Bac baculovirus expression manual.

*Expression and Purification of CCTα.* Wild-type and mutant enzymes were expressed in Sf9 cells grown in suspension to a density of $1 \times 10^6$ cells/mL. Virus was added to a multiplicity of infection of 2. Cells were harvested 48

Table 1: Wild-Type CCTα cDNA Sequence around the Codons for Lys122 and Arg196 and the Sequence of Mutagenic Oligonucleotides[a]

| | |
|---|---|
| wild-type | 5′-GATGAGCTAACGCACAACTTCAAGGGCTTCACTGTGATG-3′ |
| Lys122Ala | 5′-GATGAGCTAACGCACAACTTCGCCGGCTTCACTGTGATG-3′ |
| Lys122Arg | 5′-GATGAGCTAACGCACAACTTCCGCGGCTTCACTGTGATG-3′ |
| wild-type | 5′-GCTCCCACACAGAGGACAGAAGGCATCTCCACATCAGACATCATC-3′ |
| Arg196Lys | 5′-GCTCCCACACAGAAGACAGAAGGCATCAGTACTTCAGACATCATC-3′ |

[a] Shown are the sequences of the wild-type cDNA sequence around the codons for Lys122 and Arg196 and the oligonucleotides used for PCR mutagenesis. Underlined are the codons corresponding to Lys122 and Arg196 of wild-type CCTα as well as each codon change. In the Lys122Ala oligonucleotide, a NaeI restriction enzyme site (GCCGGC) was added as a silent mutation. In the Lys122Arg oligonucleotide, a SacII restriction enzyme site (CCGCGG) was added as a silent mutation. In the Arg196Lys oligonucleotides, a ScaI restriction enzyme site (AGTACT) was added as a silent mutation.

h post-infection. For purification of wild type and mutant enzymes the procedure published for recombinant wild-type rat CCTα (*14*) was used. Briefly, a high-speed Sf9 cell supernatant was loaded onto a DEAE-Sepharose column equilibrated with buffer A (10 mM Tris-Cl, pH 7.5, 2 mM DTT, 1 mM EDTA) containing 150 mM NaCl. The column was washed with 10 column volumes of buffer A containing 150 mM NaCl and 1% Nonidet P-40 followed by 10 column volumes of buffer A containing 150 mM NaCl. The enzyme was eluted from the column with a 150 to 400 mM NaCl gradient in buffer A. The fractions constituting the peak of CCTα activity were pooled, diluted 5-fold in buffer A, and loaded onto a CM-Sepharose column equilibrated with buffer A containing 30 mM NaCl. The enzyme was eluted from the column with a gradient of NaCl from 30 to 150 mM, and the fractions containing the peak of the CCTα protein eluate were pooled. SDS−PAGE analysis of the purification procedure is shown in Figure 2A. Purification was monitored using an antibody directed against amino acids 1−236 of rat CCTα.

*Enzyme Assays.* CCTα was assayed essentially as described previously (*12*). The standard reaction mixture contained 50 mM imidazole, 25 mM magnesium acetate, 0.1 mM phosphatidylcholine:oleate (1:1 molar ratio), the desired amounts of [$^{14}$C]phosphocholine (specific activity between 0.04l and 0.1 mCi/mmol), CTP, and enzyme in a final volume of 100 $\mu$L at pH 7.0. Reactions were at 37 °C for 15 min (wild-type enzyme and Arg196Lys mutant enzyme) or 60 min (Lys122 mutant enzymes) and terminated by the addition of 100 $\mu$L of 150 mM phosphocholine in 10% trichloroacetic acid. 500 $\mu$L of 10 mg/mL activated charcoal was added, and the reaction mixture was incubated on ice for 1 h. The charcoal was pelleted and washed twice with water, and the radioactivity as [$^{14}$C]CDP-choline was quantitated by liquid scintillation counting. Protein was determined by the method of Bradford (*15*) using bovine serum albumin as standard.

*Kinetic Analysis.* For wild-type enzyme and mutant enzyme Arg196Lys, the kinetic parameters $V_{max}$ and $K_m$ with respect to PoC and $V_{max}$ and $K^*$ with respect CTP were determined from secondary plots (*16*). For wild-type enzyme, PoC was varied from 0.5 to 1.8 mM, and CTP was varied form 1 to 4 mM. For Arg196Lys mutant enzyme, PoC was varied from 1 to 5 mM, and CTP was varied from 5 to 20 mM. For Lys122 mutant enzymes, the kinetic parameters $V_{max}$ and $K_m$ with respect to phosphocholine were determined from double reciprocal plots. PoC was varied from 5 to 150 mM. Data were fit to the Lineweaver−Burk equation $1/v = (V_{max}/K_m 1/[S]) + 1/V_{max}$, where $v$ is the rate of the reaction, $V_{max}$ is the maximal rate of the reaction, $K_m$ is the Michaelis

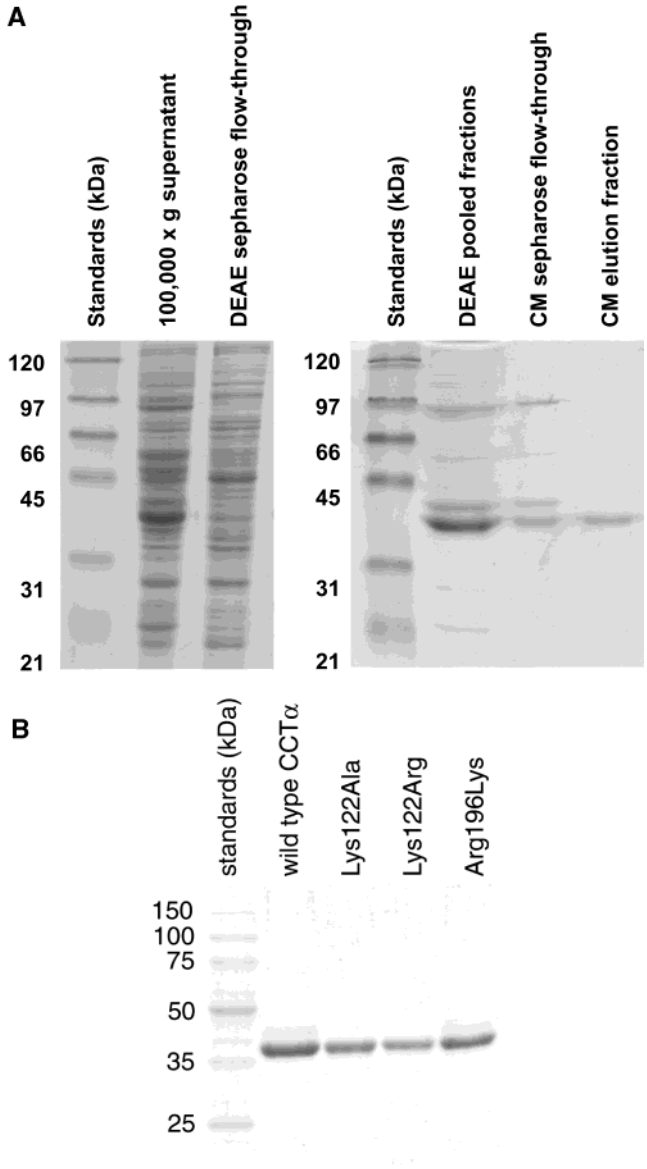


FIGURE 2: (A) SDS−PAGE of fractions from purification of Lys122Ala mutant enzyme. The enzyme was purified as described in Materials and Methods. 20 $\mu$g of protein was loaded per lane for the 100 000*g* cell supernatant and DEAE sepharose flow-through. 10 $\mu$g of protein was loaded for the DEAE pooled fraction, 5 $\mu$g for the CM column flow-through, and 3 $\mu$g for the CM elution fraction. (B) SDS−PAGE of purified wild-type CCTα or the indicated mutant enzyme. 3 $\mu$g of protein was loaded per lane.

constant, and [S] is the substrate concentration. Because of the observed cooperativity of mutant enzyme Lys122Arg toward CTP, kinetic data were analyzed by nonlinear regression analysis using SigmaPlot. CTP concentration was varied from 1 to 20 mM. Initial velocity versus [S] data were

Table 2: Kinetic Parameters of Purified Wild-Type and Mutant Enzymes[a]

| | $V_{max}$ (eu/mg)[b] | $K_m$ (mM) PoC[c] | $K^*$ (mM) CTP | $V_{max}/K_m$ PoC | $V_{max}/K^*$ CTP | Hill coefficient CTP |
|---|---|---|---|---|---|---|
| wild-type | 19 672 ± 5092 | 0.45 ± 0.11 | 1.54 ± 0.31 | 43 715 | 12 774 | 1.4 ± 0.1 |
| Lys122Ala | 59 ± 22 | 212 ± 104 | 2.09 ± 0.55[d] | 0.28 | 28 | 1.0 ± 0.2 |
| Lys122Arg | 18 ± 1 | 36 ± 6 | 4.78 ± 0.75[d] | 0.50 | 3.8 | 2.1 ± 0.4 |
| Arg196Lys | 4655 ± 309 | 2.35 ± 0.09 | 35.7 ± 0.6 | 1981 | 130 | nd[e] |

[a] Kinetic data were obtained as described in Materials and Methods. [b] One enzyme unit (eu) is the nmol of CDP-choline produced per minute. [c] Phosphocholine is abbreviated PoC. [d] The CTP $K^*$ value is an apparent kinetic constant obtained by varying CTP concentration in the presence of 40 mM PoC. Saturating concentrations of PoC were not attainable in the assay due to the high PoC $K^*$ value of Lys122 mutant enzymes. [e] Cooperativity toward CTP was not determined for mutant enzyme Arg196Lys since the high $K^*$ for CTP made saturating concentrations of CTP in the assay nonattainable.

fit to the sigmoidal Hill equation $v = (V_{max}[S]^n)/(K^{*n} + [S]^n)$, where $v$ is the rate of the reaction, $K^*$ is the Hill constant, and $n$ is the Hill coefficient. Mutant enzyme Lys122Ala did not exhibit cooperativity toward CTP, and kinetic data were fit to the hyperbolic equation $v = (V_{max}[S])/(K_m + [S])$.

## RESULTS

*Site-Directed Mutagenesis.* One way to study the role of an amino acid side chain in the function of a protein is to replace the residue with one of the other 19 naturally occurring amino acids. This can be accomplished at the genetic level by replacing the codon for the native amino acid with one encoding a desired amino acid, a procedure termed site-directed mutagenesis. Site-directed mutagenesis was conducted in this study to replace the codon for either lysine 122 or arginine 196, employing PCR-mediated overlap extension mutagenesis (*13*) and oligonucleotides encoding the desired nucleotide changes (Table 1). Mutation to alanine employs the simplest strategy for examining the function of a side chain by removing both the bulk and the hydrogen bonding capability of the side chain. Therefore, the codons for Lys122 and Arg196 were replaced by a codon encoding alanine.[3] Substitution of arginine for lysine, or vice-versa, may be considered a more conservative change, retaining the positive charge of the amino acid side chain at physiological pH. cDNA constructs encoding lysine instead of arginine at position 196 and arginine at position 122 instead of lysine were also created.

*Expression and Purification of Wild-Type and Mutant Enzymes.* CCTα was expressed in Sf9 insect cells with a baculovirus expression system. Expression of CCTα in this system is very efficient, with an overexpressed band evident by SDS−PAGE of a whole cell supernatant (Figure 2A). Assays conducted on whole cell supernatants under standard assay conditions (4 mM PoC and 10 mM CTP) revealed considerable activity for wild-type CCTα, 1550 nmol of CDP-choline produced per minute per milligram of protein. Sf9 insect cells expressing Arg196Lys mutant enzyme, assayed using standard assay conditions, had considerably lower activity, 201 nmol of CDP-choline produced per minute per milligram protein. Sf9 insect cells expressing Lys122 mutant enzymes, however, possessed activity no greater than cells infected with virus not expressing CCTα or uninfected Sf9 cells, approximately 4 nmol of CDP-choline produced per minute per milligram protein. Mutant enzymes Lys122Ala, Lys122Arg, and Arg196Lys were expressed at levels similar to the wild type enzyme, as judged by Western blot, and behaved identically to wild-type CCTα during purification. Purification of CCTα is dependent on the ability of the enzyme to bind to both anion-exchange resin (DEAE-sepharose) and cation-exchange resin (CM-sepharose) at pH 7.5, suggesting the protein is folded such that one portion, or domain, of the protein has a net negative charge and the remaining portion has a net positive charge. A grossly misfolded protein molecule would most likely not bind to both DEAE and CM sepharose. Since mutant enzymes Lys122Ala, Lys122Arg, and Arg196Lys were successfully purified, this suggests there were no gross alterations in overall protein folding. The final purity of expressed mutant forms of CCTα was judged by SDS−PAGE to be equivalent to wild-type CCTα, with yields of between 5 and 15 mg of pure protein per liter of Sf9 cells (Figure 2B).

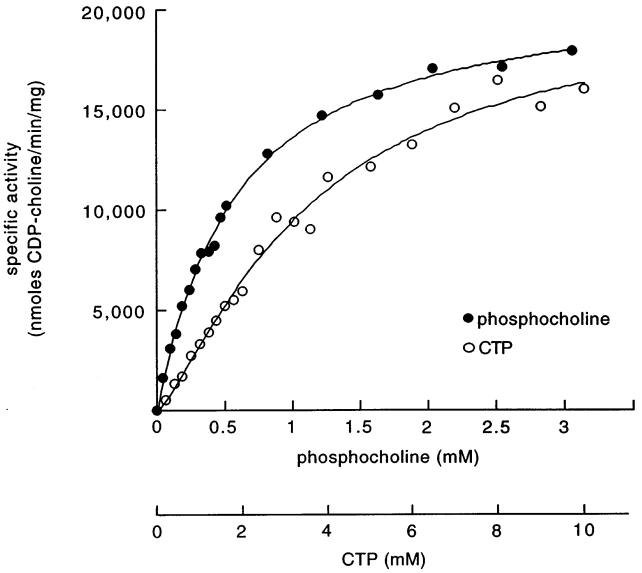


FIGURE 3: Initial velocity vs [S] plot illustrating the cooperativity of wild-type CCTα toward CTP. Phosphocholine dependence shows a hyperbolic relationship, while CTP dependence is sigmoidal. Data were fit to the Hill equation using nonlinear regression analysis as described in Materials and Methods. For CTP, the Hill coefficient is calculated to be 1.4 and for phosphocholine 1.0.

*Cooperativity with Respect to CTP.* In enzymes consisting of multiple identical protein subunits, cooperative binding of substrate can occur, resulting in the effective presence of

[3] Mutant enzyme Arg196Ala was not produced in insect cells using the baculovirus expression system, as judged by Western blot and enzyme assay. That Arg196Ala did not express suggests that arginine 196 may fulfill a role in protein folding or stability. Mutation of arginine 196 to alanine may have rendered the protein susceptible to intracellular protease attack.

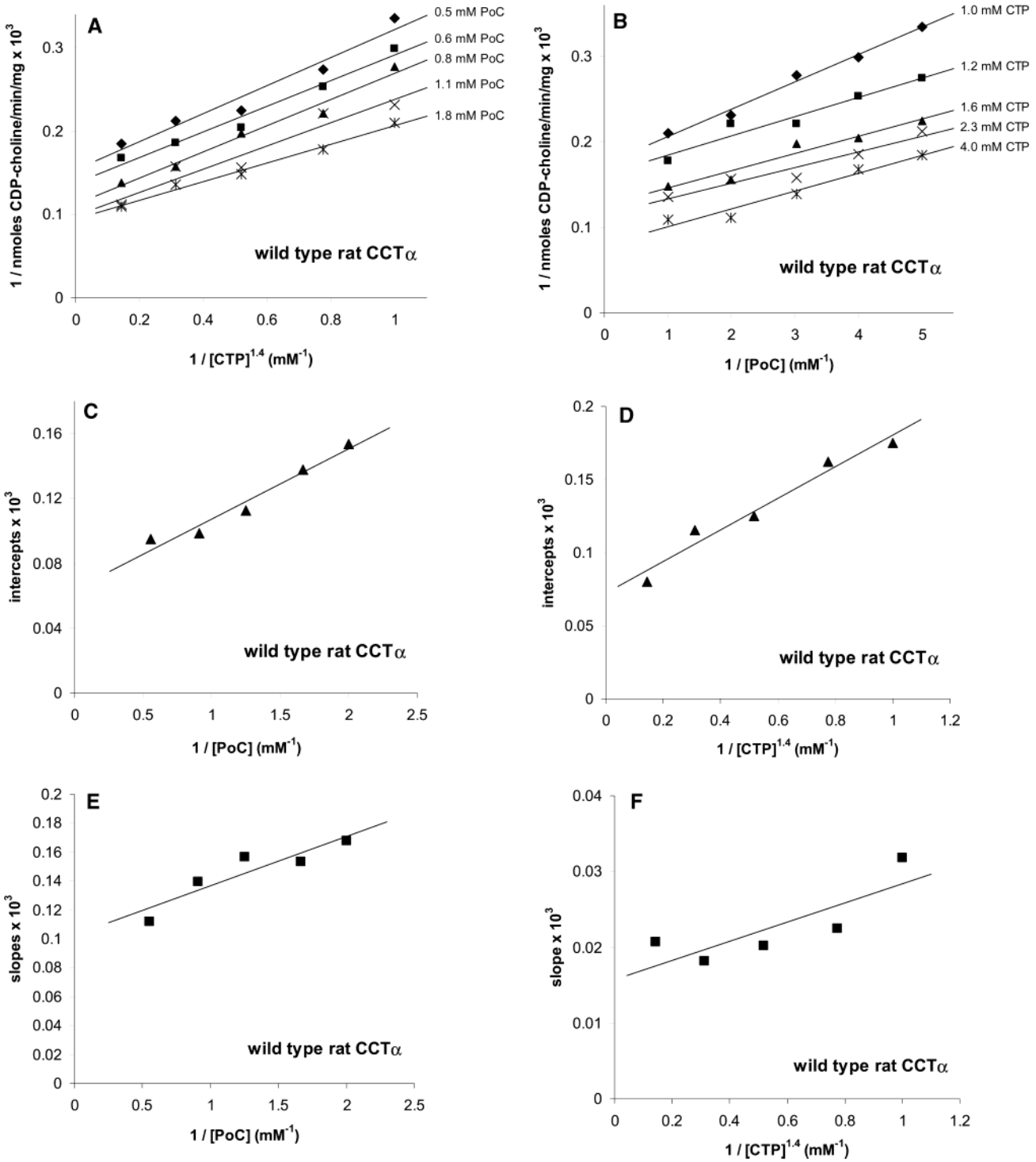


FIGURE 4: Kinetic plots for wild-type enzyme. (A) Wild-type CCTα $1/v$ vs $1/[CTP]^{1.4}$ plots; (B) $1/v$ vs $1/[PoC]$ plots; (C, D) replot of intercepts; and (E, F) slopes vs the reciprocal of the opposite substrate concentration. PoC was varied from 0.5 to 1.8 mM, and CTP was varied from 1 to 4 mM. Data were collected and analyzed as described in Materials and Methods.

low and high affinity binding sites. The binding of substrate to one subunit can induce conformational changes that result in altered substrate affinity in the remaining vacant sites. Mammalian CCT is thought to be a multimeric enzyme, postulated to form an elongated dimer (*17*, *18*) or possibly higher order aggregates (*14*). Kinetic parameters of wild-type CCT determined from nonlinear regression analysis of initial velocity versus [S] plots with data fit to the Hill equation [$v = (V_{max}[S]^n)/(K^{*n} + [S]^n)$] show the dependence of CCT activity on CTP is cooperative, with a Hill coefficient for wild-type CCT of 1.4 (Table 2). A primary plot of initial velocity versus CTP concentration exhibits a sigmoidal relationship rather than the hyperbolic relationship seen for PoC (Figure 3). Kinetic parameters for wild-type (Figure 4) and mutant enzyme Arg196Lys (Figure 5) were ultimately determined from replots of slopes and intercepts calculated from double reciprocal plots (*16*). For CTP, substrate concentrations were multiplied by the Hill coefficient ($n$) to account for the observed cooperativity (*16*). Lys122Arg mutant enzyme also exhibited cooperativity toward CTP, with a Hill coefficient of approximately 2; however, Lys122Ala showed no cooperativity toward CTP, with a Hill

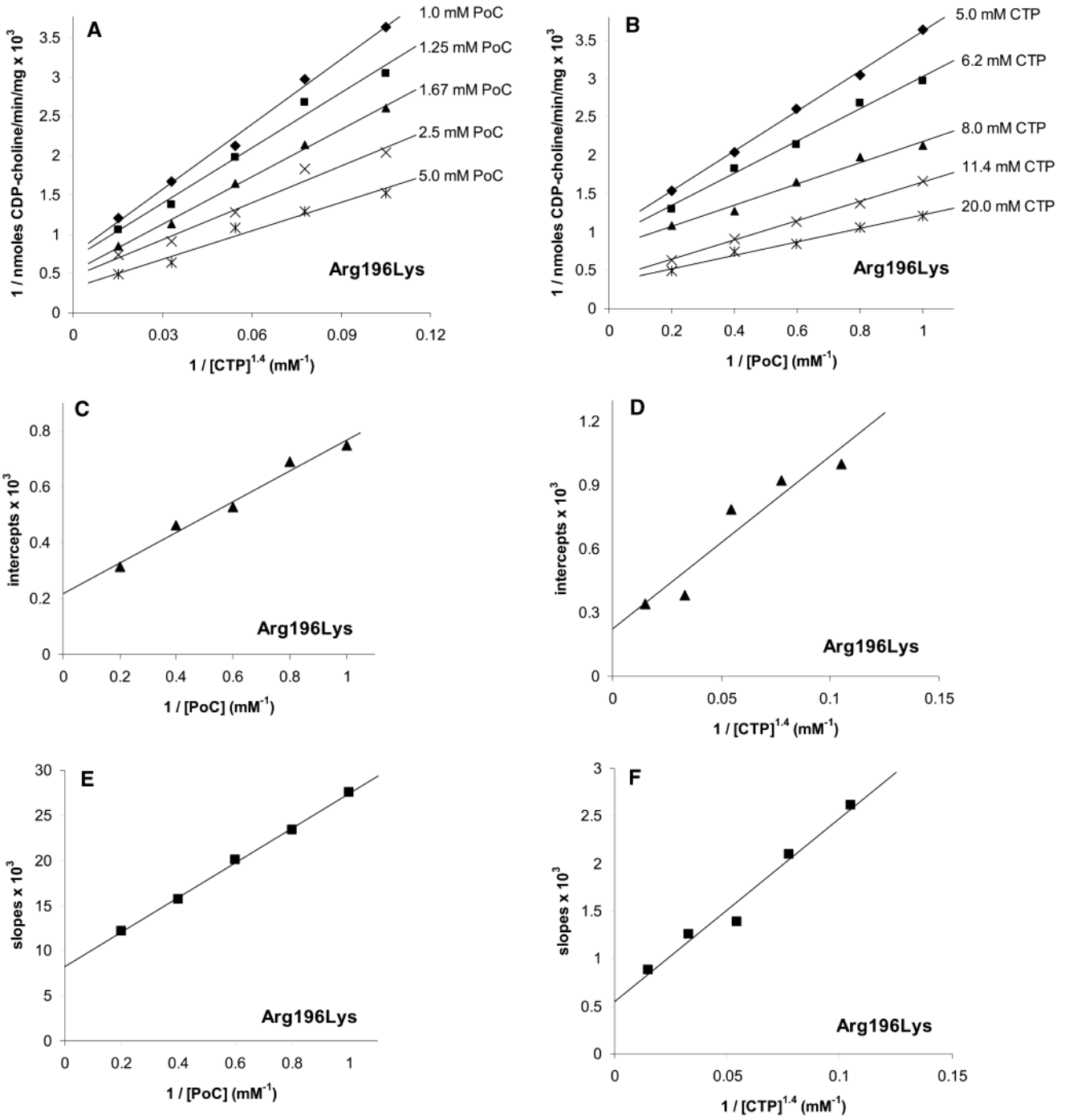


FIGURE 5: Kinetic plots for mutant enzyme Arg196Lys. (A) Arg196Lys $1/v$ vs $1/[CTP]^{1.4}$ plots; (B) $1/v$ vs $1/[PoC]$ plots; (C, D) replot of intercepts; and (E, F) slopes vs the reciprocal of the opposite substrate concentration. PoC was varied from 1 to 5 mM, and CTP was varied from 5 to 20 mM. Data were collected and analyzed as described in Materials and Methods.

coefficient near 1. Neither wild-type CCT nor any mutant enzyme showed cooperativity toward phosphocholine, with Hill coefficients near 1 in all cases.

*Mutation of Arginine 196 Impairs Catalytic Efficiency.* Replacement of Arg196 with lysine is detrimental to the catalytic function of CCTα. The catalytic efficiency ($V_{max}/K_m$) of Arg196Lys with respect to PoC decreased 22-fold. However, an even greater change was seen in the catalytic efficiency ($V_{max}/K^*$) of Arg196Lys with respect to CTP, which decreased 98-fold. Arg196Lys retains 24% of the wild-type $V_{max}$ value with a modest change in the $K_m$ value for phosphocholine of 5-fold. However, the $K^*$ value for CTP is increased 30-fold (Table 2 and Figure 5). The high $K^*$ for CTP made obtaining saturating concentrations of CTP in the enzyme assay an insurmountable obstacle. Cooperativity toward CTP was not able to be accurately determined for mutant enzyme Arg196Lys, but nevertheless, activity versus CTP plots exhibited a curvilinear shape, suggesting cooperativity with respect to CTP. To directly compare the kinetic parameters of mutant enzyme Arg196Lys to wild type a Hill coefficient equal to wild-type enzyme, 1.4, was assumed.

*Lysine 122 Is Important for Efficient Catalysis.* Replacement of lysine 122 with either alanine or arginine severely impairs the catalytic function of CCTα. The catalytic efficiency ($V_{max}/K_m$) of Lys122Ala and Lys122Arg with

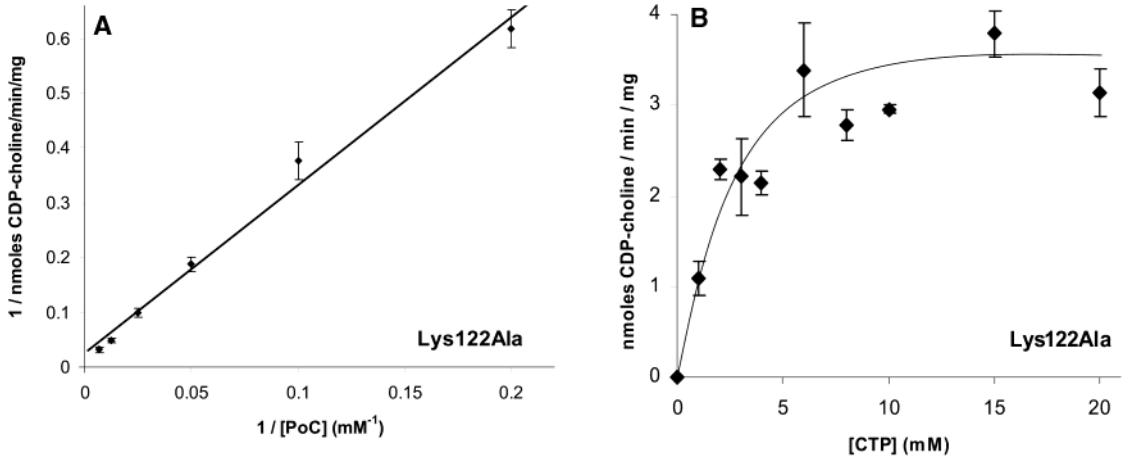


FIGURE 6: Kinetic plots for mutant enzyme Lys122Ala. (A) Lys122Ala $1/v$ vs 1/[PoC] plot and (B) $v$ vs [CTP] plot. PoC concentration was varied from 5 to 150 mM, CTP concentration was varied from 1 to 20 mM, and data were fit to the Lineweaver−Burk equation $1/v = (V_{max}/K_m*1/[S]) + 1/V_{max}$. Data are averages of triplicate determinations, and error bars on the appropriate data points indicate standard error. Experiments were repeated with similar results. Data were acquired and analyzed as described in Materials and Methods.

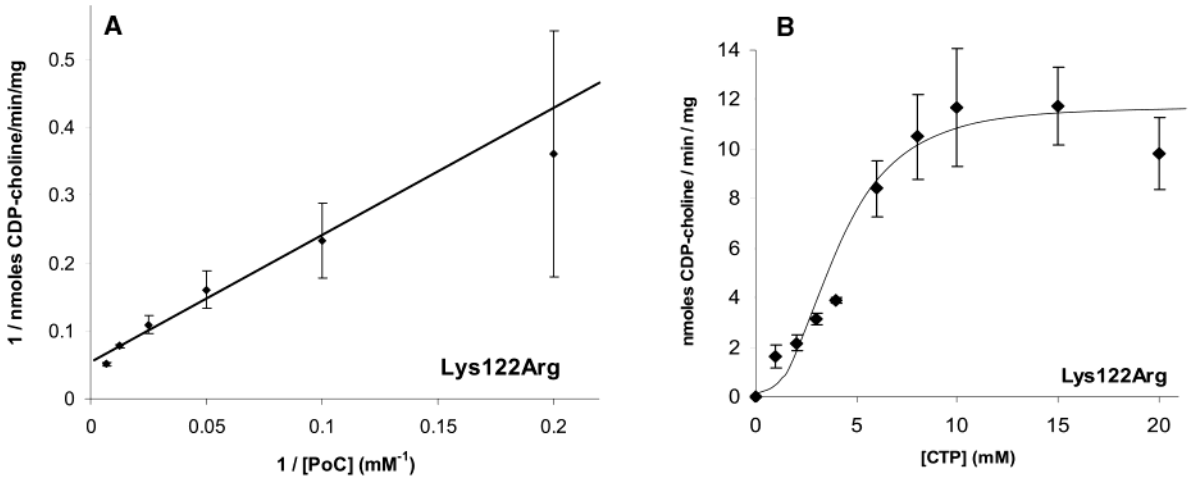


FIGURE 7: Kinetic plots for mutant enzyme Lys122Arg. (A) Lys122Arg $1/v$ vs 1/[PoC] plot and (B) $v$ vs [CTP] plot. PoC concentration was varied from 5 to 150 mM, and data were fit to the Lineweaver−Burk equation $1/v = (V_{max}/K_m*1/[S]) + 1/V_{max}$. CTP concentration was varied from 1 to 20 mM, and kinetic data were analyzed by nonlinear regression analysis using SigmaPlot. Data are averages of triplicate determinations, and error bars on the appropriate data points indicate standard error. Experiments were repeated with similar results. Data were acquired and analyzed as described in Materials and Methods.

respect to PoC is decreased by approximately $10^5$. Lys122Ala retains only 0.30% of the wild-type $V_{max}$ value, with an increase in the $K_m$ value for phosphocholine of 471-fold but no significant change in the $K^*$ value for CTP. (Table 2 and Figure 6). Lys122Arg retains only 0.09% of the wild-type $V_{max}$ value, with an increase in the $K_m$ value for phosphocholine of 80-fold but only a 3-fold change in the $K^*$ value for CTP. (Table 2 and Figure 7). Because of the extremely high $K_m$ values for PoC exhibited by the Lys122 mutant enzymes, saturating concentrations of PoC were not attainable in the assays. In the kinetic analysis, $V_{max}$ values were determined by varying PoC from 5 to 150 mM, the greatest concentration practically attainable, in the presence of CTP at 20 mM, a concentration at, or close to, saturation. The CTP $K^*$ value, therefore, is an approximation of an apparent kinetic constant obtained by varying CTP concentration from 1 to 20 mM in the presence of 40 mM PoC, a concentration of PoC below saturation.

## DISCUSSION

*Contribution of Arginine 196 of Rat CCTα to Catalysis.* Mutation of arginine 196 of CCTα, the first amino acid of the conserved RTEGIST motif, though not drastically affecting the maximal rate of formation of CDP-choline from CTP and phosphocholine, has a profound effect on the substrate concentration at which maximal rate is achieved. Removal of the arginine side chain at position 196 by mutation to lysine decreases the catalytic efficiency as much as 98-fold, predominantly due to the 23-fold increase in the $K^*$ value for the substrate CTP. Lysine retains the positive charge of arginine 196; however, the charged amino group is now present on the $\epsilon$-carbon of the side chain rather than the $\phi$-carbon, shortening the potential overall length of the side chain by approximately 1 Å, assuming an extended conformation of the side chain. Interactions formed by the terminal positive charge of the side chain of arginine 196 may not be retained upon mutation to lysine, but contacts made by the α, $\beta$, and $\gamma$ methylene groups will potentially remain. That mutation of arginine 196 to lysine decreases the $V_{max}$ value just 4-fold argues against the hypothesis that Arg196 is an active site amino acid directly involved in catalysis. However, the large increase in the $K^*$ value for CTP suggests that this residue is important for CTP binding, perhaps interacting with a phosphate of the CTP. Another

possibility is that Arg196 is distal to the active site, involved in an ionic interaction that maintains the integrity of the CTP binding pocket.

*Contribution of Lysine 122 of Rat CCTα to Catalysis.* Mutation of lysine 122 of CCTα to either alanine or arginine dramatically decreases the maximal rate of formation of CDP-choline as well as considerably increases the phosphocholine concentration necessary for catalysis to occur. Removal of the lysine side chain at position 122 decreases the catalytic efficiency more than 5 orders of magnitude with respect to phosphocholine, largely due to increases in the $K_m$ value of 2 orders of magnitude. Retention of positive charge at position 122 by replacement of lysine with arginine results in no greater retention of catalytic activity than removal of the majority of the lysine side chain by replacement with alanine. If the positive charge of lysine 122 is critical for catalysis, it must be precisely positioned for function since placing charged amino groups on the $\phi$-carbon of the side chain rather than the $\epsilon$-carbon, increasing the overall length of the side chain by approximately one angstrom, was not sufficient to retain reasonable catalytic efficiency. The decrease in $V_{max}$ of 3 orders of magnitude upon mutation of lysine 122 is consistent with this amino acid having a possible role in catalysis. However, the conclusion that Lys122 is essential for catalysis is not unequivocal, as activity was detectable for both Lys122Ala and Lys122Arg if PoC concentrations were increased tremendously in the assay.

*Conservation of Lys122 and Arg196 of CCTα.* As shown by computer generated amino acid sequence alignments, lysine is highly conserved among forms of CCT and ECT at the position corresponding to Lys122 of CCTα but is not conserved in GCT (Figure 1). GCT, however, does contain two lysine residues, lysine 44 and lysine 46, aligned on either side of lysine 122 of CCTα. Arginine is consistently present in members of the cytidylyltransferase family at the position corresponding to Arg196 of CCTα, conserved among all members of the cytidylyltransferase family, including CCTα, CCT$\beta$, ECT, and GCT (Figure 1). It is interesting to note, however, that the recently characterized cholinephosphate cytidylyltransferase from *Streptococcus pnemoniae* lacks both a HxGH and a RTEGIST motif and belongs in a family of enzymes catalyzing activation of sugar phosphates (*19*, *20*).

*Cooperativity toward CTP*. In this study, the CTP dependence of wild-type CCTα exhibits cooperativity, with a Hill coefficient of 1.4. This is a phenomenon that has not been reported previously in the literature for the wild-type enzyme. CCT purified from rat liver does not exhibit cooperativity with respect to CTP (*21*). The cooperativity of recombinant CCT purified from insect cells is not explainable at this point but is an interesting observation that warrants further investigation. CCT is extensively phosphorylated at the C-terminus (*13*), and it is possible that the phosphorylation state of CCT purified from insect cells is different than that from rat liver. The purification of recombinant rat CCT from insect cells has been reported twice (*12*, *22*), but in both cases no kinetic analysis was reported. It is notable that truncations of CCT that remove the phosphorylation or membrane-binding domain do exhibit cooperativity with respect to CTP (*23*), consistent with the concept that the state of the phosphorylation region influences the cooperativity.

*Comparison to Bacterial GCT.* In the three-dimensional structure of the GCT:CTP complex from *Bacillus subtilis*, arginine 113, the arginine of the RTEGIST motif, appears to play two roles in binding CTP (*11*). First, the positive charge of the arginine is positioned such that it stabilizes the negative charge of a $\beta$-phosphate oxygen of CTP. Second, interaction of the arginine side chain with CTP contributes to substrate binding by stacking against the nucleotide ring. Shortening the side chain of this critical amino acid upon replacement of arginine 196 of CCTα with lysine likely results in an inability of terminal positive charge to interact with the negatively charged α-phosphate oxygen of CTP, increasing $K^*$. Nonetheless, the lysine side chain is potentially long enough that it may have retained stacking interactions with the nucleotide ring of CTP, minimizing additional increases in $K^*$. It is also interesting to note that in the GCT:CTP complex, arginine 113 is approximately 7 Å from either histidine 14 or 17 of the conserved HxGH motif. The role of arginine 113 of GCT has previously been investigated using site-directed mutagenesis (*9*). In GCT, mutation of arginine 113 to alanine or lysine decreases $k_{cat}$ to 1.8 or 0.05% of the wild-type value without a significant change in $K_m$ values for CTP or glycerol phosphate. The opposite effect seen in the Arg196Lys CCTα mutant enzyme in this study, no change in maximally attainable catalytic rate but a significant increase in the $K^*$ value for CTP, suggests arginine 196 and the conserved RTEGIST motif may not fulfill the same function in CCTα as in GCT.

In the original GCT:CTP binary structure, lysine 44 and 46 are exposed to solvent on one side of an active site bowl opposite the bound cytidine nucleotide (*11*). In the recently solved crystal structure of GCT with bound product (CDP-glycerol), lysine 44 and 46 interact with the $\beta$-phosphate oxygen of the product.[2] A comparison of the GCT:CTP binary complex to the GCT:CDP-glycerol product complex reveals a major rearrangement of amino acids 37−47, which includes lysine 44 and 46 as well as a short helix from amino acids 37−43, which acts as a flap to close over the substrate glycerol phosphate. Results from the present study are consistent with a hypothesis that lysine 122 of CCTα interacts with the choline phosphate substrate and may be part of a larger region of primary sequence defining a critical portion of the active site. The difference between the GCT and the CCT reactions is the use of glycerol-3-phosphate as a substrate instead of choline phosphate. The presence of two critical active site lysines in GCT, but only one in CCT, may be a result of utilization of the different phosphorylated alcohol during catalysis.

*Comparison to Class I Aminoacyl-tRNA Synthetases.* The members of the cytidylyltransferase family have often been compared to class I aminoacyl-tRNA synthetases, primarily with respect to the modes of nucleotide binding (*24*). Class I aminoacyl-tRNA synthetases catalyze a nucleotidyl transfer of adenylyl from ATP to form aminoacyl adenylate. The principal catalytic factor in the activation of amino acids is improved binding of ATP in the pentacoordinate transition state, generated by the attack of the carboxylate of the amino acid on the α-phosphate of ATP (*25*). Class I aminoacyl-tRNA synthetases, like the cytidylyltransferases, contain a conserved HxGH motif but possess a KMSKS motif that has been postulated to play analogous structural roles to the RTEGIST motif of the cytidylyltransferases. Specifically, the

MSK portion of the KMSKS motif has been proposed as structurally equivalent to the IST portion of the RTEGIST motif (*11*). An analogous basic residue of aminoacyl-tRNA synthetases corresponding to arginine 196 of CCTα is nonetheless difficult to assign. The lysines of the KMSKS motif of aminoacyl-tRNA synthetases have been determined to be critical for catalytic function. Replacement of the lysine side chains by alanine reduces catalytic efficiency as much as 3 orders of magnitude (*26*). The lysines of the KMSKS motif participate in substrate binding (*27*, *28*) and stabilization of the pentacoordinate transition state (*29*). Results from this study, in conjunction with the GCT:CDP-glycerol structure,[2] suggest the role of the aminoacyl-tRNA synthetase KMSKS lysines may be fulfilled in the cytidylyltransferase family by lysines 44 and 46 of GCT and lysine 122 of rat CCTα, not by amino acids of the RTEGIST motif.

*Catalytic Mechanism of CCT.* As more studies reveal amino acids critical for catalysis by members of the cytidylyltransferase family, the proposed mechanism for catalysis can be refined. In the formation of CDP-choline the phosphate oxygen of phosphocholine is postulated to perform a nucleophilic attack on the α phosphate of CTP, forming a pentacoordinate transition state, a mechanism previously proposed for GCT (*9*). Histidine 89 of the CCTα HxGH motif, shown previously to be critical for catalysis (*10*), is proposed to interact with the $\beta$ phosphate of CTP. From this study, arginine 196 does not appear to play a direct catalytic role but likely contributes interactions that are critical for effective binding of CTP in the active site. As a result, mutation of arginine 196 results in an increase in the $K^*$ for CTP rather than a drastic decrease in $V_{max}$. Lysine 122 is potentially involved in catalysis, perhaps contributing energy to phosphocholine binding or stabilization of the proposed pentacoordinate transition state. Since the vital amino acids lysine 44 and 46 interact with a $\beta$ phosphate oxygen of the GCT reaction product, CDP-glycerol, lysine 122 of CCTα may interact with the phosphate moeity of phosphocholine, positioning the phosphate oxygen of phosphocholine for nucleophilic attack on the α phosphate of CTP. Removal of lysine 122, therefore, disrupts phosphocholine binding as well as prevents formation of the pentacoordinate transition state.

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