Aspartate-107 and leucine-109 facilitate efficient coupling of glutamine hydrolysis to CTP synthesis by *Escherichia coli* CTP synthase

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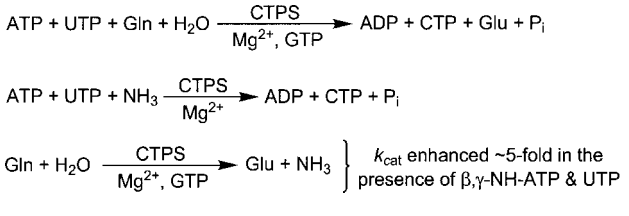
CTP synthase catalyses the ATP-dependent formation of CTP from UTP using either NH_3 or L-glutamine as the nitrogen source. GTP is required as an allosteric effector to promote glutamine hydrolysis. In an attempt to identify nucleotide-binding sites, scanning alanine mutagenesis was conducted on a highly conserved region of amino acid sequence (residues 102–118) within the synthase domain of *Escherichia coli* CTP synthase. Mutant K102A CTP synthase exhibited wild-type activity with respect to NH_3 and glutamine; however, the R105A, D107A, L109A and G110A enzymes exhibited wild-type NH_3-dependent activity and affinity for glutamine, but impaired glutamine-dependent CTP formation. The E103A, R104A and H118A enzymes exhibited no glutamine-dependent activity and were only partially active with NH_3. Although these observations were compatible with impaired activation by GTP, the apparent affinity of the D107A, L109A and G110A enzymes for GTP was reduced only 2–4-fold, suggesting that these residues do not play a significant role in GTP binding. In the presence of GTP, the k_{cat} values for glutamine hydrolysis by the D107A and L109A enzymes were identical with that of wild-type CTP synthase. Overall, the kinetic properties of L109A CTP synthase were consistent with an uncoupling of glutamine hydrolysis from CTP formation that occurs because an NH_3 tunnel has its normal structure altered or fails to form. L109F CTP synthase was prepared to block totally the putative NH_3 tunnel; however, this enzyme's rate of glutamine-dependent CTP formation and glutaminase activity were both impaired. In addition, we observed that mutation of amino acids located between residues 102 and 118 in the synthase domain can affect the enzyme's glutaminase activity, suggesting that these residues interact with residues in the glutamine amide transfer domain because they are in close proximity or via a conformationally dependent signalling mechanism.

Key words: amidotransferase, glutaminase, mutagensis, tunnel.

INTRODUCTION

CTP synthase [CTPS; EC 6.3.4.2; UTP:ammonia ligase (ADP-forming)] catalyses the ATP-dependent formation of CTP from UTP using either L-glutamine or NH_3 as the nitrogen source (Scheme 1) [1,2]. This glutamine amidotransferase is a single polypeptide chain consisting of two domains (Figure 1A). The C-terminal glutamine amide transfer (GAT) domain catalyses the hydrolysis of glutamine, and the nascent NH_3 derived from this glutaminase activity is transferred to the N-terminal synthase domain where the amination of UTP is catalysed [3,4]. Amino acid sequence similarities between GAT domains have been used to classify amidotransferases into two well-characterized families [5,6]. CTPS belongs to the Triad family of glutamine amidotransferases which utilize a Cys-His-Glu triad to catalyse glutamine hydrolysis. The N-terminal nucleophile amidotransferases utilize an N-terminal cysteine to catalyse glutamine hydrolysis.



Scheme 1 Reactions catalysed by CTPS

β,γ-NH-ATP, adenosine 5′-(β,γ-imido)triphosphate.

CTPS catalyses the final step in the *de novo* synthesis of cytosine nucleotides. Because CTP has a central role in the biosynthesis of nucleic acids [7] and membrane phospholipids [8], CTPS is a recognized target for the development of anti-neoplastic [7,9], anti-viral [9–11] and anti-protozoal [12–14] agents.

CTPS from *Escherichia coli* is the most thoroughly characterized CTPS with respect to its physical and kinetic properties, and is regulated in a complex fashion [1]. GTP is required as a positive allosteric effector to increase the efficiency (k_{cat}/K_m) of glutamine-dependent CTP synthesis 45-fold, but has a negligible effect on the reaction when NH_3 is the substrate [15,16]. In addition, the enzyme is inhibited by the product CTP [17], exhibits negative co-operativity for glutamine and GTP [18], and displays positive co-operativity for ATP and UTP [17–19]. ATP and UTP act synergistically to promote tetramerization of the enzyme to its active form [19]. Although the CTPS dimer can catalyse glutamine hydrolysis, enhanced glutaminase activity has been demonstrated in the presence of UTP and the non-hydrolysable ATP analogue β,γ-NH-ATP [adenosine 5′-(β,γ-imido)triphosphate; Scheme 1] [16]. Clearly, CTPS exhibits exquisite specificity in its ability to distinguish between CTP, UTP, ATP and GTP.

The structure of CTPS has not yet been determined and hence little is known about the enzyme's tertiary structure. However,

Abbreviations used: CPS, carbamoyl phosphate synthase; CTPS, CTP synthase; GAT, glutamine amide transfer; GF-HPLC, gel-filtration HPLC; GPATase, glutamine phosphoribosylpyrophosphate amidotransferase.

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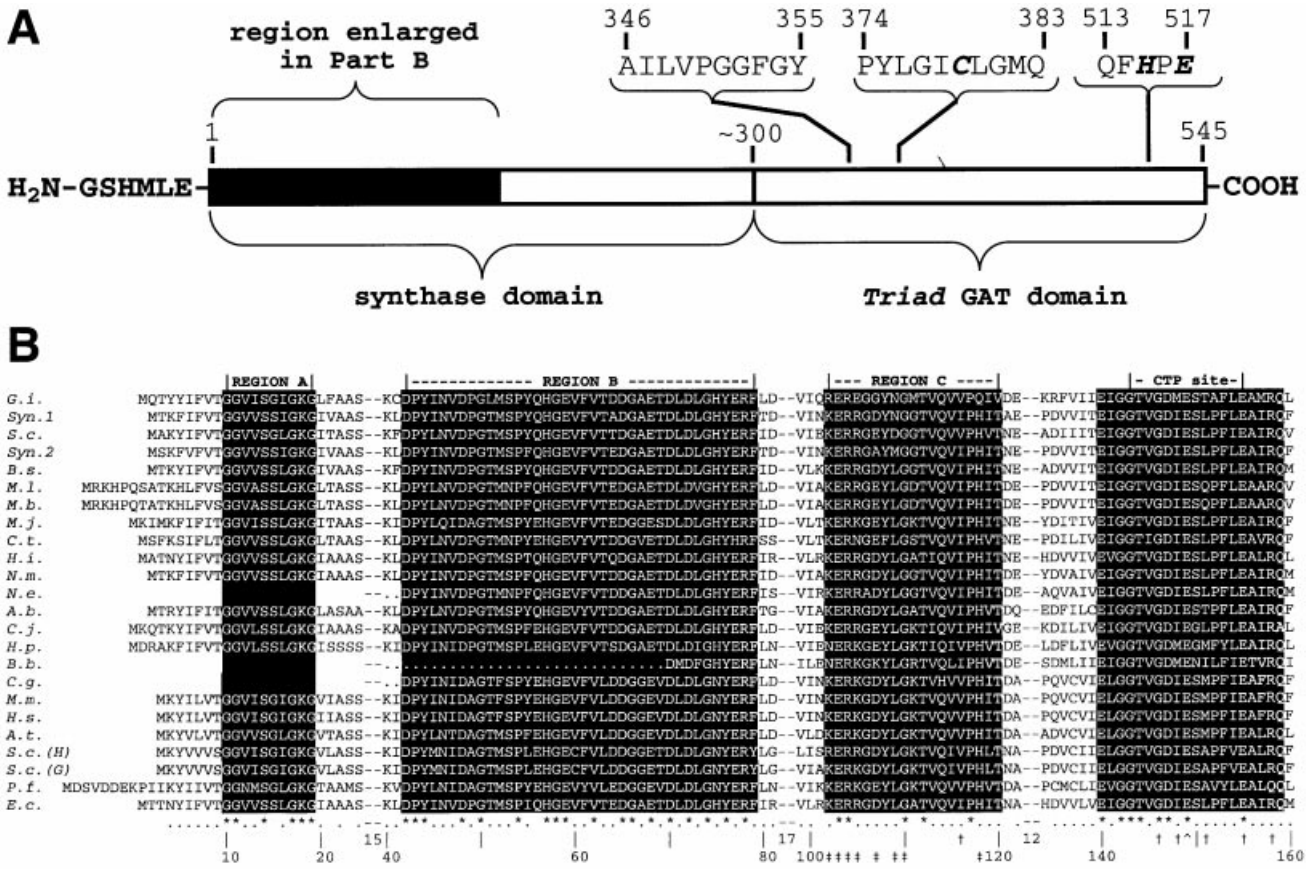


Figure 1 Primary structure of *E. coli* CTPS

(**A**) Domain structure of recombinant *E. coli* CTPS after cleavage of the hexahistidine tag. The diagram shows conserved regions of the GAT domain (including the Cys-His-Glu catalytic triad; letters in bold italics), as described in [3,4], and the synthase domain. The numbers above the diagram denote the amino acid positions according to the numbering for the wild-type enzyme. (**B**) Sequence comparison of a portion of the N-terminus (synthase domain) of 24 representative CTPSs. For the protein sequences shown, invariant residues (*) and residues which are $\geqslant$ 80% identical or conservative substitutions (.) are indicated. The previously described mutations in Chinese hamster ovary cells conferring resistance to the cytotoxic effects of arabinosylcytosine and 5-fluorouracil, and eliminating feedback inhibition by CTP [24], are indicated (†) along with the mutation identified in a *Chlamydia trachomatis* strain resistant to both cyclopentenylcytosine and feedback inhibition by CTP (^) [23]. With the exception of the mutations at residues 116 and 229 (not shown), most mutations clustered between residues 146 and 158 (shaded region). In addition to the CTP/UTP-binding site, three other highly conserved regions of amino acids are present (labelled A, B and C). Residues mutated in the present study are also indicated (‡ in region C). In descending order the proteins included in the alignment are as follows (with accession numbers): *Girardia intestinalis* (AAB41453.1), *Synechococcus* (Q54775), *Spiroplasma citri* (P52200), *Synechocystis* (P74208), *Bacillus subtilis* (P13242), *Mycobacterium leprae* (S72961), *Mycobacterium bovis* (AAB48045.1), *Methanococcus jannaschii* (Q58574), *C. trachomatis* (Q59321), *Haemophilus influenzae* (P44341), *Neisseria meningitidis* (CAB84970.1), *Nitrosomonas europaea* (AAC33441.1), *Azospirillum brasilense* (P28595), *Campylobacter jejuni* (CAB72520.1), *Helicobacter pylori* (O25116), *Borrelia burgdorferi* (O51522), *Cricetulus griseus* (P50547), *Mus musculus* (P70698), *Homo sapiens* (NP_001896.1), *Arabidopsis thaliana* (AAC78703.1), *Saccharomyces cerevisiae H* (*URA*-8 encoded; P38627), *S. cerevisiae G* (*URA*-7 encoded; P28274), *Plasmodium falciparum* (ACC36385.1) and *E. coli* (AAA69290.1). The numbering for the *E. coli* sequence is shown.

analysis of crystal structures of the Triad amidotransferases GMP synthase and carbamoyl phosphate synthase (CPS) has suggested that the structures of the GAT domains of all Triad enzymes are probably closely related [20,21]. Site-directed mutagenesis studies and sequence comparisons have revealed structural and catalytic roles for several amino acid residues within the GAT domain of CTPS, including residues of the catalytic triad (Cys-379, His-515 and Glu-517) [3], residues comprising the oxyanion hole (Gly-351, Gly-377, Gly-381 and possibly adjacent hydrophobic residues) [22], and residues between Ala-346 and Tyr-355 that appear to play an important structural role [4]. Our knowledge about the synthase domain, however, is much more limited. Analyses of active mutant CTPSs from *Chlamydia trachomatis* [23], hamster [24] and yeast [25] have revealed that mutations which render cells resistant to both the cytotoxic effects of cyclopentenylcytosine and feedback inhibition by CTP, occur between residues 116 and 229 (*E. coli* numbering), with many of the mutations clustering between residues 146 and 158 as shown in Figure 1(B). Hence, this region of the synthase domain is believed to form part of the CTP-binding site. In addition, competitive inhibition experiments have suggested that for *E. coli* CTPS this site is also the UTP-binding site [17]. The locations of the ATP- and GTP-binding sites, however, have not been identified.

In addition to the conserved region of amino acid sequence adjacent to and including the CTP/UTP-binding site, several other highly conserved regions reside within the N-terminus of the synthase domain (Figure 1B). The present report describes scanning alanine mutagenesis of the highly conserved region between residues 102 and 118 (region C) within the synthase domain. Surprisingly, kinetic characterization of mutant CTPSs (K102A, E103A, R104A, R105A, D107A, L109A/F, G110A and H118A) revealed that several residues located centrally within this conserved region function to ensure efficient coupling of glutamine-dependent NH_3 formation to the synthase activity. Most interestingly, replacement of Leu-109 by alanine yields an

enzyme for which glutamine hydrolysis and NH_3-dependent CTP formation occur at wild-type rates, but glutamine-dependent CTP formation is markedly impaired. This observation is consistent with a failure of NH_3 derived from glutamine to be transferred to the synthase domain. In addition, some mutations in the synthase domain also exhibited a marked effect on both the glutaminase activity and glutamine-dependent CTP formation, providing additional evidence that the synthase and the GAT domains interact within the tertiary structure of the enzyme.

EXPERIMENTAL

General materials and methods

Restriction enzymes were purchased from Gibco-BRL Life Technologies (Burlington, ON, Canada) and New England Biolabs (Mississauga, ON, Canada). His·Bind® resin, thrombin cleavage capture kits and the pET-15b expression system were purchased from Novagen (Madison, WI, U.S.A.). *Pfu* Turbo® DNA polymerase was purchased from Stratagene (La Jolla, CA, U.S.A.). All other chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Oligonucleotide primers for sequencing reactions and site-directed mutagenesis were commercially synthesized by ID Labs (London, ON, Canada). All plasmid preparations for mutagenesis and transformation were conducted using the QIAprep® spin plasmid purification kit (Qiagen, Mississauga, ON, Canada). Zorbax Bio Series GF-250 columns were from Chromatographic Specialities (Brockville, ON, Canada). Sequence alignments were conducted using ClustalX (v. 1.8) [26]. DNA sequencing was conducted at the Dalhousie University NRC Institute for Marine Biosciences Joint Laboratory (Halifax, NS, Canada) and Robarts Research Institute (London, ON, Canada). For HPLC experiments, a Waters 510 pump and 680 controller were used for solvent delivery. Injections were made using a Rheodyne 7725i sample injector fitted with either a 20 or 50 μl injection loop.

Enzyme expression and purification

Wild-type recombinant *E. coli* CTPS was expressed in and purified from *E. coli* strain BL21(DE3) cells transformed with the plasmid pET15b-CTPS1 as described previously [15]. This construct encodes the CTPS gene product with an N-terminal hexahistidine tag. The BL21(DE3) cells were grown in Luria–Bertani medium at 37 °C, induced using isopropyl β-D-thiogalactoside according to the Novagen expression protocol [27], and lysed using sonication on ice (5×10 s bursts with 30 s intervals at output setting 5 using a Branson Sonifier 250). The crude lysate was clarified by centrifugation (39000 ***g***, 20 min, 4 °C) and the soluble histidine-tagged CTPS was purified using metal-ion affinity chromatography as described in the Novagen protocols [27]. The resulting enzyme solution was concentrated using an Amicon® Centriprep-30 concentrator (Fisher Scientific, Nepean, ON, Canada) and then dialysed into Hepes buffer (70 mM, pH 8.0) containing EDTA (0.5 mM) and $MgCl_2$ (10 mM; assay buffer). All enzyme purification procedures were conducted at 4 °C.

Thrombin-catalysed cleavage of the histidine tag from the soluble enzyme (new N-terminus, GSHMLEM[1] ...) was conducted in Hepes buffer (70 mM, pH 8.0) containing EDTA (0.5 mM) using a thrombin ratio of 0.5 units/mg of target protein. After 16 h at 25 °C, cleavage was complete and the biotinylated thrombin was removed from the reaction mixture using streptavidin–agarose resin (Novagen) at a ratio of 16 μl of settled resin/unit of thrombin following the Novagen protocol [27]. Cleaved CTPS, free of biotinylated thrombin, was then dialysed against assay buffer. The results of the purification and cleavage procedures were routinely monitored using SDS/PAGE. The amino acid residues in the recombinant wild-type and mutant enzymes are numbered according to the sequence of the wild-type *E. coli* enzyme starting with M^1 as position one.

Table 1 Oligonucleotide primers used for site-directed mutagenesis

All oligonucleotides are shown in the 5′ → 3′ orientation. Positions of mismatches are indicated by underlining. Both forward (F) and reverse (R) primers are shown.

CTPS mutant	Oligonucleotide primer	Primer type
K102A	CTCTGACGTTCTGCGTGCAGAACGCCGCGGTGAC	F
	GTCACCGCGGCGTTCTGCACGCAGAACGTCAGAG	R
E103A	GACGTTCTGCGTAAAGCACGCCGCGGTGACTAC	F
	GTAGTCACCGCGGCGTGCTTTACGCAGAACGTC	R
R104A	GACGTTCTGCGTAAAGAAGCCCGCGGTGACTACCTCGGC	F
	GCCGAGGTAGTCACCGCGGGCTTCTTTACGCAGAACGTC	R
R105A	CTGCGTAAAGAACGCGCCGGTGACTACCTCGGCGC	F
	GCGCCGAGGTAGTCACCGGCGCGTTCTTTACGCAG	R
D107A	AAAGAACGCCGCGGTGCCTACCTCGGCGCAACC	F
	GGTTGCGCCGAGGTAGGCACCGCGGCGTTC	R
L109A	CGCGGTGACTACGCCGGCGCAACCGTGCAG	F
	CCTGCACGGTTGCGCCGGCGTAGTCACCGCGGCG	R
L109F	$^{2-}O_3$PO-CGCGGTGACTACTTCGGCGCAACCGGCAG	F
G110A	GGTGACTACCTCGCCGCAACCGTGCAG	F
	CTGCACGGTTGCGGCGAGGTAGTCACC	R
H118A	GTGCAGGTTATTCCGGCCATCACTAACGCAATC	F
	GATTGCGTTAGTGATGGCCGGAATAACCTGCAC	R

Site-directed mutagenesis

pET15b-CTPS1, a pET-15b plasmid containing the recombinant CTPS gene [15], was used as the template for site-directed mutagenesis. *E. coli* strain DH5α cells containing the pET15b-CTPS1 plasmid were grown overnight in Luria–Bertani medium at 37 °C supplemented with ampicillin (400 μg/ml). Cells were harvested and the pET15b-CTPS1 plasmid was isolated. Site-directed mutagenesis was conducted using either the QuikChange™ kit from Stratagene or the MORPH™ kit from Eppendorf-5 Prime (Boulder, CO, U.S.A.). The procedures followed were those described by the manufacturers. The synthetic oligonucleotide primers used to construct the mutants are shown in Table 1. Potential mutant plasmids were isolated and heat shock [28] was used to transform competent DH5α cells. These cells were used for plasmid maintenance and for all sequencing reactions. The entire mutant genes were sequenced to verify that no other alterations in the nucleotide sequence had been introduced. Competent *E. coli* strain BL21(DE3) cells were used as the host for target gene expression. The histidine-tagged mutant enzymes were purified and their histidine tags were removed using the same procedures as described for the recombinant wild-type enzyme.

Enzyme assays and protein determinations

CTPS activity was determined at 37 °C using a continuous spectrophotometric assay by following the rate of increase in absorbance at 291 nm resulting from the conversion of UTP into CTP ($\Delta\epsilon = 1338\ M^{-1} \cdot cm^{-1}$) [17]. The standard assay mixture consisted of Hepes buffer (70 mM, pH 8.0) containing EDTA (0.5 mM), $MgCl_2$ (10 mM), CTPS and saturating concentrations of UTP (1 mM) and ATP (1 mM) in a total volume of 1 ml. Enzyme and nucleotides were pre-incubated together for 2 min

at 37 °C followed by addition of substrate (NH_4Cl or glutamine) to initiate the reaction. Total NH_4Cl concentrations in the assays were 5, 10, 20, 30, 50, 60, 80 and 100 mM and CTPS concentrations were: wild-type, 0.14–0.2 units/ml; K102A, 0.15–0.16 units/ml; E103A, 0.052–0.069 units/ml; R104A, 0.09–0.12 units/ml; R105A, 0.15–0.19 units/ml; D107A, 0.15–0.17 units/ml; L109A, 0.33–0.38 units/ml; L109F, 0.13–0.18 units/ml; G110A, 0.11–0.13 units/ml, and H118A, 0.044–0.052 units/ml. For assays of glutamine-dependent CTP formation, concentrations of glutamine were 0.1, 0.2, 0.3, 0.5, 1, 2, 3 and 6 mM and CTPS concentrations were: wild-type, 0.09–0.1 units/ml; K102A, 0.1–0.11 units/ml; R105A, 0.1–0.14 units/ml; D107A, 0.09–0.1 units/ml; L109A, 0.11 units/ml; L109F, 0.03 units/ml, and G110A, 0.08–0.11 units/ml. The concentration of GTP was maintained at 0.25 mM for all assays when glutamine was used as the substrate. In addition, the ionic strength was maintained at 0.25 M in all spectrophotometric assays by the addition of KCl. One unit is defined as the amount (μmol) of CTP produced/min at saturating concentrations of all substrates.

All kinetic parameters were determined in triplicate and average values are reported. Initial rate kinetic data was fit to eqn (1) by non-linear regression analysis using the program EnzymeKinetics v. 1.5 (1996) from Trinity Software (Plymouth, NH, U.S.A.):

$$v_i = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

Where v_i is the initial velocity, V_{max} ($= k_{cat}[E]_T$; $[E]_T$ is the total enzyme concentration) is the maximal velocity at saturating substrate concentrations, [S] is the substrate concentration (i.e. glutamine or NH_3) and K_m is the Michaelis constant for the substrate. Values of K_m for ammonia were calculated using the concentration of NH_3 present at pH 8.0 {pK_a (NH_4^+) = 9.24 [29]}. Values of k_{cat} (per subunit) were calculated for CTPS variants with the hexahistidine tag removed using the molecular masses of 61029 Da (wild-type), 60972 Da (K102A), 60971 Da (E103A), 60944 Da (R104A and R105A), 60985 Da (D107A), 60987 Da (L109A), 61063 Da (L109F), 61043 Da (G110A) and 60963 Da (H118A). The reported errors are S.D. Unless mentioned otherwise, protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with BSA as a standard.

Glutaminase assay

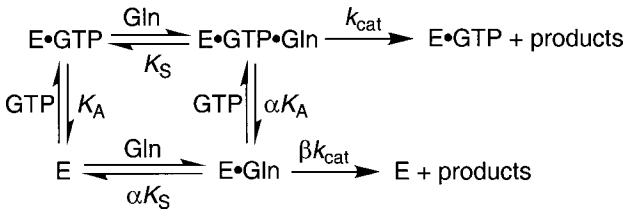
The abilities of wild-type and mutant forms of CTPS to catalyse glutamine hydrolysis were determined by following the production of glutamate using reversed-phase HPLC separation of the *o*-phthaldialdehyde derivatives of glutamate and glutamine with fluorescence detection as described previously [30]. Assays contained ATP (1 mM), UTP (1 mM), GTP (0.25 mM), glutamine (6 mM) and CTPS (wild-type, 3.5–5.5 μg/ml; mutants, 4–12 μg/ml) in a total volume of 2.5 ml.

Apparent activation constants for GTP

The values of the apparent activation constants (K'_A) for wild-type and mutant CTPSs were determined by measuring the initial rates of CTP formation at varying GTP concentrations (10, 20, 30, 40, 60, 80, 100, 120, 160, 200, 250 and 300 μM) with a fixed saturating glutamine concentration (10 mM). All other assay conditions were the same as those described for the standard assay with ATP (1 mM) and UTP (1 mM) at saturating concentrations. Typical CTPS concentrations were 0.12–0.16 units/ml (wild-type), 0.15–0.19 units/ml (R105A), 0.14–0.16 units/ml (D107A), 0.15–0.19 units/ml (L109A), 0.15–0.18 units/ml (L109F) and 0.08–0.1 units/ml (G110A). Plots of the observed initial velocities with respect to GTP concentration were hyperbolic, and in the absence of GTP, no significant enzymic activity was observed. Activation by GTP was therefore treated using the general modifier kinetic mechanism [31] shown in Scheme 2 (i.e. with $\beta \approx 0$) and the K'_A values were determined by fitting data from plots of observed velocity against [GTP] at saturating glutamine concentration to eqn (2) using non-linear regression analysis as described above:

$$v_i = \frac{V'_{max}[GTP]}{K'_A + [GTP]} \quad (2)$$

V'_{max} is the apparent maximal velocity at saturating concentrations of GTP. Hill plots were constructed to evaluate if co-operativity effects of GTP were present within the range of concentrations used in the assay.



Scheme 2 Kinetic mechanism for the activation of CTPS by GTP

E, enzyme complexed with ATP and UTP; K_A and K_S are dissociation constants, $\beta \approx 0$, and $K'_A = K_A(K_S + \alpha[Gln])/(K_S + [Gln])$. The term α is a factor by which K_A and K_S are altered in the thermodynamic cycle.

CD spectra

CD spectra were obtained using a JASCO J-810 spectropolarimeter. CD spectra were recorded for both the wild-type and mutant enzymes (E103A, L109F and H118A) over the range 190–280 nm in the absence of nucleotides. A marked decrease in buffer transparency was observed below 190 nm and therefore all spectra were truncated at this wavelength. The resulting CD spectra obtained from enzyme solutions ($\approx$ 0.2 mg/ml) in Bis-Tris propane buffer (10 mM, pH 8.0) containing $MgSO_4$ (10 mM) were analysed for the percentage of α-helical and β-sheet structure using CDNN CD Spectra Deconvolution v. 2.1 developed by G. Böhm et al. [31a] (http://bioinformatik.biochemtech.uni-halle.de/index.html). Protein concentrations were determined spectrophotometrically using a molar absorption coefficient equal to 37800 $M^{-1} \cdot cm^{-1}$ which was determined for the wild-type enzyme at 280 nm.

Tetramerization of CTPS

The ability of mutant CTPSs to form tetramers was evaluated using gel-filtration HPLC (GF-HPLC). Wild-type and mutant (D107A, L109A, L109F and G110A) CTPSs, and standard proteins (50 μl injection volume) were eluted under isocratic conditions using sodium phosphate buffer (0.2 M, pH 7.0) containing $MgCl_2$ (10 mM) at a flow rate of 0.5 ml/min on a Zorbax Bio Series GF-250 (4 μm, 4.6 mm $\times$ 250 mm) column. The eluted proteins were detected by native protein fluorescence (λ_{ex}, 285 nm; λ_{em}, 335 nm) using a PerkinElmer LS-50 luminescence spectrophotometer. GF-HPLC of both wild-type and mutant enzymes was conducted in the presence and absence of ATP

(1 mM) and UTP (1 mM), and the retention times were compared with those observed for the wild-type enzyme. The column was standardized using the following proteins (1.5 mg/ml): bovine thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa). The retention time of bovine thyroglobulin was used to estimate the column void volume (V_o).

RESULTS

Using site-directed mutagenesis the genes encoding the mutant enzymes listed in Table 1 were constructed, and their corresponding histidine-tagged proteins expressed and purified using metal-ion affinity chromatography. The histidine tag was then removed from the proteins using thrombin-catalysed cleavage and the resulting proteins were greater than 95% pure as judged using SDS/PAGE. The kinetic parameters k_{cat} and K_m for both the wild-type and mutant CTPSs utilizing NH_3 and glutamine as substrates are shown in Tables 2 and 3. Representative double-reciprocal plots are shown in Figure 2. In general, mutations within the highly conserved region of amino acids between residues 102 and 118 could be divided into two groups: (i) those enzymes that had either wild-type or reduced ability to utilize glutamine as a substrate for CTP formation (Table 2), and (ii) those enzymes that were inactive when glutamine was employed as a substrate (Table 3).

Mutants with wild-type or reduced glutamine-dependent rates of CTP formation

The R105A, D107A, L109A, L109F and G110A enzymes all exhibited reduced rates of CTP synthesis when glutamine was employed as the substrate in the presence of GTP. This reduction in catalytic efficiency (k_{cat}/K_m) with glutamine was manifested solely as a reduction in the value of k_{cat}, with the value of K_m for the mutant enzymes being very similar to that observed for the wild-type enzyme.

The R105A, D107A, L109A and L109F enzymes had values of k_{cat}, K_m and k_{cat}/K_m that were similar to those observed for the wild-type enzyme when NH_3 was the substrate. For G110A CTPS, the values of K_m and k_{cat} with respect to NH_3 were reduced $\approx$ 3-fold and 2-fold, respectively, relative to the wild-type enzyme, but the overall efficiency (k_{cat}/K_m) for the NH_3-dependent formation of CTP was similar to that observed for wild-type CTPS. Interestingly, G110A displayed substrate inhibition at NH_4Cl concentrations greater than 50 mM (Figure 2A). Glycine may exhibit a much broader range of Φ and Ψ dihedral angles than other amino acids such as the alanine residue present in the mutant protein [32]. Therefore, it is possible that Gly-110 has a structural role with which the introduction of an alanine residue is not compatible. Consequently, the binding site for exogenous NH_3 may be perturbed, giving rise to the altered kinetics with respect to NH_3.

The K102A mutation had little effect on the enzyme's ability to utilize either glutamine or NH_3 as substrate.

Table 2 Kinetic parameters for wild-type and mutant CTPSs displaying glutamine-dependent CTP formation

The values of k_{cat} are per enzyme subunit. K_m values determined for the substrate NH_3 have been calculated based on the total concentration of NH_4Cl used in the assay conducted at pH 8.0; i.e. since the pK_a (NH_4^+) = 9.24 [29], $[NH_3] = 0.0575 \times [NH_4Cl]_{Total}$. The value of k_{cat} for L-glutamine hydrolysis was estimated using initial rate data obtained at a saturating concentration of L-glutamine (6 mM) and with [GTP] = 0.25 mM. n.d., not determined.

		CTPS variant						
Experiment	Kinetic parameter	Wild-type	K102A	R105A	D107A	L109A	L109F	G110A
NH_3-dependent CTP formation	K_m (mM)	2.08 ± 0.03	2.08 ± 0.15	2.36 ± 0.35	1.78 ± 0.83	1.59 ± 0.15	2.21 ± 0.28	0.71 ± 0.13
	k_{cat} (s^{-1})	9.4 ± 2.7	12.2 ± 0.9	8.88 ± 1.23	8.70 ± 0.08	7.97 ± 0.52	11 ± 1.2	4.18 ± 0.44
	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	4.6 ± 1.3	5.87 ± 0.71	3.84 ± 0.92	4.89 ± 0.11	5.03 ± 0.23	5.1 ± 1.1	5.95 ± 0.49
L-Glutamine-dependent CTP formation	K_m (mM)	0.32 ± 0.01	0.17 ± 0.01	0.38 ± 0.05	0.25 ± 0.01	0.38 ± 0.02	0.30 ± 0.06	0.27 ± 0.01
	k_{cat} (s^{-1})	6.1 ± 0.3	4.22 ± 0.27	1.53 ± 0.13	3.5 ± 0.16	1.64 ± 0.05	1.03 ± 0.08	2.17 ± 0.23
	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	19.2 ± 0.6	24.2 ± 2.5	3.8 ± 1.0	14.6 ± 1.2	4.33 ± 0.18	3.5 ± 0.5	8.19 ± 0.73
L-Glutamine hydrolysis	k_{cat} (s^{-1})	4.98 ± 0.21	4.8 ± 1.2	2.00 ± 0.38	4.31 ± 0.71	5.15 ± 0.34	0.86 ± 0.09	2.85 ± 0.17
GTP as an activator	K'_A (mM)	0.037 ± 0.018	n.d.	0.116 ± 0.01	0.06 ± 0.021	0.139 ± 0.015	0.18 ± 0.02	0.065 ± 0.016
	Hill number (n)	0.98 ± 0.09	n.d.	1.01 ± 0.02	0.98 ± 0.06	0.96 ± 0.04	0.92 ± 0.04	1.03 ± 0.03

Table 3 Kinetic parameters for wild-type and mutant CTPSs with no glutamine-dependent CTP formation

The values of k_{cat} are per enzyme subunit. K_m values determined for the substrate NH_3 have been calculated based on the total concentration of NH_4Cl used in the assay conducted at pH 8.0 (see Table 2 legend). n.a. refers to mutants for which no activity could be detected (i.e. $< 1\%$ wild-type activity). The value of k_{cat} for L-glutamine hydrolysis was estimated using initial rate data obtained at a saturating concentration of L-glutamine (6 mM) and with [GTP] = 0.25 mM.

		CTPS variant			
Experiment	Kinetic parameter	Wild-type	E103A	R104A	H118A
NH_3-dependent CTP formation	K_m (mM)	2.08 ± 0.03	0.14 ± 0.01	0.48 ± 0.16	1.52 ± 0.19
	k_{cat} (s^{-1})	9.4 ± 2.7	0.92 ± 0.06	1.96 ± 0.27	0.18 ± 0.01
	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	4.6 ± 1.3	6.6 ± 0.6	4.29 ± 0.8	0.12 ± 0.01
L-Glutamine-dependent CTP formation	K_m (mM)	0.32 ± 0.01	n.a.	n.a.	n.a.
	k_{cat} (s^{-1})	6.1 ± 0.3	n.a.	n.a.	n.a.
	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	19.2 ± 0.6	n.a.	n.a.	n.a.
L-Glutamine hydrolysis	k_{cat} (s^{-1})	4.98 ± 0.21	0.14 ± 0.04	0.18 ± 0.06	0.1 ± 0.03

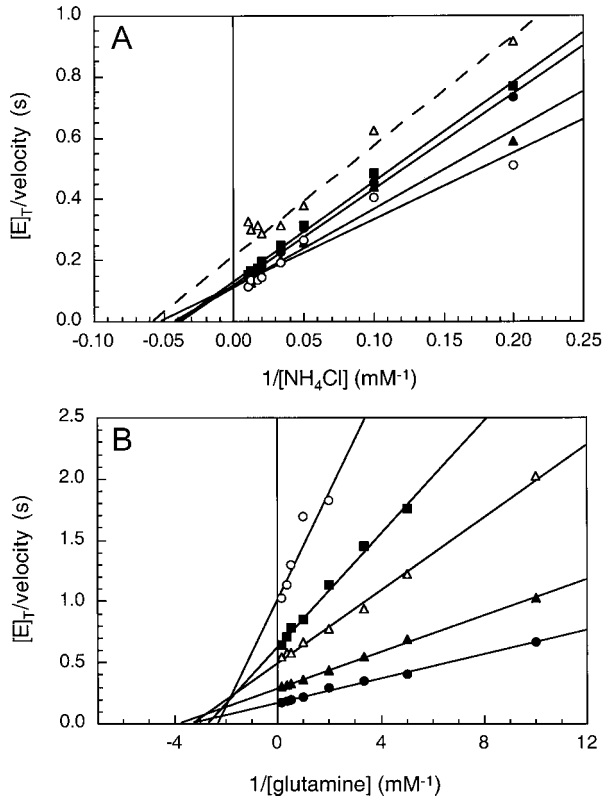


Figure 2 Catalytic activity of wild-type and mutant CTPSs

Representative double-reciprocal plots for wild-type (●) and mutant [D107A (▲), L109A (■), L109F (○) and G110A (△)] CTPSs utilizing either NH_3 (**A**) or glutamine (**B**) as the substrate are shown. The assay conditions are as described in the Experimental section. Kinetic parameters for each CTPS variant are given in Table 2.

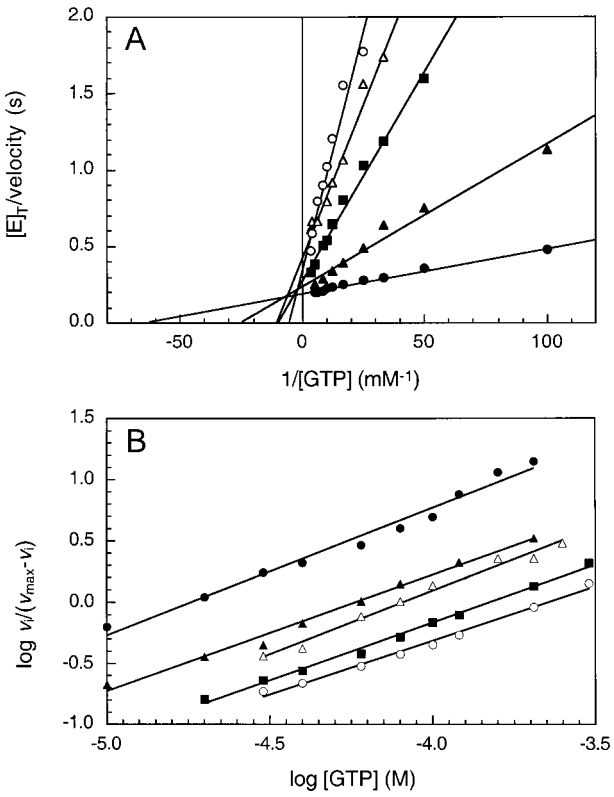


Figure 3 Apparent affinity of wild-type and mutant CTPSs for GTP

(**A**) Representative double-reciprocal plots for the activation of wild-type (●) and mutant [D107A (▲), L109A (■), L109F (○) and G110A (△)] CTPSs by GTP are shown. (**B**) Hill plots for the activation of wild-type and mutant CTPSs by GTP are shown (symbols are as in **A**). The assay conditions are as described in the Experimental section. The values of the apparent dissociation constants (K'_A) for each CTPS variant with respect to GTP are given in Table 2.

Mutants with no glutamine-dependent CTP formation

Several mutations (E103A, R104A and H118A) caused a complete loss of detectable ($< 1\%$) glutamine-dependent CTP formation. For the E103A and R104A mutants, the values of K_m for NH_3 were reduced $\approx$ 15-fold and 4-fold, respectively, relative to wild-type CTPS. The values of k_{cat} for these mutants were reduced 10-fold and 5-fold, respectively, so that the overall efficiency (k_{cat}/K_m) for the NH_3-dependent formation of CTP remained similar to that observed for wild-type CTPS. Interestingly, R104A CTPS displayed substrate inhibition at NH_4Cl concentrations above 50 mM, similar to the G110A mutant (Figure 2A). Whether this phenomenon arises because of a structural change or the loss of the catalytic function inherent to the conserved Arg-104 residue is not clear. Finally, the H118A mutant displayed a 4-fold reduction in k_{cat}/K_m with respect to NH_3 relative to wild-type CTPS. The kinetics for NH_3-dependent amination of UTP catalysed by this mutant may be altered because of the proximity of His-118 to the UTP/CTP-binding site (Figure 1).

Activation by GTP

The activation of glutamine-dependent CTP formation by GTP was examined as a function of GTP concentration for wild-type CTPS and those mutant CTPSs that displayed glutamine-dependent CTP formation (R105A, D107A, L109A, L109F and G110A). In all cases, rectangular hyperbolae giving rise to linear double-reciprocal plots were observed (Figure 3A). Although Levitzki and Koshland [16,18] reported that GTP exhibits negative co-operativity (i.e. binding of the first molecule of GTP makes binding of the second molecule more difficult), co-operativity of GTP binding was not observed over the range of GTP concentrations examined in the present study. Double-reciprocal plots were linear with no downward curvature (Figure 3A), in agreement with the GTP-activation results reported by Robertson and Villafranca for the *E. coli* enzyme [33], and more recently for the *Lactococcus lactis* enzyme [34]. In addition, linear Hill plots with slopes equal to one were observed for both wild-type and mutant enzymes (Figure 3B, Table 2). The values of the apparent dissociation constants for GTP activation (K'_A) obtained from the hyperbolic plots were $\approx$ 2–4-fold higher for the mutant enzymes relative to wild-type CTPS (Table 2).

Tetramerization of CTPS variants

Both mutant (D107A, L109A, L109F and G110A) and wild-type CTPSs gave similar retention times when examined using GF-HPLC in the absence of nucleotides. When GF-HPLC was conducted in the presence of ATP and UTP, the retention times were decreased for each of the mutant enzymes to the same extent that the retention time was decreased for the wild-type enzyme (results not shown). Thus these mutations did not appear

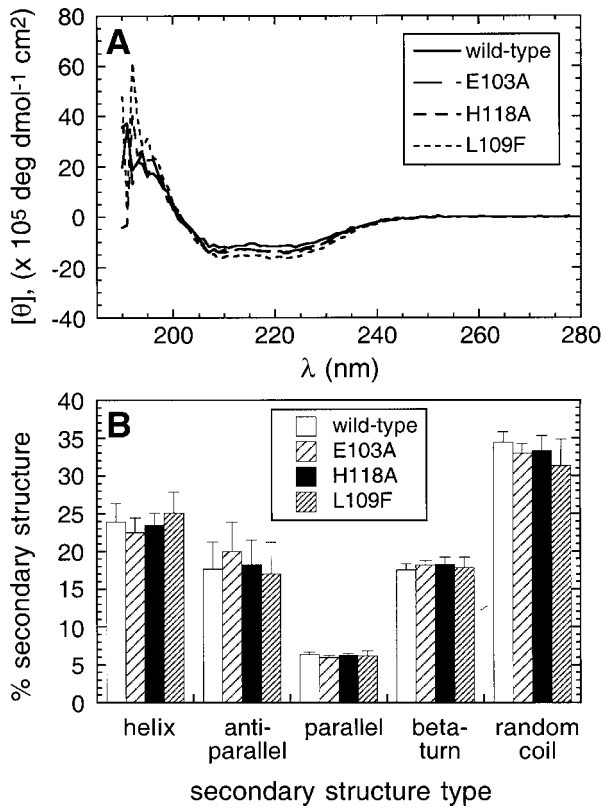


Figure 4 CD analysis of wild-type and mutant CTPSs

(**A**) Spectra of wild-type, E103A, H118A and L109F CTPSs were recorded for 0.2 mg/ml solutions in Bis-Tris buffer (10 mM, pH 8.0) containing $MgSO_4$ (10 mM) in the absence of nucleotide triphosphates. The average of three scans over a wavelength range of 190–280 nm is shown for each CTPS variant. (**B**) The relative amount of each type of secondary structure is indicated for wild-type, E103A, H118A and L109F CTPSs. Error bars represent the S.D. from three independent trials.

to alter the ability of CTPS to oligomerize in the presence of ATP and UTP.

CD studies

The secondary structural content of wild-type CTPS and the mutant enzymes E103A, H118A and L109F were analysed using CD spectroscopy. Figure 4 shows that the secondary structure content of all these mutant proteins was experimentally identical with that of the wild-type enzyme. Although no gross perturbations in secondary structure were evident in the mutant proteins, the possibility that the mutations caused a localized perturbation of secondary structure or change in conformation could not be ruled out.

Glutaminase activity

The ability of the wild-type and mutant enzymes to catalyse glutamine hydrolysis was assessed in the presence of ATP, UTP and GTP by following the production of glutamate (Tables 2 and 3). Because the apparent K_m values for glutamine were similar for wild-type and all mutant CTPSs which exhibited glutamine-dependent CTP formation, we used a single saturating concentration of glutamine (6 mM) to estimate k_{cat} values for the glutaminase activity of the wild-type and mutant enzymes. Using this approach, the value of k_{cat} for wild-type CTPS was estimated to be 4.98 s^{-1}. (This value, obtained at a single saturating glutamine concentration, is slightly lower than the value of 6.01 s^{-1} obtained from a Michaelis–Menten plot constructed using a series of glutamine concentrations [30].) The value of k_{cat} for the K102A mutant was 4.8 s^{-1}, consistent with this mutant's ability to catalyse glutamine-dependent CTP formation with wild-type efficiency. The values of k_{cat} for glutamine hydrolysis catalysed by the mutant enzymes D107A and L109A were experimentally equal to that observed for wild-type CTPS (Table 2). Although the L109A mutant had wild-type glutaminase activity, no lag in CTP production relative to glutamate production was observed despite an $\approx$ 4-fold reduction in the value of k_{cat} for glutamine-dependent CTP formation.

The values of k_{cat} for the R105A, L109F and G110A enzymes were reduced $\approx$ 2.5-, 6- and 1.7-fold respectively, relative to wild-type CTPS, whereas the glutaminase activities of the E103A, R104A and H118A enzymes were significantly impaired.

DISCUSSION

Both purine (ATP and GTP) and pyrimidine (UTP and CTP) nucleotides are ligands of CTPS, and the ability of CTPS to utilize these different nucleotides for unique roles implies that the enzyme is able to discriminate effectively between them at the molecular level. Although the details of this precise molecular discrimination are not known, a putative UTP/CTP-binding site within the synthase domain has been identified (Figure 1) [23,24]. The locations of the ATP- and GTP-binding sites, however, have not yet been identified. In the present study, we sought to characterize one of several highly conserved regions of amino acid sequence found within the synthase domain of *E. coli* CTPS with the hope of identifying a nucleotide-binding site.

Scanning alanine mutagenesis of the highly conserved sequence between residues 102 and 118 yielded mutant CTPSs which could be divided into two groups: (i) those mutants that retained some ability to catalyse glutamine-dependent CTP formation (K102A, R105A, D107A, L109A and G110A), and (ii) those mutants that could not catalyse glutamine-dependent CTP formation (E103A, R104A and H118A). The turnover numbers (k_{cat}) and efficiencies (k_{cat}/K_m) of the mutant enzymes are compared in Figure 5. The present study describes the detailed kinetic characterization of these mutant CTPSs and suggests that Asp-107 and Leu-109 play a role in ensuring efficient coupling of glutamine-dependent NH_3 formation to the synthase activity.

Replacement of Arg-105, Asp-107, Leu-109 and Gly-110 by alanine causes an impairment of the enzyme's ability to catalyse glutamine-dependent CTP formation but does not affect the NH_3-dependent activity. With the exception of K102A, alanine mutagenesis of residues preceding Arg-105 and following Gly-110 (i.e., Asp-103, Arg-104 and His-118) causes a marked reduction in the k_{cat} values for NH_3-dependent CTP formation and obviates glutamine-dependent CTP formation. Interestingly, the changes in k_{cat} for NH_3-dependent CTP formation are compensated for by changes in K_m such that the catalytic efficiencies (k_{cat}/K_m) of all the mutant CTPSs (except H118A) are similar to that observed for the wild-type enzyme. This implies that, with the exception of His-118, the structure of the site for ATP-dependent amination of UTP is not significantly perturbed by mutation of these residues.

For all the mutants exhibiting reduced efficiencies of glutamine-dependent CTP-formation, the apparent K_m values for glutamine are essentially unchanged from that observed for the wild-type enzyme; only the k_{cat} values are reduced. The hydrolysis of glutamine is the rate-limiting step in CTPS-catalysed CTP formation [35] even in the presence of GTP which increases k_{cat}

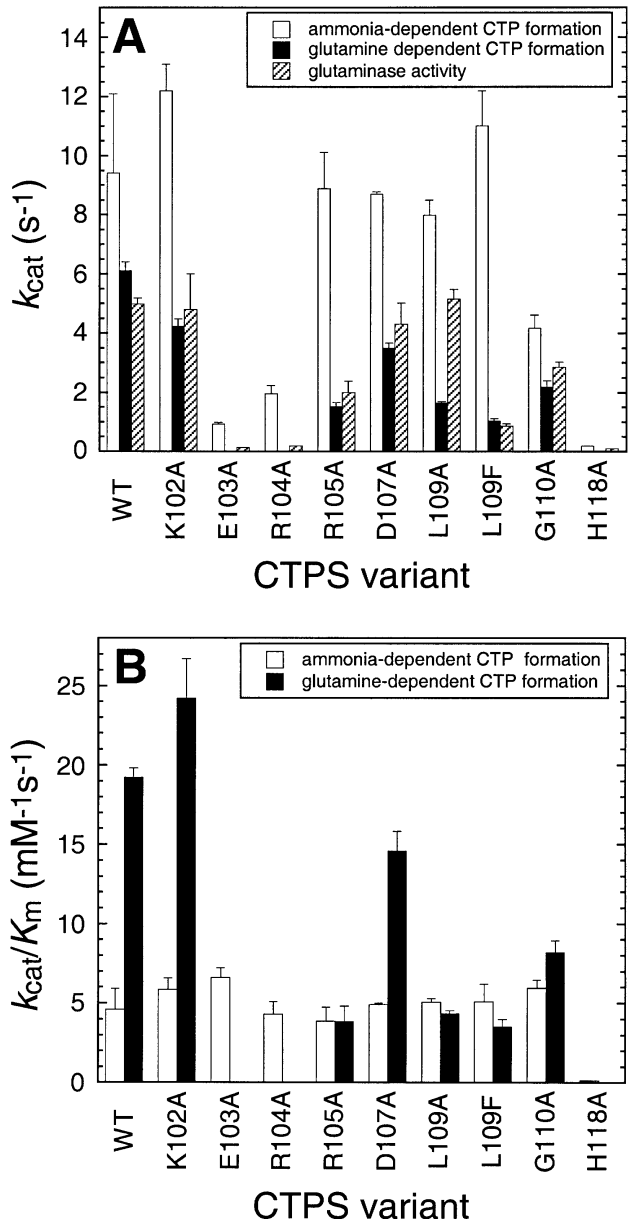


Figure 5 Comparison of turnover numbers and efficiencies for wild-type and mutant CTPSs

Values of k_{cat} for NH_3-dependent CTP formation, glutamine-dependent CTP formation, and glutamine hydrolysis are shown (**A**). Values of k_{cat}/K_m for NH_3-dependent CTP formation and glutamine-dependent CTP formation are shown in (**B**). Data are from Tables 2 and 3.

by 7-fold and decreases K_m by 6-fold [16]. Our observations that mutations between Arg-105 and Gly-110 in the synthase domain (apparently remote from the GAT domain) did not affect glutamine binding (i.e. K_m) would appear to rule out the hypothesis that the observed reduction in glutamine-dependent CTP formation was caused by impaired GTP binding. However, it is conceivable that the mutations impaired GTP binding such that only the rate of glutamine-dependent CTP formation (i.e. k_{cat}) was reduced. In addition, our observations are consistent with several other hypotheses, including mutations that result in either (i) failure of the enzyme to aggregate to form the active tetramer, (ii) an inability to undergo the appropriate conformational change upon binding GTP that stabilizes tetrahedral intermediates formed during glutamine hydrolysis [15], (iii) constriction or blockage of a putative NH_3 tunnel or (iv) failure to form a putative NH_3 tunnel. Several experiments were conducted to distinguish between these hypotheses.

Activation by GTP

In general, three regions of conserved amino acid sequence which constitute GTP-binding sites have been identified in a variety of GTP-binding proteins and the α subunits of receptor-mediated G-proteins. These regions have the consensus sequences GXXXXGK(T/S), DXXG and (N/T)KXD [36–40]. The GXXXXGK(T/S) (or 'P-loop') and DXXG elements are known to interact with the phosphate groups of guanine nucleotides, whereas the (N/T)KXD element is responsible for the specificity of guanine recognition. The spacing of these consensus sequences is $\approx$ 40–80 amino acids between the first and second sequence elements and between the second and third sequence elements. Of these consensus sequences, only DXXG is found in *E. coli* CTPS and it occurs once in the synthase domain (DYLG, residues 107–110) and twice in the GAT domain (DENG, residues 421–424; DLGG, residues 434–437). Only the DYLG sequence is a putative consensus sequence because it is highly conserved between species, resides within a highly conserved region of the synthase domain (residues 102–120; Figure 1B) and is located 88 amino acids C-terminal to a conserved GXXXXXGK (residues 11–18) sequence which closely resembles the first consensus sequence element. It is not surprising that the (N/T)KXD element does not appear to be present in CTPS since ITP can activate glutamine-dependent CTP formation, although less efficiently than GTP (J. E. MacDonnell and S. L. Bearne, unpublished work).

We determined the apparent activation constants of the wild-type and mutant enzymes (R105A, D107A, L109A and G110A) with respect to GTP in order to ascertain what effect the mutations had on GTP binding. Surprisingly, all the mutant enzymes have K'_A values for GTP that are only 2–4-fold greater than that of the wild-type enzyme, indicating that in fact GTP binding is not significantly impaired. When the aspartate residues found in the DXXG consensus sequences of typical G-proteins are altered by site-directed mutagenesis, a more significant reduction in GTP binding affinity is usually observed. For example, the D80N mutation in elongation factor Tu [41] and the D57A mutation in p21 H-Ras [42] yield 23-fold and 105-fold reductions in GTP binding affinity, respectively. Mutations of the glycine residue, however, do not usually alter the affinity for GTP [43–46]. Interestingly, a highly conserved sequence at the N-terminus of CTPS (G_{11}VVSSLGK$_{18}$ in Figure 1B) is very similar to the first consensus sequence that is usually present in ATP- and GTP-binding proteins (i.e. GXXXXGK), and it is appropriately located 88 residues N-terminal to the DYLG sequence. Mutation of the lysine residue within such sequences has been shown to eliminate completely GTPase activity in human MxA protein (K$\rightarrow$N) [47] and increase the apparent K_m for GTP 1.7-fold in *E. coli* adenylosuccinate synthase (K$\rightarrow$R) [48]. We have found that K18A CTPS displays neither glutamine- nor NH_3-dependent activities (A. Iyengar and S. L. Bearne, unpublished work), consistent with Lys-18 playing either a structural role or a role in catalysing the amination reaction but not a unique role in GTP binding.

Because the DYLG sequence does not seem to play a role in GTP binding, it appears that none of the known GTP binding consensus sequences are present in CTPS. This implies that CTPS has evolved unique sequences for recognition of GTP and

discrimination between its other nucleoside triphosphate ligands. A similar proposal has been put forward for ornithine decarboxylase, which also utilizes GTP as a positive allosteric effector and appears to have a unique GTP-binding site [49,50].

Enzyme oligomerization

We then considered the possibility that the mutations might cause a structural perturbation that interferes with the ability of the enzyme to form active tetramers. The observed kinetic results could arise if the dimeric form of the mutant enzyme, in the presence of ATP and UTP, could only utilize NH_3 as its substrate, as opposed to wild-type CTPS which forms a tetramer and utilizes both NH_3 and glutamine. This possibility seemed unlikely since Levitzki and Koshland [16] showed that dimeric wild-type CTPS (i.e. in the absence of ATP and UTP) could utilize glutamine as a substrate although the k_{cat} value for GTP-stimulated glutaminase activity was about 5-fold less than that observed for tetrameric CTPS (i.e. in the presence of ATP and UTP). Using GF-HPLC, we found that the mutant enzymes D107A, L109A and L109F were able to oligomerize in the presence of ATP and UTP similar to wild-type CTPS.

The tunnel hypothesis

It is possible that those mutations that impair glutamine-dependent CTP formation do so by either directly interacting with the GAT domain or by stopping CTPS from undergoing the GTP-induced change to a conformation that stabilizes the tetrahedral intermediates formed during glutamine hydrolysis. Although either of these scenarios may apply to the R105A, G110A and L109F enzymes, they can be ruled out for the D107A and L109A mutants since these enzymes hydrolyse glutamine at wild-type rates in the presence of GTP (Table 2). The most plausible explanation for the kinetic properties of these latter two mutants is that replacement of Asp-107 or Leu-109 by alanine alters the normal structure of a putative NH_3 tunnel (i.e. the tunnel is leaky or constricted and impedes passage of the nascent NH_3), or causes a structural perturbation that prevents the formation of a transient tunnel, the effect being much more pronounced for the L109A mutation.

The X-ray crystal structures have been solved for a number of amidotransferases including CPS [21,51,52], glutamine phosphoribosylpyrophosphate amidotransferase (GPATase) [53,54], GMP synthase [20], glucosamine-6-phosphate synthase [55–57], asparagine synthase B [58] and anthranilate synthase [59,60]. In nearly all cases, the structures contain tunnels of varying lengths for sequestering the nascent NH_3, derived from glutamine hydrolysis, from the bulk solvent and shuttling it from the GAT domain to the synthase domain [61]. Because the presence of NH_3 tunnels seems to be a general theme among the amidotransferases [62], it is not unreasonable to expect that such a tunnel might exist in CTPS. Indeed, the observed lack of equilibration of the nascent NH_3 derived from CTPS-catalysed glutamine hydrolysis with the solvent is compatible with the existence of an NH_3 tunnel [35]. In addition, NH_4^+ is not a substrate for CTPS [35] and sequestration of NH_3 in a tunnel would prevent protonation of NH_3 derived from glutamine hydrolysis. The possibility that transient formation of a tunnel might occur is suggested by the structures of GMP synthase [6,20] and GPATase [53], which appear to require ligand binding for formation of an NH_3 tunnel.

Our observations that L109A CTPS exhibits wild-type NH_3-dependent activity, wild-type affinity for glutamine and wild-type glutaminase activity but has impaired glutamine-dependent CTP formation are fully consistent with the hypothesis that Leu-109 plays a role in either the structure or formation of an NH_3 tunnel and ensures efficient coupling of the glutamine hydrolysis and amination reactions. The hypothesis that replacement of the side chain of Leu-109 with the smaller side chain of alanine could cause a tunnel blockage has precedent. For example, the F334A mutant of GPATase exhibited the kinetic properties expected for a blocked or disrupted NH_3 tunnel [63].

Comparison with other amidotransferases

At first glance, the reduction in the efficiency of glutamine-dependent CTP formation catalysed by L109A CTPS may not appear highly significant (i.e. both k_{cat} and k_{cat}/K_m are decreased only about 4-fold). However, mutations of the amidotransferases CPS [64,65] and GPATase [66,67] which generate either constricted or leaky NH_3 tunnels have produced enzymes that exhibit wild-type NH_3-dependent activity but impaired glutamine-dependent activity, much like the mutant CTPSs described in the present study. Unlike the present study, however, the mutated residues in CPS and GPATase were selected using the X-ray crystal structures as a guide. For example, 12 different mutant forms of CPS were prepared with mutations of residues lining the NH_3 tunnel chosen so that either passage of NH_3 would be restricted or the NH_3 would be protonated to yield the less reactive ammonium cation. Even with this more informed approach, eight of the 12 CPS mutants retained wild-type kinetic properties and significant alteration of the kinetic properties was observed with only two of the mutant enzymes. The CPS mutants G359Y and G359F exhibited wild-type NH_3-dependent activity, while k_{cat} for glutamine-dependent activity was reduced 18-fold and 65-fold respectively [64,65]. Unlike the D107A and L109A mutants characterized in the present study for which the K_m for glutamine was not altered by mutagenesis, the K_m values of the G359Y and G359F CPS mutants for glutamine were increased > 500-fold because of structural perturbation of the glutamine-binding site [68]. Raushel and co-workers [65,69] have estimated the rate of diffusion of NH_3 through the NH_3 tunnel in CPS to be $\approx 5 \times 10^8$ Å/s (1 Å $\equiv$ 0.1 nm). Reduction of this rate by even several orders of magnitude may not have an appreciable effect on the overall rate of product formation by the enzyme. Hence, amidotransferases with impaired migration of NH_3 through a tunnel may still exhibit wild-type steady-state kinetic parameters [65]. This explanation also accounts for why L109A CTPS did not show a lag in CTP formation. For the G359Y and G359F CPS mutants, a lag in carbamoyl phosphate formation was observed. Recent X-ray crystallographic studies on G359F CPS have revealed that this mutation introduces a hole in the NH_3 tunnel of CPS that uncouples glutamine hydrolysis and carbamate formation, and permits escape of NH_3 to the bulk solvent, causing a lag in the rate of product formation [65,68]. Subsequent utilization of NH_3 from bulk solution as its concentration increases during the course of the reaction causes an increase in rate of product formation [65].

In an attempt to more effectively block NH_3 passage through the putative NH_3 tunnel, we replaced the side chain of Leu-109 with the more bulky hydrophobic side chain of phenylalanine. Like the mutants containing alanine substitutions, L109F CTPS exhibited a reduced efficiency of glutamine-dependent CTP formation relative to the efficiency of NH_3-dependent CTP formation. However, L109F CTPS also displayed a marked reduction in the glutaminase activity. The origin of this effect is not clear but could arise because the phenylalanine side chain causes a local perturbation of the tertiary architecture of the protein

thereby altering the glutamine site such that glutamine binding is not affected but its rate of hydrolysis is reduced. Such a structural alteration must be subtle since the CD spectrum of the L109F mutant was very similar to that of wild-type CTPS. An alternative explanation is that the reduction in glutaminase activity results from failure of GTP-binding to induce the appropriate conformational change that promotes glutamine hydrolysis. This explanation seems quite plausible because the K'_A value of L109F CTPS is very similar to that of L109A CTPS (which exhibits wild-type glutaminase activity), indicating that GTP binding is not impaired. A similar kinetic profile was observed for R105A CTPS, implying that Arg-105 also has an important catalytic and/or structural role in effecting efficient glutamine turnover.

Conclusions

Kinetic investigations of alanine mutations of amino acid residues between Arg-105 and Gly-110 suggest that these residues are important for efficient coupling of glutamine hydrolysis in the GAT domain to CTP formation in the synthase domain. Our observations suggest that Leu-109 plays a role in either the structure or formation of an NH_3 tunnel and ensures efficient coupling of the glutamine hydrolysis and amination reactions. This study provides further evidence that residues within the synthase domain of CTPS can play a significant role in the enzyme's glutamine-dependent activity. This observation suggests that the synthase and GAT domains interact because they are in close proximity or via a conformationally dependent signalling mechanism. Site-directed mutagenesis of amino acid residues residing in the other conserved regions shown in Figure 1(B) is currently underway.

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