Inhibition of *Escherichia coli* CTP synthase by glutamate γ -semialdehyde and the role of the allosteric effector GTP in glutamine hydrolysis

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Cytidine 5'-triphosphate synthase catalyses the ATP-dependent formation of CTP from UTP with either ammonia or glutamine as the source of nitrogen. When glutamine is the substrate, GTP is required as an allosteric effector to promote catalysis. *Escherichia coli* CTP synthase, overexpressed as a hexahistidine-tagged form, was purified to high specific activity with the use of metal-ion-affinity chromatography. Unfused CTP synthase, generated by the enzymic removal of the hexahistidine tag, displayed an activity identical with that of the purified native enzyme and was used to study the effect of GTP on the inhibition of enzymic activity by glutamate γ -semialdehyde. Glutamate γ -semialdehyde is expected to inhibit CTP synthase by reacting reversibly with the active-site Cys-379 to form an analogue of a tetrahedral intermediate in glutamine hydrolysis. Indeed, glutamate γ -semialdehyde is a potent linear mixed-type inhibitor of CTP synthase with respect to glutamine (K_{is} 0.16 ± 0.03 mM; K_{ii} 0.4 ± 0.1 mM) and a competitive inhibitor with respect to am-

monia (K_i 0.39 ± 0.06 mM) in the presence of GTP at pH 8.0. The mutant enzyme (C379A), which is fully active with ammonia but has no glutamine-dependent activity, is not inhibited by glutamate γ -semialdehyde. Although glutamate γ -semialdehyde exists in solution primarily in its cyclic form, Δ^1 -pyrroline-5-carboxylate, the variation of inhibition with pH, and the weak inhibition by cyclic analogues of Δ^1 -pyrroline-5-carboxylate (L-proline, L-2-pyrrolidone and pyrrole-2-carboxylate) confirm that the rare open-chain aldehyde species causes the inhibition. When ammonia is employed as the substrate in the absence of GTP, the enzyme's affinity for glutamate γ -semialdehyde is decreased approx. 10-fold, indicating that the allosteric effector, GTP, functions by stabilizing the protein conformation that binds the tetrahedral intermediate(s) formed during glutamine hydrolysis.

Key words: amidotransferase, tetrahedral intermediate, transition state stabilization.

INTRODUCTION

CTP synthase [EC 6.3.4.2; UTP: ammonia ligase (ADP-forming)] is a glutamine amidotransferase that catalyses the ATP-dependent formation of CTP from UTP by using either L-glutamine or ammonia as the source of nitrogen (Scheme 1) [1]. CTP synthase is a single polypeptide chain consisting of two domains: the C-terminal glutamine amide transfer (GAT) domain catalyses the hydrolysis of glutamine; the N-terminal synthase domain

catalyses the amination of UTP [2,3]. Amidotransferases have been divided into two subfamilies on the basis of sequence alignments of the GAT domains [4]. CTP synthase belongs to the Triad (or class I) type, which also includes anthranilate synthase, carbamoyl phosphate synthase (CPS), formylglycinamide synthase, GMP synthase, imidazole glycerol phosphate synthase and aminodeoxychorismate synthase [4,5]. The class I enzymes use a cysteine-histidine-glutamate triad to catalyse glutamine hydrolysis. The other type of amidotransferase, known as Ntn (or class II), uses an N-terminal cysteine residue to catalyse glutamine hydrolysis.

CTP synthase catalyses the final step in the *de novo* synthesis of cytosine nucleotides. Because the nucleotide CTP has a central role in the biosynthesis of nucleic acids [6] and membrane phospholipids [7], the mechanism and regulation of CTP synthase have been the focus of numerous investigations [4]. The enzyme from *Escherichia coli* is the best-characterized CTP synthase with regard to its physical and kinetic properties and is regulated in a complex fashion. It is inhibited by the product CTP [8], exhibits negative co-operativity for glutamine and GTP (an effector) [9] and displays positive co-operativity for ATP and UTP [8–10]. ATP and UTP act synergistically to promote tetramerization of the enzyme to its active form. In addition to these non-covalent modes of regulation, the yeast enzyme encoded by the *URA7* gene is regulated by phosphorylation [11]. Elevated CTP synthase activity is common in some forms of leukaemia and some solid tumours [12]. Consequently the enzyme is a recognized target for the development of anti-viral and anti-

Scheme 1 Reaction catalysed by CTP synthase

R represents ribose 5'-triphosphate.

Abbreviations used: CPS, carbamoyl phosphate synthase; DON, 6-diazo-5-oxonorleucine; GAT, glutamine amide transfer; GlmS, glucosamine-6-phosphate synthase; GSA, glutamate γ -semialdehyde; His₆-CTPS, CTP synthase containing an N-terminal hexahistidine tag; P5C, Δ^1 -pyrroline-5-carboxylate.

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Scheme 2 Reaction of Cys-379 with glutamine and GSA

(A) Glutamine hydrolysis; (B) thiohemiacetal formation. Enzyme-bound species are shown in brackets.

neoplastic agents [13]. In addition, the regulation of CTP synthase activity is important in the regulation of phospholipid biosynthesis [14].

In the sequence of reactions catalysed by CTP synthase, glutamine is first hydrolysed to yield glutamate and nascent ammonia. The GAT domain of *E. coli* CTP synthase contains a reactive cysteine residue at position 379 that is believed to participate in the formation of a γ -glutamyl *S*-ester intermediate, as shown in Scheme 2(A) [2,4,15,16]. The nascent ammonia generated via glutamine hydrolysis reacts with UTP that has been activated by phosphorylation at the 4-position, to yield CTP [15,17]. GTP is an allosteric effector that stimulates the enzyme's glutaminase activity but has no effect on the reaction when ammonia is the substrate [18]. An understanding of how GTP promotes glutamine hydrolysis might provide insights into novel ways of selectively inhibiting this amidotransferase. Our understanding of CTP synthase catalysis was greatly enhanced by the comprehensive kinetic and mechanistic studies conducted by Koshland and co-workers in the early 1970s [1]. Levitzki and Koshland [18] found that when ammonia was the substrate, the values of both K_m and k_{cat} were independent of GTP concentration. However, when glutamine was employed as the substrate, the efficiency (k_{cat}/K_m) of the glutaminase reaction was increased 45-fold by the presence of GTP because of a decrease in K_m and an increase in k_{cat} . These authors proposed that the simplest explanation for this effect was that GTP enhanced the rate of formation of the covalent glutamyl enzyme

(steps 2 and 3 in Scheme 2A, leading to the *S*-ester) [18]. To examine this possibility in more detail, we have used the aldehyde inhibitor glutamate γ -semialdehyde (GSA) to mimic the tetrahedral intermediates formed during glutamine hydrolysis (Scheme 2B).

Aldehydes that are structurally related to the acyl portion of substrates act as potent reversible inhibitors of proteases containing a reactive cysteine [19] or serine [20] residue at the active site. Despite the fact that the glutamine analogue GSA exists in unfavourable equilibrium with Δ^1 -pyrroline-5-carboxylate (P5C) [21], it is a potent reversible inhibitor of the amidotransferases glucosamine-6-phosphate synthase (GlmS) [21,22] and CPS [23]. Inhibition of these enzymes involves the formation of a thiohemiacetal that resembles the tetrahedral intermediates in the formation and breakdown of the covalently bound *S*-ester intermediate in glutamine hydrolysis (Scheme 2B). We reasoned that GSA might also be a strong reversible inhibitor of CTP synthase and serve as a probe to determine whether the allosteric effector GTP acts to stabilize an enzyme conformation that binds the tetrahedral intermediates formed during the glutamylation and subsequent hydrolysis of the *S*-ester intermediate (Scheme 2). This paper describes the potent inhibition of CTP synthase by the intermediate analogue inhibitor GSA and the effect of the allosteric effector GTP on this inhibition. We demonstrate that GTP promotes glutamine amidotransferase activity, in part, by enhancing interactions between the enzyme and tetrahedral intermediate(s) formed during glutamine hydrolysis. In addition,

we provide experimental evidence confirming the role of Cys-379 in catalysis.

EXPERIMENTAL

General materials

Restriction enzymes were purchased from Gibco BRL (Burlington, Ontario, Canada). His-Bind resin, thrombin cleavage capture kits and the pET-15b expression system were purchased from Novagen (Madison, WI, U.S.A.). δ -Hydroxylysine, L-proline, L-2-pyrrolidone-5-carboxylate, pyrrole-2-carboxylate, 2-amino-5-phosphonobutyric acid and all other chemicals were purchased from Sigma (Oakville, Ontario, Canada). Amicon Centriprep-30 concentrators were purchased from Fisher Scientific (Nepean, Ontario, Canada). Synthetic oligonucleotide primers for DNA sequencing, PCR and site-directed mutagenesis were purchased from ID Labs (London, Ontario, Canada).

P5C

P5C was synthesized by the oxidation of a mixture of D,L- and D,L-allo- δ -hydroxylysine with periodic acid and purified by ion-exchange chromatography as described by Williams and Frank [24]. The concentration of P5C was determined by the colorimetric method of Mezl and Knox [25], taking the molar absorption coefficient of the condensation product of P5C with *o*-aminobenzaldehyde (ϵ 2580 M⁻¹·cm⁻¹) reported by these authors.

Purification of wild-type CTP synthase

The plasmid pMW5, a 5.3 kbp vector containing the *E. coli pyrG* gene, which encodes CTP synthase [2], was obtained from Professor Howard Zalkin (Purdue University, West Lafayette, IN, U.S.A.). This plasmid was used to transform *E. coli* JF1754 cells [26]. These cells were then grown in Luria-Bertani medium and lysed by sonication on ice (five 3 min bursts with 1 min intervals at output setting 5, with a Branson Sonifier 250). The wild-type enzyme was then purified by the method of Lewis and Villafranca [27].

Construction of pET15b-CTPS1 expression plasmid

The *E. coli* gene encoding CTP synthase was subcloned from plasmid pMW5 into the *Xho*I and *Bam*HI sites of expression vector pET-15b (Novagen), which attaches a hexahistidine tag (MGSSHHHHHSSGLVPR↓GSHMLEM₁...CTPS) to the N-terminus of the CTP synthase polypeptide. Plasmids containing the gene encoding CTP synthase (pET15b-CTPS1 or mutant genes) were propagated in *E. coli* strain DH5 α cells and introduced into *E. coli* strain BL21(DE3) cells as the host for target gene expression. Standard techniques were used for DNA isolation and cloning and the gel electrophoresis of both proteins and DNA [28]. Transformed BL21(DE3) cells were grown as described below and the crude lysates from four different colonies were assayed for CTP synthase activity with both ammonia and glutamine as substrates. A colony that displayed enzymic activity similar to that of the wild-type enzyme was selected and cloning of the gene encoding CTP synthase was confirmed by restriction mapping and sequencing by the dideoxy chain termination method with T7 polymerase (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). The sequence of the subcloned gene encoding CTP synthase was identical with the published sequence for *E. coli* CTP synthase (GenBank® accession number M12843).

Expression and purification of recombinant CTP synthase

Recombinant CTP synthase was expressed and purified with a procedure similar to that described by Lim et al. [29] for the purification of CTP synthase from *Giardia intestinalis*. BL21-(DE3) cells transformed with pET15b-CTPS1 were grown in Luria-Bertani medium at 37 °C and induced with isopropyl β -D-thiogalactoside in accordance with the Novagen expression protocol [30]. Soluble hexahistidine-tagged CTP synthase (His₆-CTPS) was then purified by metal-ion-affinity chromatography as described in the Novagen protocols [30]. All enzyme purification procedures were conducted at 4 °C. The resulting enzyme solution was concentrated with a Centriprep-30 concentrator and then dialysed into assay buffer (70 mM Hepes, pH 8.0, containing 0.5 mM EDTA and 10 mM MgCl₂) or storage buffer [200 mM Hepes, pH 8.0, containing 1 mM EDTA, 2 mM dithiothreitol, 10 mM MgCl₂ and 20% (v/v) glycerol].

Thrombin-catalysed cleavage of the hexahistidine tag from soluble enzyme (new N-terminus, GSHMLEM₁...) was conducted in 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, with a thrombin ratio of 2 units/mg of target protein. Cleavage was complete after 22 h at 25 °C; the biotinylated thrombin was removed from the reaction mixture using streptavidin-agarose resin (Novagen) at a ratio of 16 μ l of settled resin per unit of thrombin. Cleaved CTP synthase free of biotinylated thrombin was then dialysed against the assay buffer.

Site-directed mutagenesis

The pET-15b plasmid containing the recombinant gene encoding CTP synthase was used as the template for PCR-based site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, U.S.A.). The procedure followed was that described by the manufacturer. The two synthetic primers used to construct the C379S mutant were 5'-dCCTTA-TCTGGGCATTAGCCTGGGTATGCAGGTG-3' and 5'-dC-ACCTGCATACCCAGGCTAATGCCCAGATAAGG-3', where the positions of the mismatches are indicated by the underlined bases. The two synthetic primers used to construct the C379A mutant were 5'-dCCTTATCTGGGCATTGCGCT-GGGTATGCAGGTG-3' and 5'-dCACCTGCATACCCAGG-GCAATGCCCAGATAAGG-3'. The entire mutant genes were sequenced to verify that no other alterations in the nucleotide sequence had been introduced. The hexahistidine-tagged mutant enzymes were purified and had their hexahistidine tags removed with the same procedures as those described for the recombinant wild-type enzyme.

Enzyme assay and protein determinations

CTP synthase activity was determined at 37 °C with a continuous spectrophotometric assay by following the rate of increase in A_{291} resulting from the conversion of UTP to CTP ($\Delta\epsilon$ 1338 M⁻¹·cm⁻¹) [27]. The standard assay mixture consisted of 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 10 mM MgCl₂, CTP synthase (0.09–0.14 unit/ml) and a saturating concentration of nucleotides (1 mM UTP and 1 mM ATP) in a total volume of 1 ml. The total NH₄Cl concentrations ranged between 5 mM and 100 mM when ammonia was the substrate and the ionic strength was maintained at 0.20 M by the addition of KCl. K_m values were calculated for the concentration of ammonia present at pH 8.0. The concentration of GTP was saturating at 0.25 mM and the concentrations of glutamine ranged between 0.1 and 10 mM when glutamine was the substrate. Enzyme and nucleotides were preincubated together for 5 min at 37 °C and then the reaction was initiated by the addition of either NH₄Cl or

Scheme 3 Open chain and cyclic forms of GSA

glutamine. Specific activities were determined at saturating concentrations of all nucleotides by using glutamine as the substrate. One unit is defined as the activity producing 1 μ mol of CTP/min per mg of protein. Values of k_{cat} were calculated for His₆-CTPS and CTP synthase with the His₆ tag removed by using molecular masses of 62911 and 61029 Da respectively. Reported errors are S.D. Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with BSA standards.

Enzyme inhibition

All inhibition assays were conducted with enzyme from which the hexahistidine tag had been removed. Assays were conducted at 37 °C in 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 1 mM UTP, 1 mM ATP and 10 mM MgCl₂, except where noted. Inhibition assay solutions also contained either glutamine (0.1, 0.2, 0.5, 1.0 and 6.0 mM) and GTP (0.25 mM) or NH₄Cl (5.0, 10.0, 20.0, 50.0 and 100.0 mM). This fixed, saturating concentration of GTP was chosen to avoid the deviations from the Michaelis–Menten hyperbola that occur at lower GTP concentrations [9], which would have complicated the inhibition experiments. The inhibition of the glutamine-dependent CTP synthase activity by solutions of P5C was examined at pH 6.5, 7.3 and 8.0. P5C is stable in acidic solutions but not in neutral solutions, in which polymerization is believed to occur [24,25]. For this reason, acidic preparations of P5C were neutralized with aqueous KOH only immediately before use. Except where noted otherwise, the ionic strength of all inhibition assay mixtures was maintained at 0.25 M by the addition of KCl.

In solution, GSA exists in equilibrium with its cyclic form, P5C, and its *gem*-diol (hydrate) (Scheme 3). Other minor species might also be present depending on the pH of the solution [21,31]. In neutral aqueous solutions the equilibria strongly favour the cyclic form but at lower pH the equilibria favour cyclization less strongly [21]. The aminobenzaldehyde assay measures the total concentration of both cyclic and open-chain species present [25]. For these reasons, unless otherwise stated, the term P5C is used to represent all the equilibrium species present at a given pH. The total P5C concentrations used at each pH were as follows: 0, 0.0125, 0.025 and 0.050 mM for pH 6.5;

0, 0.025, 0.050 and 0.100 mM for pH 7.3; and 0, 0.05, 0.10 and 0.20 mM for pH 8.0. When the inhibition of the ammonia-dependent CTP synthase activity was examined at pH 8.0, the total P5C concentrations were 0, 0.025, 0.10 and 0.20 mM. Enzyme (0.09–0.14 unit/ml) and nucleotides were incubated for 5 min at 37 °C; inhibitor was then added and the mixture was incubated for a further 2 min at 37 °C. Reactions were then initiated by the addition of either glutamine or NH₄Cl. The effects of L-proline (20 mM), L-2-pyrrolidone-5-carboxylate (0, 5.0, 10.0 and 15.0 mM), pyrrole-2-carboxylate (0, 5.0, 10.0 and 15.0 mM) and D,L-2-amino-4-phosphonobutyric acid (0, 2.5, 5.0 and 10.0 mM) on CTP synthase activity with both glutamine and ammonia as the substrate were examined at the concentrations indicated. When ammonia was the substrate, inhibition was examined in both the presence and the absence of GTP (0.25 mM). When inhibition was observed, complete Michaelis–Menten plots were constructed at all inhibitor concentrations by using the concentrations of glutamine or NH₄Cl given above. In all inhibition studies, Michaelis–Menten plots were hyperbolic and kinetic data were analysed by non-linear regression analysis of the Michaelis–Menten plots with the program Enzymekinetiks (1990) from Trinity Software (Compton, NH, U.S.A.).

Inhibition data were fitted to either

$$v_i = \frac{V_{max}[S]}{K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})} \quad (1)$$

or

$$v_i = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \quad (2)$$

which describe linear mixed-type or competitive inhibition respectively [32]. In these equations, v_i is the initial velocity, V_{max} is the maximal velocity at saturating substrate concentration, S is the substrate (glutamine or ammonia), K_m is the Michaelis constant for the substrate, and I is the inhibitor. The inhibition constants were determined in triplicate and average values are reported. The reported errors are S.D.

RESULTS

Enzyme purification

The *E. coli* gene encoding CTP synthase was originally inserted into the plasmid pMW5, a pUC8 derivative, and traditionally the enzyme has been overexpressed as a single polypeptide chain by using this plasmid [2]. The enzyme has been purified with a variety of techniques including ion-exchange chromatography, hydroxyapatite chromatography, gel filtration and hydrophobic chromatography [1,8,27,33] to give specific activities ranging between 5.8 and 8.7 μ mol of CTP/min per mg [27,33]. In the present study the gene encoding *E. coli* CTP synthase was subcloned into the pET-15b vector and the resulting recombinant gene encoding a fusion protein containing an N-terminal hexahistidine tag was overexpressed, purified by metal-ion-affinity chromatography and treated with thrombin to remove the hexahistidine tag. SDS/PAGE analysis of the soluble proteins extracted from BL21(DE3) cells harbouring the pET15b-CTPS1 plasmid grown under inducing conditions revealed the presence of a protein band with an apparent mobility similar to that expected for CTP synthase, which was not present in extracts from cells grown under non-inducing conditions (Figure 1A). The specific activity of the crude lysate obtained after sonication of the induced BL21(DE3) cells was 1.0 ± 0.1 unit/mg. Typically, the specific activity of the affinity-purified enzyme bearing the hexahistidine tag was 8 ± 1 units/mg with respect to glutamine.

Figure 1 SDS/PAGE analyses of recombinant CTP synthase

(A) Analysis of recombinant CTP synthase expression in BL21(DE3) cells transformed with pET15b-CTPS1. SDS/PAGE was conducted with the total protein extracted from cells transformed with pET15b (lane 1) or pET15b-CTPS1 grown in conditions under which expression was uninduced (lane 2) or induced with isopropyl β -D-thiogalactoside (lane 3). In all cases glucose was included in the growth medium. The arrow indicates the position of the CTP synthases bearing an N-terminal hexahistidine tag. (B) SDS/PAGE analysis of protease cleavage of the hexahistidine tag from CTP synthase. Lane 1, molecular mass markers; lane 2, wild-type CTP synthase purified with the protocol of Lewis and Villafranca [27]; lane 3, affinity-purified His₆-CTPS; lane 4, purified His₆-CTPS treated with biotinylated thrombin. (C) SDS/PAGE analysis of affinity-purified recombinant CTP synthase mutants. Lane 1, molecular mass markers; lane 2, wild-type recombinant CTP synthase; lane 3, C379S CTP synthase; lane 4, C379A CTP synthase. In all panels the sizes of molecular mass markers are indicated at the left.

Table 1 Kinetic properties of recombinant *E. coli* CTP synthase

Values of k_{cat} are per enzyme subunit at an ionic strength of 0.20 M. Assay conditions were as described in the Experimental section. Kinetic parameters for glutamine were determined in the presence of 0.25 mM GTP. The corresponding parameters in the absence of GTP could not be determined accurately because of the low activities of both the wild-type and recombinant enzymes. K_m values determined with NH₄Cl as the substrate have been adjusted to reflect the concentration of ammonia (pK_a 9.24 [48]) at pH 8.0. Wild-type CTP synthase was purified by the method of Lewis and Villafranca [27]. The K_m values were determined in the present study but because the purification did not yield pure wild-type enzyme (see Figure 1B), accurate k_{cat} values could not be calculated. The k_{cat} value shown is that reported by Lewis and Villafranca [27] with respect to glutamine.

Enzyme	Substrate ... NH ₃		Glutamine	
	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)
His ₆ -CTPS	2.3 ± 0.2	16 ± 2	0.29 ± 0.04	8.1 ± 0.9
Thrombin-treated CTP synthase (lacking His ₆)	2.0 ± 0.1	13 ± 2	0.30 ± 0.02	6.6 ± 0.8
Wild-type CTP synthase	2.3 ± 0.1	—	0.32 ± 0.02	6.7

Removal of the hexahistidine tag (Figure 1B) yielded enzyme with a specific activity of 6.5 ± 0.8 units/mg. This value is in excellent agreement with the value 6.7 units/mg reported for the pure wild-type enzyme [27].

The kinetic properties of hexahistidine-tagged CTP synthase and CTP synthase lacking the hexahistidine tag are shown in Table 1. Cleavage of the hexahistidine tag with thrombin did not alter the enzyme's kinetic parameters. The values of K_m and k_{cat} with respect to both glutamine and ammonia for the thrombin-treated enzyme were identical with those for the wild-type enzyme. Typically, 0.35 mg of hexahistidine-tagged enzyme was obtained from 1 g of BL21(DE3) cells. Thus this protocol provides rapid access to the milligram quantities of highly purified preparations of CTP synthase required for detailed studies of the enzyme's catalytic mechanism and regulation.

Table 2 Kinetic properties of recombinant wild-type and mutant *E. coli* CTP synthases at an ionic strength of 0.25 M

Values of k_{cat} are per enzyme subunit at an ionic strength of 0.25 M. Assay conditions were as described in the Experimental section. Kinetic parameters for glutamine were determined in the presence of 0.25 mM GTP. Values of K_m determined with NH₄Cl as the substrate have been adjusted to reflect the concentration of ammonia (pK_a 9.24 [48]) at pH 8.0. Abbreviation: n.d., activity too low to be measured accurately.

CTP synthase variant	Substrate ... NH ₃		Glutamine	
	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)
Wild-type	1.83 ± 0.08	8.6 ± 0.3	0.32 ± 0.01	6.1 ± 0.3
C379S	1.8 ± 0.3	10.4 ± 0.4	n.d.	n.d.
C379A	1.9 ± 0.3	9.9 ± 0.2	n.d.	n.d.

Activity of the C379S and C379A mutants

Sequence alignments of the Type I glutamine amidotransferase domains revealed that Cys-379 of *E. coli* CTP synthase was probably the cysteine residue that forms the S-ester with glutamine [2,4]. The C379S and C379A mutant enzymes were prepared by site-directed mutagenesis (Figure 1C). Indeed, the conversion of Cys-379 into serine abolished the glutamine-dependent formation of CTP but left the ammonia-dependent activity intact (Table 2). Because the replacement of cysteine by serine is a conservative mutation, it is unlikely that structural perturbations caused the alteration in activity. Conversion of Cys-379 into alanine resulted in a similar loss of glutamine-dependent activity, whereas the ammonia-dependent activity was not significantly altered. These observations support the catalytic role of Cys-379 in glutamine hydrolysis.

Inhibition by P5C and analogues

The inhibition of recombinant wild-type CTP synthase activity by solutions of P5C was examined with either glutamine or ammonia as the substrate. When glutamine was used as the

Figure 2 Double-reciprocal plots showing the inhibition of CTP synthase activity by P5C and L-2-pyrrolidone-5-carboxylate

Typical data are shown that illustrate (A) the linear mixed-type inhibition observed for P5C (●, 0 mM; ▲, 0.05 mM; ○, 0.10 mM; △, 0.20 mM) with respect to glutamine, (B) the competitive inhibition observed for P5C (●, 0 mM; ▲, 0.025 mM; ○, 0.10 mM; △, 0.20 mM) with respect to ammonia and (C) the weak competitive inhibition observed for L-2-pyrrolidone-5-carboxylate (●, 0 mM; ▲, 5.0 mM; ○, 10.0 mM; △, 20.0 mM) with respect to ammonia. All plots are for data obtained at pH 8.0 with a GTP concentration of 0.25 mM. Other conditions were as described in the Experimental section. The inset in (A) shows the replot of the slope [apparent K_m/V_{max} (s/ μ M)] and the y-intercept [apparent $1/V_{max}$ (nmol/s)] values as a function of inhibitor concentration. The value of the x-intercept is $-K_{is}$ or $-K_{ii}$ respectively. The inset in (B) shows a replot of the slope [apparent K_m/V_{max} (s/ μ M)] values as a function of inhibitor concentration and the value of the x-intercept is $-K_i$. The inset in (C) shows the replot of the slope [apparent K_m/V_{max} (s/ μ M)] values as a function of inhibitor concentration. A slight parabolic curvature (solid line) is typically observed for all cyclic P5C analogues and D,L-2-amino-4-phosphonobutylate at high inhibitor concentrations. Apparent K_i values were determined by using the three lowest inhibitor concentrations as indicated by the dotted line. The average inhibition constants obtained from three trials are reported in Tables 3 and 4.

Table 3 Kinetic parameters for linear mixed type and competitive inhibition of recombinant wild-type CTP synthase by P5C

Inhibition constants are the observed values based on the total concentrations of P5C and open-chain species present in the assay; the ionic strength was 0.25 M. Adjustment of these values to reflect the concentration of GSA as the true inhibitor is described in the Discussion section. The values of the apparent inhibition constants K_{is} (app) and K_{ii} (app) are calculated from slope replots and intercept replots respectively [32]. K_m values determined with NH_4Cl as the substrate have been adjusted to reflect the concentration of ammonia (pK_a 9.24 [48]) at pH 8.0.

Substrate	pH	[GTP] (mM)	K_m (mM)	K_{is} (app) (mM)	K_{ii} (app) (mM)
Glutamine	8.0	0.25	0.37 ± 0.04	0.16 ± 0.03	0.4 ± 0.1
	7.3	0.25	0.35 ± 0.03	0.052 ± 0.007	0.15 ± 0.05
	6.5	0.25	0.43 ± 0.05	0.026 ± 0.003	0.13 ± 0.05
Ammonia	8.0	0.25	2.6 ± 0.6	0.39 ± 0.06	—
	8.0	0	1.5 ± 0.2	$3.4 \pm 0.7^*$	—

* K_i (app) was calculated from triplicate determinations of the ratio ($v_i/v_0 = 0.90 \pm 0.03$) of the velocity observed in the presence of 1.0 mM P5C (v_i) and in the absence of inhibitor (v_0) at an NH_4Cl concentration of 50 mM, on the assumption that the type of inhibition remained competitive (eqn 2); the ionic strength was 0.40 M.

substrate in the presence of GTP (0.25 mM), linear mixed-type inhibition was observed at all pH values studied (Figure 2A and Table 3), with the inhibition being more pronounced when the pH was decreased from 8.0 to 6.5. Inhibition studies at pH values outside this range were not feasible because of the lowered activity of the enzyme. In addition, when glutamine was employed as the substrate in the absence of GTP, the velocities were too low for reliable inhibition data to be obtained.

The inhibition of CTP synthase activity was competitive (Figure 2B) with respect to ammonia in the presence of GTP (K_i 0.39 mM). However, in the absence of GTP, only weak inhibition was observed. Using an NH_4Cl concentration equal to 50 mM (ionic strength 0.40 M), the initial rates were measured in the absence and the presence of 1 mM P5C; assuming that the inhibition remained competitive in the absence of GTP, a K_i of approx. 3.4 mM was calculated. In addition, when the ammonia-dependent activity of the C379A mutant was examined in the presence of 1 mM P5C, no significant inhibition was observed. Unfortunately, the large increase in ionic strength caused by the high salt concentration in the neutralized P5C solutions prevented the use of higher concentrations of P5C in the inhibition assays when ammonia was the substrate in the absence of GTP. We found that both wild-type and recombinant CTP synthases were sensitive to changes in ionic strength, losing activity at higher ionic strengths. In general, the ionic strength was maintained at 0.25 M to permit comparison between the ammonia-dependent and glutamine-dependent activities (see Table 3).

To obtain an indication of whether P5C is bound to the enzyme in a cyclic form, analogues of P5C were tested for their effect on CTP synthase activity. The effects of the cyclic analogues L-proline, L-2-pyrrolidone-5-carboxylate and pyrrole-2-carboxylate are shown in Table 4. When glutamine was the substrate, no significant inhibition of CTP synthase was observed. When ammonia was the substrate, L-proline did not inhibit the enzyme, whereas L-2-pyrrolidone-5-carboxylate and pyrrole-2-carboxylate were weak competitive inhibitors. However, none of these compounds was as potent an inhibitor as P5C. Figure 2(C) shows typical inhibition data for these analogues. A slight curvature of the replots was observed, which could be fitted with a parabolic function. This slight curvature might represent binding of the analogues at two independent sites on the free enzyme [32]. Apparent K_i values were estimated from the lowest three inhibitor

Table 4 Effect of cyclic analogues and 2-amino-4-phosphonobutyrate on the activity of *E. coli* CTP synthase

In all cases the observed inhibition was competitive with respect to the substrate. Except for the cases indicated below, apparent inhibition constants [K_i (app)] were calculated from replots with the three lowest inhibitor concentrations, at which the curvature of the replots was negligible (see Figure 2C). Experiments were conducted at pH 8.0.

Compound	Substrate	[GTP] (mM)	K_i (app) (mM)
L-Proline	Glutamine	0.25	$\geq 139 \pm 17^*$
	Ammonia	0.25	$626 \pm 69^\dagger$
		0	$198 \pm 16^\dagger$
Pyrrole-2-carboxylate	Glutamine	0.25	$136 \pm 16^\dagger$
	Ammonia	0.25	11.0 ± 0.3
		0	11 ± 1
L-2-Pyrrolidone-5-carboxylate	Glutamine	0.25	$343 \pm 45^\dagger$
	Ammonia	0.25	12.6 ± 0.7
		0	12 ± 1
D,L-2-Amino-4-phosphonobutyrate	Glutamine	0.25	$125 \pm 15^\dagger$
	Ammonia	0.25	5.6 ± 0.6
		0	5.5 ± 0.2

* L-Proline did not produce any detectable inhibition at a concentration of 20 mM. The lower limit for the K_i is calculated on the assumption that a minimum of 5% inhibition could have been detected.

† The K_i values were calculated from triplicate determinations of the ratio (v_i/v_0) of the velocity observed in the presence of 20 mM inhibitor (v_i) and in the absence of inhibitor (v_0) at glutamine and NH_4Cl concentrations of 0.50 and 50 mM respectively and on the assumption that the type of inhibition was competitive; the ionic strength was 0.25 M.

Figure 3 Effect of ammonium ion on glutamine activity

Reaction mixtures contained 1 mM ATP, 1 mM UTP, 0.25 mM GTP, 15 $\mu\text{g/ml}$ CTP synthase and 10 mM MgCl_2 in 70 mM Hepes, pH 7.2, and increasing concentrations of either NH_4Cl (●), NH_4Cl with 15 mM glutamine (○) or $(\text{NH}_4)_2\text{SO}_4$ with 15 mM glutamine (▲). Assays were conducted at 37 °C and the ionic strength attributable to the ammonium salts was maintained at 0.1 M by the addition of KCl.

concentrations, which corresponded to the linear region of the replot. These apparent K_i values did not depend on the presence or absence of GTP. D,L-2-Amino-4-phosphonobutyrate, analogous in structure to the linear GSA hydrate, did not inhibit CTP synthase with respect to glutamine (Table 4). When ammonia was employed as the substrate, the inhibition results were similar to those obtained for the cyclic analogues except that 2-amino-4-phosphonobutyrate was a slightly more effective competitive inhibitor. As with the cyclic analogues, inhibition by 2-amino-4-phosphonobutyrate was independent of GTP concentration.

Relationship between glutamine and NH_4^+ sites

To determine the relationship between sites for nascent ammonia and exogenous ammonium, Levitzki and Koshland [16] conducted a competition experiment at pH 7.2 (i.e. NH_3 existing almost exclusively as NH_4^+) in which they found that high concentrations of $(\text{NH}_4)_2\text{SO}_4$ inhibited the glutamine-dependent activity of CTP synthase. These authors concluded that the nascent ammonia arising from glutamine hydrolysis and exogenous NH_4^+ competed for a single site. Figure 3 shows the results of our replication of this experiment in which constant ionic strength was maintained with KCl. We also examined the effect of NH_4^+ derived from NH_4Cl . Indeed, high concentrations of $(\text{NH}_4)_2\text{SO}_4$ inhibited the glutamine-dependent activity but the inhibition was caused by the SO_4^{2-} . In contrast, high concentrations of NH_4Cl did not inhibit the rate of glutamine-dependent CTP production. In fact, the added NH_4Cl increased the rate slightly. This rate increase, however, was much less than would have been predicted from the sum of the individual rates of CTP production observed when either NH_4Cl or glutamine was used as the substrate at pH 7.2. Thus exogenous NH_4^+ does not seem to compete with the nascent ammonia released from glutamine. Zalkin and Truitt obtained a similar result for GMP synthase [34].

DISCUSSION

The thiol function of Cys-379 of *E. coli* CTP synthase is believed to participate in the formation of a γ -glutamyl *S*-ester intermediate during glutamine hydrolysis (Scheme 2A) [2]. GSA, related in structure to the acyl portion of glutamine, is a potent reversible inhibitor of the amidotransferases GlnS [21,22] and CPS [23]. Kinetic and X-ray crystallographic studies have provided evidence that the free aldehyde inhibits these enzymes because it forms a thiohemiacetal within the active site resembling bound tetrahedral intermediates in the formation and breakdown of acyl-enzyme intermediates [21,22] (Scheme 2B). Accordingly, we reasoned that GSA should inhibit CTP synthase in an analogous manner. If the allosteric activator, GTP, promotes glutamine hydrolysis by inducing a conformational change that enhances interactions with bound tetrahedral intermediates, then inhibition by GSA should be sensitive to the presence of GTP.

GSA exists in equilibrium with its intramolecular cyclization product P5C. We found that P5C is a potent inhibitor of CTP synthase activity with respect to glutamine, giving rise to linear mixed-type inhibition. The glutamine-dependent activity of CTP synthase is too low for inhibition studies to be conducted in the absence of GTP; however, this experimental difficulty was overcome by examining the inhibition by P5C with ammonia as the substrate. P5C is a competitive inhibitor with respect to ammonia and, in the presence of GTP, the enzyme's apparent affinity for the inhibitor is increased approx. 10-fold (Table 2). This correlates well with the 6-fold decrease in the K_m value for glutamine that is observed in the presence of GTP [18]. Thus GTP enhances the affinity of CTP synthase for the inhibitor.

Inhibition of amidotransferases by the rarer open-chain semi-aldehyde and not the cyclic P5C has been well established for GlnS [21] and CPS [23]. To demonstrate that this is also true for CTP synthase, we examined the effect of analogues of P5C and GSA on enzyme activity (Table 4). No significant inhibition by the cyclic analogues L-proline, L-2-pyrrolidone-5-carboxylate and pyrrole-2-carboxylate was observed with respect to glutamine in the presence of GTP. However, when ammonia was the substrate, both L-2-pyrrolidone-5-carboxylate and pyrrole-2-carboxylate, which mimic P5C in having an sp^2 -hybridized carbon atom adjacent to the ring nitrogen, behaved as weak competitive

by DON and glutamine [15,37] and are believed to give rise to the 'half-of-the-sites' reactivity observed when DON reacts with only half of the total glutamine sites yet abolishes all of the glutamine activity of the enzyme and leaves the ammonia activity intact [37]. Distinct differences between the native, glutamylated and DON-modified enzyme have been demonstrated by their differing reactivities towards 5,5'-dithiobis-(2-nitrobenzoic acid) [37]. In addition, ammonia derived from the hydrolysis of glutamine does not equilibrate with the solvent [16] and this sequestration of the nascent ammonia has been attributed to conformational changes in the glutamylated enzyme. Indeed, sequestration of the nascent ammonia derived from glutamine hydrolysis seems to be a common theme in amidotransferases [38] and might arise, in part, through either conformational changes or solvent-inaccessible ammonia channels [39–44]. Similarly, in Scheme 4(B), a conformational change induced by ammonia might account for the inability of the enzyme–ammonia complex to bind GSA.

The apparent inhibition constant [K_i (app), where $-K_i$ (app) is the x -intercept from a replot of apparent K_m values as a function of inhibitor concentration] for competitive inhibition by GSA, as depicted in Scheme 4(B), is described by

$$K_i(\text{app}) = \left(\frac{K_{\text{GTP}}K_iK'_i + K_iK'_i[\text{GTP}]}{K_{\text{GTP}}K'_i + K_i[\text{GTP}]} \right) \left(1 + \frac{[\text{P5C}]}{K'_i} \right) \quad (3)$$

and is derived by using the rapid equilibrium assumption [32]. Both E and E·GTP bind ammonia and catalyse amination to the same degree, which is consistent with the observations of Levitzki and Koshland [18]. When $[\text{GTP}] = 0$, K_i (app) = $K_i(1 + [\text{P5C}]/K'_i)$ and when $[\text{GTP}]$ is high, K_i (app) = $K'_i(1 + [\text{P5C}]/K'_i)$. Because added GTP (0.25 mM) decreased K_i (app) 10-fold, we conclude that $K'_i < K_i$ and that E·GTP has a higher affinity for GSA. Our inhibition results suggest that alternative forms of P5C bind to the ammonia site of E or E·GTP with the same affinity (K'_i). Simultaneous binding of these species and GSA does not alter our conclusion that $K'_i < K_i$.

Implications for allosteric activation by GTP

Two steps comprise the glutamylation of CTP synthase: formation of the tetrahedral intermediate (k_2 in Scheme 2) and its collapse to form the S -ester (k_3 in Scheme 2). The second step is probably rate-limiting if the catalysis of glutamylation resembles that described for the cysteine protease papain [45–47]. The present work demonstrates that CTP synthase binds GSA at the glutamine site and that this binding is enhanced in the presence of GTP. Recently, our understanding of how class I amidotransferases stabilize the tetrahedral intermediates formed during glutamine hydrolysis has been vastly improved by elegant crystallographic studies on *E. coli* GMP synthase [43] and *E. coli* CPS [23]. The structures of both of these amidotransferases, which share three conserved regions in the GAT domain with CTP synthase [4], show the presence of a catalytic triad and an oxyanion hole that stabilizes the tetrahedral intermediate [5]. CTP synthase probably possesses a similar oxyanion hole and the binding of GTP induces a conformational change that strengthens the interactions between this oxyanion hole and the tetrahedral intermediate, thereby promoting catalysis. To the extent that the tetrahedral intermediates resemble the transition states for the formation and collapse of the tetrahedral intermediates, it is also likely that the GTP-induced conformation stabilizes these transition states, although recent investigations have indicated that thiohemiacetals might be poor transition-state models for cysteine-protease-catalysed reactions [19]. Demonstration of the presence of an oxyanion hole through structural

studies, combined with the direct kinetic evidence for stabilization of the tetrahedral intermediate(s) described here, should give a much clearer picture of the overall role of GTP as an allosteric effector in CTP synthase catalysis.

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REFERENCES

- Koshland, D. E. and Levitzki, A. (1974) CTP synthetase and related enzymes. *Enzymes* **10**, 539–559.
- Weng, M., Makaroff, C. A. and Zalkin, H. (1986) Nucleotide sequence of *Escherichia coli* *pyrG* encoding CTP synthetase. *J. Biol. Chem.* **261**, 5568–5574.
- Weng, M. and Zalkin, H. (1987) Structural role for a conserved region in the CTP synthetase glutamine amide transfer domain. *J. Bacteriol.* **169**, 3023–3028.
- Zalkin, H. (1993) The amidotransferases. *Adv. Enzymol.* **66**, 203–309.
- Zalkin, H. and Smith, J. L. (1998) Enzymes utilizing glutamine as an amide donor. *Adv. Enzymol. Relat. Areas Mol. Biol.* **72**, 87–144.
- Traut, T. W. (1988) Physiological concentrations of purines and pyrimidines. *CRC Crit. Rev. Biochem.* **23**, 121–169.
- Kennedy, E. P. (1986) The biosynthesis of phospholipids. In *Lipids and Membranes: Past, Present and Future* (Op den Kamp, J. A. F., Roelofs, B. and Wirtz, K. W. A., eds.), pp. 171–206. Elsevier Scientific Publishers, Amsterdam.
- Long, C. W. and Pardee, A. B. (1967) Cytidine triphosphate synthetase of *Escherichia coli* B. *J. Biol. Chem.* **242**, 4715–4721.
- Levitzki, A. and Koshland, D. E. (1969) Negative cooperativity in regulatory enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **62**, 1121–1128.
- Levitzki, A. and Koshland, D. E. (1972) Ligand-induced dimer-to-tetramer transformation in cytidine triphosphate synthetase. *Biochemistry* **11**, 247–252.
- Park, T.-S., Ostrander, D. B., Pappas, A. and Carman, G. M. (1999) Identification of Ser⁴²⁴ as the protein kinase A phosphorylation site in CTP synthetase from *Saccharomyces cerevisiae*. *Biochemistry* **38**, 8839–8848.
- Williams, J. C., Kizaki, H. and Weber, G. (1978) Increased CTP synthetase activity in cancer cells. *Nature (London)* **271**, 71–72.
- Kensler, T. W. and Cooney, D. A. (1989) Inhibitors of the *de novo* pyrimidine pathway. In *Design of Enzyme Inhibitors as Drugs* (Sandler, M. and Smith, H. J., eds.), pp. 379–401. Oxford University Press, New York.
- Ostrander, D. B., O'Brien, D. J., Gorman, J. A. and Carman, G. M. (1998) Effect of CTP synthetase regulation by CTP on phospholipid synthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 18992–19001.
- Long, C. W., Levitzki, A. and Koshland, D. E. (1970) The subunit structure and subunit interactions of cytidine triphosphate synthetase. *J. Biol. Chem.* **245**, 80–87.
- Levitzki, A. and Koshland, D. E. (1971) Cytidine triphosphate synthetase. Covalent intermediates and mechanisms of action. *Biochemistry* **10**, 3365–3371.
- von der Saal, W., Anderson, P. M. and Villafranca, J. J. (1985) Mechanistic investigations of *Escherichia coli* cytidine-5'-triphosphate synthetase. *J. Biol. Chem.* **260**, 14993–14997.
- Levitzki, A. and Koshland, D. E. (1972) Role of an allosteric effector. Guanosine triphosphate activation in cytidine triphosphate synthetase. *Biochemistry* **11**, 241–246.
- Dufour, É., Storer, A. C. and Ménard, R. (1995) Peptide aldehydes and nitriles as transition state analog inhibitors of cysteine proteases. *Biochemistry* **34**, 9136–9143.
- Thompson, R. C. (1977) Peptide aldehydes: potent inhibitors of serine and cysteine proteases. *Methods Enzymol.* **46**, 220–225.
- Bearne, S. L. and Wolfenden, R. (1995) Glutamate γ -semialdehyde as a natural transition state analogue inhibitor of *Escherichia coli* glucosamine-6-phosphate synthase. *Biochemistry* **34**, 11515–11520.
- Badet-Denisot, M.-A., Leriche, C., Massière, F. and Badet, B. (1995) Nitrogen transfer in *E. coli* glucosamine-6P synthase. Investigations using substrate and bisubstrate analogues. *Bioorg. Med. Chem. Lett.* **5**, 815–820.
- Thoden, J. B., Huang, X., Rauschel, F. M. and Holden, H. M. (1999) The small subunit of carbamoyl phosphate synthetase: snapshots along the reaction pathway. *Biochemistry* **38**, 16158–16166.
- Williams, I. and Frank, F. (1975) Improved chemical synthesis and enzymatic assay of Δ^1 -pyrroline-5-carboxylic acid. *Anal. Biochem.* **64**, 85–97.
- Mezl, V. A. and Knox, W. E. (1976) Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. *Anal. Biochem.* **74**, 430–440.
- Chung, C. T., Niemela, S. L. and Miller, R. H. (1989) One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2172–2175.

- 27 Lewis, D. A. and Villafranca, J. J. (1989) Investigation of the mechanism of CTP synthetase using rapid quench and isotope partitioning methods. *Biochemistry* **28**, 8454–8459
- 28 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, pp. 1.21–1.52, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 29 Lim, R. L. H., O'Sullivan, W. J. and Stewart, T. S. (1996) Isolation, characterization and expression of the gene encoding cytidine triphosphate synthetase from *Giardia intestinalis*. *Mol. Biochem. Parasitol.* **78**, 249–257
- 30 Novagen (1997) *pET System Manual*, 7th edn, TB055, pp. 18–64, Novagen, Madison, Wisconsin
- 31 Lewis, M. L., Rowe, C. J., Sewald, N., Suterland, J. D., Wilson, E. J. and Wright, M. C. (1993) The effect of pH on the solution structure of Δ^1 -pyrroline-2-carboxylic acid as revealed by NMR and electrospray mass spectroscopy. *Bioorg. Med. Chem. Lett.* **3**, 1193–1196
- 32 Segel, I. H. (1975) *Enzyme Kinetics*, pp. 102 and 170–178, John Wiley & Sons, New York
- 33 Anderson, P. M. (1983) CTP synthetase from *Escherichia coli*: an improved purification procedure and characterization of hysteretic and enzyme concentration effects on kinetic properties. *Biochemistry* **22**, 3285–3292
- 34 Zalkin, H. and Truitt, C. D. (1977) Characterization of the glutamine site of *Escherichia coli* guanosine 5'-monophosphate synthetase. *J. Biol. Chem.* **252**, 5431–5436
- 35 Lienhard, G. E. and Jencks, W. P. (1966) Thiol addition to the carbonyl group. Equilibria and kinetics. *J. Am. Chem. Soc.* **88**, 3982–3995
- 36 Badet, B., Vermoote, P., Haumont, P.-Y., Lederer, F. and Le Goffic, F. (1987) Glucosamine synthetase from *Escherichia coli*: purification, properties, and glutamine-utilizing site location. *Biochemistry* **26**, 1940–1948
- 37 Levitzki, A., Stallcup, W. B. and Koshland, D. E. (1971) Half-of-the-sites reactivity and the conformational states of cytidine triphosphate synthetase. *Biochemistry* **10**, 3371–3378
- 38 Massière, F. and Badet-Denisot, M.-A. (1998) The mechanism of glutamine-dependent amidotransferases. *Cell. Mol. Life Sci.* **54**, 205–222
- 39 Mullins, L. S. and Raushel, F. M. (1999) Channeling of ammonia through the intermolecular tunnel contained within carbamoyl phosphate synthetase. *J. Am. Chem. Soc.* **121**, 3803–3804
- 40 Huang, X. and Raushel, F. M. (2000) An engineered blockage within the ammonia tunnel of carbamoyl phosphate synthetase prevents the use of glutamine as a substrate but not ammonia. *Biochemistry* **39**, 3240–3247
- 41 Krahn, J. M., Kim, J. H., Burns, M. R., Parry, R. J., Zalkin, H. and Smith, J. L. (1997) Coupled formation of an amidotransferase interdomain ammonia channel and a phosphoribosyltransferase active site. *Biochemistry* **36**, 11061–11068
- 42 Muchmore, C. R. A., Krahn, J. M., Kim, J. H., Zalkin, H. and Smith, J. L. (1998) Crystal structure of glutamine phosphoribosylpyrophosphate amidotransferase from *Escherichia coli*. *Protein Sci.* **7**, 39–51
- 43 Tesmer, J. J. G., Klem, T. J., Deras, M. L., Davisson, V. J. and Smith, J. L. (1996) The crystal structure of GMP synthetase reveals a novel catalytic triad and is a structural paradigm for two enzyme families. *Nat. Struct. Biol.* **3**, 74–86
- 44 Larsen, T. M., Boehlein, S. K., Schuster, S. M., Richards, N. G. J., Thoden, J. B., Holden, H. M. and Rayment, I. (1999) Three-dimensional structure of *Escherichia coli* asparagine synthetase B: a short journey from substrate to product. *Biochemistry* **38**, 16146–16157
- 45 Lowe, G. and Yuthavong, Y. (1971) Kinetic specificity in papain-catalysed hydrolyses. *Biochem. J.* **124**, 107–115
- 46 Lowe, G. and Yuthavong, Y. (1971) pH-dependence and structure-activity relationships in the papain-catalysed hydrolysis of anilides. *Biochem. J.* **124**, 117–122
- 47 O'Leary, M. H., Urberg, M. and Young, A. P. (1974) Nitrogen isotope effects on the papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide. *Biochemistry* **13**, 2077–2081
- 48 Jencks, W. P. and Regenstein, J. (1968) Ionization constants of acids and bases. In *Handbook of Biochemistry* (Sober, H. A., ed.), pp. J150–J189, The Chemical Rubber Co., Cleveland, OH

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