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Cytidine Triphosphate Synthetase. Covalent Intermediates and Mechanisms of Action*

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ABSTRACT: The mechanism of CTP-synthetase from *Escherichia coli* B was elucidated and appears to be composed of the steps given in the adjacent column.

Ammonia can replace glutamine as the amino donor. Evidence is presented that externally added ammonia and the nascent ammonia released from the glutamine reaction occupy the same site.

$$\text{enzyme} + \text{glutamine} \xrightarrow{\text{GTP}} \text{glutamyl-enzyme} + NH_3 \text{ (bound)}$$

$$\text{glutamyl-enzyme} + H_2O \longrightarrow \text{enzyme} + \text{glutamate}$$

$$NH_3 + \text{UTP} \rightleftharpoons H_2N\text{–UTP (tetrahedral adduct)}$$

$$H_2N\text{–UTP} + \text{ATP} \rightleftharpoons H_2N(\text{P})\text{>UTP (phosphorylated tetrahedral adduct)}$$

$$H_2N(\text{P})\text{>UTP} \longrightarrow \text{CTP} + P_i$$

CTPS[1] was first described by Lieberman (1955, 1956) and was shown to catalyze the formation of CTP from NH_3 and ATP (eq 1). Chakraborty and Hurlbert (1961) and Long and

$$\text{UTP} + NH_3 + \text{ATP} \xrightarrow{Mg^{2+}} \text{CTP} + \text{ADP} + P_i \qquad (1)$$

Pardee (1967) established that the enzyme can also utilize glutamine as a nitrogen donor when GTP is present as an allosteric effector.

$$\text{UTP} + \text{Glu-NH}_2 + \text{ATP} \xrightarrow[\text{GTP}]{Mg^{2+}} \text{CTP} + \text{Glu·OH} + \text{ADP} + P_i \qquad (2)$$

The ever increasing literature on allosteric proteins is revealing more and more of their behavior. On the other hand the complete explanation of the binding and catalytic behavior of a protein in terms of its structure is yet to be performed for any one enzyme. CTP-synthetase seemed to offer a particularly advantageous case for such a study. Its structure is relatively simple since its subunits are identical (Levitzki *et al.*, 1971), and its association–dissociation behavior from a tetramer to dimer also is conveniently simple (Long *et al.*, 1970). Its interaction with ligands showed both negative (Levitzki and Koshland, 1970) and positive cooperativity (Long and Pardee, 1967; Levitzki and Koshland, 1969) and it is one of that fairly large group of enzymes which can use either glutamine or ammonia as a nitrogen source. It thus appeared sufficiently complicated to exhibit properties which are illustrative for many allosteric proteins and yet sufficiently simple to allow the hope that its properties could be described in terms of its molecular structure. In this paper the covalent chemistry of the enzyme and the relation between the ammonia and glutamine sites are elucidated. In subsequent papers the allosteric and structural properties will be examined.

Experimental Section

The nucleotides and other chemicals were obtained commercially and were of the highest purity available. In cases in which precise stoichiometric determinations were needed, chromatographic tests were performed to establish the purity of the reagents. β,γ-NH-ATP (referred to as ADPNP) was the gift of Dr. Ralph Yount of Washington State University, Pullman, Wash. [^{14}C]Glutamine (New England Nuclear or

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[1] Abbreviations used are: cytidine 5′-triphosphate synthetase, CTPS; tetrahedral adduct of NH_3 to 4′ position of UTP, NH_2-UTP; intermediate in which OH at 4′ position of NH_2-UTP is phosphorylated, $NH_2(\text{P})$>—UTP; β,γ-imido-ATP, ADPNP; polyethyleneimine, PEI; 6-diazo-5-oxonorleucine, DON; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, HEPES.

TABLE I: Stoichiometry of the CTP-synthetase Reaction.[a]

	Products Formed (Normalized to mmoles of CTP Actually Formed)		
	CTP	ADP	P_i
Glu-NH_2 reaction	1.0	1.09	1.0
NH_3 reaction	1.0	0.85	0.80

[a] The glutamine reaction mixture contained 1×10^{-3} M ATP, 1×10^{-3} M UTP, 1×10^{-2} M $MgCl_2$, 0.02 M Tris-acetate (pH 7.2), 6×10^{-3} M Glu-NH_2, and 2.5×10^{-4} M GTP. The ammonia reaction mixture contained 1×10^{-3} M ATP, 1×10^{-3} M UTP, 1×10^{-2} M $MgCl_2$, 0.02 M Tris-acetate (pH 8.2), and 0.025 M $(NH_4)_2SO_4$. The two reaction mixtures were, respectively, incubated with 0.1 unit of CTP-synthetase at 37°. In both cases controls without enzyme were run in parallel. The reaction was followed by monitoring CTP formation reading A_{291} (Long and Pardee, 1967). At 80% conversion of UTP into CTP the reaction was stopped by incubating the samples for 3 min in boiling water and Dowex 50 (H^+ form) was added to remove Mg^{2+} ions. Determination of products as described in the Experimental Section.

Schwarz) was chromatographed on a Bio-Gel P-2 column to remove high molecular weight material.

CTP-synthetase was purified from *Escherichia coli* using a modification (Levitzki and Koshland, 1970) of the method described earlier by Long and Pardee (1967). Protein was determined by the method of Lowry *et al.* (1951). P_i was assayed according to the method of Fiske and Subbarow (1925). Enzymatic determination of ADP was performed according to Adam (1963). γ-Glutamyl hydroxamate was determined using the ferric chloride reaction (Pamiljans *et al.*, 1962), and ammonia was analyzed with the glutamate dehydrogenase submicro assay (Levitzki, 1970). Thin-layer chromatography was performed either on commercial PEI-cellulose plates (Brinkman and Co.), or freshly prepared ones using 1 M lithium chloride in 1 M formic acid as elution solvent systems (Randerath, 1964).

Carboxymethylation of glutamine-free enzyme was carried out as follows. CTP-synthetase (2.8 mg, specific activity 5.5 units/mg) was incubated with 0.1 ml of a mixture of 5×10^{-3} M dithioerythritol, 2.5×10^{-4} M GTP, 7.5×10^{-4} M UTP, 7.5×10^{-4} M ATP, 0.01 M $MgCl_2$, and 0.02 M imidazole-acetate buffer at pH 7.2 for 40 min at 37°. The incubation mixture was cooled to 0° and 219 mg of $(NH_4)_2SO_4$ were added to precipitate the protein. The supernatant after centrifugation was carefully removed and the precipitate resuspended in 60% saturated solution of $(NH_4)_2SO_4$ and recentrifuged. This process was repeated once more. The precipitate was dissolved in 0.35 ml of 0.02 M sodium phosphate (pH 7.4), containing 1 mM EDTA. Tris-acetate buffer (0.2 ml of 2 M Tris-acetate at pH 8.0) containing [^{3}H]iodoacetate (15.05 mg/ml, 41,910 cpm/μmole, pH 8.0) was added. The mixture was allowed to incubate at 25° until less than 1% of the activity remained (1 hr). Then the sample was cooled to 0° and 0.2 ml of trichloroacetic acid 50% was added. The mixture was allowed to stand for 30 min and centrifuged for 15 min at 10,000 rpm at 0°. The precipitate was resuspended in cold 5% trichloroacetic acid and recentrifuged three times. Finally the precipitate was dissolved in 0.3 ml of 0.1 M Na_2CO_3. Protein and radioactivity were determined.

The glutamine–enzyme mixture was carboxymethylated in a similar way except that 2.8 mg of CTP-synthetase (specific activity 5.4) was first incubated with 0.03 M L-glutamine at pH 7.2 for 15 min at 37°. Then 0.2 ml of [^{3}H]iodoacetate (15.05 mg/ml) was added and the rest of the procedure was similar to the treatment of the glutamine-free enzyme.

The 4′-^{18}O-labeled UTP was synthesized as follows. Li_4CTP (600 μmoles) and 2 g $NaNO_2$ were dissolved in 7.05 ml of [^{18}O]-H_2O, 1.54% atom excess. The pH was brought to 4.0 by adding concentrated H_2SO_4 (0.1 ml). The mixture was sealed and incubated at 37° for 24 hr. The reaction mixture was diluted to 350 ml in distilled water and applied to a Dowex 1 (1.5 × 1.8 cm) column previously equilibrated with 0.01 M Tris-HCl (pH 8.5). The column was eluted first with 300 ml of 0.01 M HCl–0.05 M NaCl. The UTP was eluted with 0.1 N HCl (Lieberman, 1956). The pooled UTP fractions were neutralized to pH 7.0 with NaOH and lyophilized. The solid residue was mixed thoroughly with 7.0 ml of H_2O and filtered to remove the NaCl cake. The UTP was then precipitated as the barium salt by adding barium acetate stoichiometrically. Conversion of Ba_2UTP into the sodium salt was accomplished by treating Ba_2UTP with the sodium form of Chelex-100 (Dowex A-1). The yield was 285 μmoles (77%). The product was pure according to PEI-cellulose thin-layer chromatography (Randerath, 1964) and as judged by the spectral properties.

Results

Stoichiometry of the CTP-synthetase Reaction. Purified starting materials were mixed with the enzyme and the reaction allowed to go almost to completion. The final products obtained using either ammonia or glutamine as the nitrogen source are listed in Table I. In both cases ADP and P_i were produced in equal amounts. An equivalent amount of UTP was converted into CTP and no breakdown products of GTP were found. No AMP was produced indicating that the enzyme is free of myokinase-type activity. The results establish the stoichiometry of the two reactions (eq 1 and 2).

Covalent Glutamyl-Enzyme Intermediate. The release of ammonia from glutamine to serve as a nitrogen source in the reaction could occur in several ways but one of the most logical was the formation of a covalent glutamyl-enzyme intermediate. A search for this intermediate was initiated by incubating the enzyme (5 mg/ml in 0.02 M imidazole acetate buffer at pH 7.2) with an excess of extra reactants (2.5 mM GTP, 0.75 ATP, 7.5 mM UTP, and 0.1 M magnesium chloride) at 25° for 12 hr to decompose all the nonlabeled glutamine. The enzyme was then dialyzed against 25 ml of 1.0 mM ^{14}C-labeled glutamine at 4° with changes in 24 hr. Protein, enzyme activity, and radioactivity were assayed and no significant loss in enzyme activity occurred during this period. The excess radioactivity within the dialysis was found to be equivalent to 0.9 equiv of glutamine per subunit of 52,000 molecular weight.

The next step was to determine whether part of the bound glutamine is covalently attached. A typical experiment is described in Figure 1. Pure CTP-synthetase (specific activity 5.8 units/mg) was dialyzed against 0.02 M sodium phosphate buffer (pH 7.4), containing 1 mM EDTA for 18 hr at 4°. The glutamine-free enzyme was divided into two parts. (a) One part was incubated with 10 mM [^{14}C]Glu-NH_2 at 25° for 15 min, and then quenched with 5 mM I_3^- which was found, under these conditions, to inactivate the enzyme instantaneously and

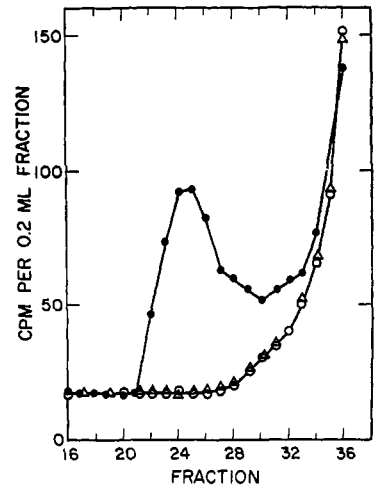


FIGURE 1: The formation of a [^{14}C]glutamyl-enzyme. (a) (●) Glutamine-free enzyme (0.2 ml; 2.85 mμmole) in 0.02 M sodium phosphate buffer containing 1 mM EDTA was incubated with 10 μl of 0.1 M [^{14}C]glutamine (1050 cpm/mμmole) for 15 min at 25°. Then 10 μl of 0.1 M I_2 (in 5% KI) was added and the sample was cooled to 0°. The sample was then applied to a Bio-Gel P-2 column (1.5 × 10 cm) equilibrated with 8 M urea in 0.02 M sodium phosphate buffer (pH 7.4), 1 mM EDTA, and 70 mM β-mercaptoethanol in the cold. (b) (△) Same except enzyme was reacted with the affinity-label DON. (c) (○) Same except the enzyme was reacted with I_3^- first and then with [^{14}C]glutamine. The fraction of the enzyme trapped as the covalent intermediate in all experiments was 0.08–0.12.

(b) the second part was inactivated first with I_3^- and then incubated with the [^{14}C]glutamine. All samples were applied to a Bio-Gel P-2 column to separate the enzyme protein and any radioactivity found from the bulk of [^{14}C]glutamine. The peak of radioactivity associated with the protein indicated a covalent intermediate or a very tightly bound noncovalent intermediate. The latter possibility could be eliminated by observing the same pattern when the P-2 column was run with the buffer containing 8 M urea and 10 mM β-mercaptoethanol.

No radioactivity was associated with the protein when 7.5×10^{-4} M ATP, 7.5×10^{-4} M UTP, and 2.5×10^{-4} M GTP were included since these convert the glutamyl-enzyme into glutamic acid and free enzyme. No breakthrough peaks were obtained when the DON-labeled enzyme was reacted with [^{14}C]glutamine or when I_2 was added prior to glutamine incubation (Figure 1).

The combination of dialysis and Sephadex experiments provide strong evidence for a covalent glutamyl-enzyme intermediate.

Nature of the Amino Acid Residue in the Glutamyl-Enzyme Intermediate. The previous finding (Long *et al.*, 1970) that the affinity-label DON, which is the structural analog of glutamine, reacts with a sulfhydryl group on the protein, led to an experiment to test the possible involvement of a sulfhydryl group in the reaction with glutamine.

When CTP-synthetase is carboxymethylated at pH 8.5 in the presence of glutamine as described in the Experimental Section, 3.9 sulfhydryl groups out of a total of 5.1/52,000 molecular weight protein are labeled. In the absence of glutamine 5.1 groups are labeled. It thus appears that approximately one free sulfhydryl group per subunit in the free enzyme is protected by glutamine. This together with the similar action of the DON, which is so similar in structure to glutamine, suggests that the glutamyl-enzyme is a thioester involving a cysteine residue.

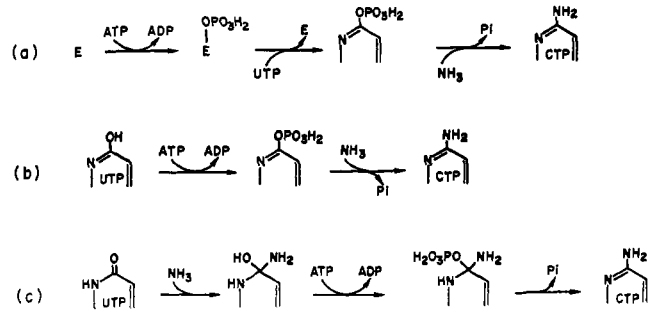


FIGURE 2: Alternative pathways for CTP synthesis.

Evidence for a Phosphorylated UTP Intermediate. Since the amination of UTP is ATP dependent and ATP is cleaved to ADP and P_i during the amination, it seemed logical to look for a phosphorylated intermediate. Three possible mechanisms seemed logical (Figure 2): (a) a phosphorylated enzyme intermediate, (b) activation of UTP by ATP followed by displacement by NH_3, and (c) amination of UTP followed by activation and elimination of P_i. Mechanism a requires an ADP–ATP-exchange reaction independent of UTP; mechanism b an ADP–ATP exchange requiring UTP be present. Neither is observed. Actually as seen in Table II there is a very slow ADP–ATP exchange which is only 1% of the overall rate and is independent of UTP concentration. Since traces of glutamine were difficult to remove, DON-labeled enzyme was tested also and gave even less exchange even though its

TABLE II: ADP–ATP-Exchange Rate.[a]

Enzyme Preparation	Added Ligand	Exchange Rate (Moles of ADP Exchanged/ Mole of Active Sites per min)
DON-modified enzyme		1.20
DON-modified enzyme	UTP	1.10
Native		0.068
Native	CTP	0.071
Turnover number (k_{cat}) at 25°		61

[a] Conditions: the reaction mixture contained 7.96×10^{-4} M [^{3}H]ADP, 7.5×10^{-4} M ATP, 7.5×10^{-4} UTP, 0.01 M $MgCl_2$, 0.02 M Borax–NaOH (pH 9.3), and 0.04 unit of native or DON-labeled CTP-synthetase which was free of glutamine and ammonia. The incubation was performed at 25°. All solutions were prepared from boiled distilled water (pH 9.0) through which nitrogen was bubbled to remove traces of ammonia. At different time intervals a sample was withdrawn and incubated at 100° (boiling water) to inactivate the enzyme. Then Dowex 50 was added to remove Mg^{2+}. Samples were applied to tlc plates of PEI-cellulose and run with 2 M LiCl in 2 M HCO_2H. The nucleotide spots were identified under a uv lamp using appropriate markers and the ATP and ADP spots were counted in 5 ml of toluene scintillation liquid containing omniflor with 5% Biosolv BBS-3. No CTP was formed even after 10.5 hr. The exchange rate was calculated as originally described by Duffield and Calvin (1946) and later by Wahl and Bonner (1951).

TABLE III: Effect of ATP Analogs on CTP-synthetase.[a]

ATP Analogs	Type of Inhibition	Diss Constant at 25°	Rate of Glutamine Cleavage (Moles of NH_3 Released/Mole of Enzyme per min (k_{cat})	Formation of CTP
β,γ-CH_2-ATP	Competitive	$K_i = 6.6 \times 10^{-4}$	145	No
ADPNP	Competitive	$K_i = 3.3 \times 10^{-4}$	150	No
ATP[b]		$K_D = 3.0 \times 10^{-4}$	150	Yes

[a] Conditions were as follows. Inhibition studies: the assay mixture contained 0.75 mM UTP, 10 mM $MgCl_2$, 20 mM acetate (pH 7.2), 6 mM glutamine, 0.1–0.7 mM ATP, and varying concentrations of the analog or ATP. K_i's were calculated from double-reciprocal plots. Activity studies: in the presence of ATP the rate of glutamine cleavage was assumed equal to the rate of CTP synthesis. [b] Direct binding measurement at 25° (A. Levitzki and D. E. Koshland, Jr., in preparation).

NH_3 reaction is unaffected by the DON. This leaves the third alternative or the possibility that there is no phosphorylated UTP intermediate.

The presence of a phosphorylated UTP was demonstrated by ^{18}O experiments. Any of the mechanisms shown above in which the OH of the UTP is phosphorylated and then displaced by NH_3 should give ^{18}O-labeled P_i if ^{18}O is incorporated into the 4′ position of UTP. ^{18}O-labeled UTP was incubated with the reaction mixture (75 μmoles of ATP, 50 μmoles of [^{18}O]UTP, 15 μmoles of GTP, and 36 μmoles of glutamine). P_i (60 μmoles) was obtained (Fiske and Subbarow, 1925) and roughly equivalent amounts of ADP (50 μmoles) and CTP (50 μmoles). All ultraviolet-absorbing material was removed with charcoal and the P_i was then converted into KH_2PO_4 (Boyer *et al.*, 1961; Sartorelli *et al.*, 1966) for ^{18}O assay. The results showed 0.6 ^{18}O atom was incorporated, slightly less than the 1.0 to be expected for quantitative conversion. In view of the possibility of exchange with the medium [^{16}O]H_2O, and contamination with unlabeled phosphate 0.6 is quite close to the expected value. The ^{18}O of UTP has been transferred to the P_i originally present as the terminal phosphate in ATP. Hence, a phosphorylated UTP intermediate is formed and mechanism c seems to be the preferred alternative.

A tetrahedral adduct as shown in mechanism c would be highly unstable. Its existence should be transitory if it exists. The alternatives, a NH_3-induced conformation change leading to the phosphorylation of UTP followed by immediate NH_3 attack or a concerted process are not excluded. All include a special role for NH_3 in generating this step and the adduct seems the most likely but certainly is not established.

Reversibility of the CTP-synthetase Reaction. The extent to which the CTP-synthetase reaction is reversible was tested (eq 1) by incubating 10^{-3} M [^{14}C]CTP (12 μCi mmole), 0.01 M P_i, 10^{-3} M ADP, and 0.01 M $MgCl_2$ in 0.02 M imidazole acetate (pH 7.2) with 0.1 unit of CTP-synthetase at 37°. No decrease at 291 mμ was detected and no [^{14}C]UTP formation could be found, even after 1-hr incubation. It seemed therefore that under the conditions used, the equilibrium constant lies almost completely in the direction of CTP synthesis.

Role of Glutamine and Ammonia. The overall stoichiometry of the reaction does not answer two problems in regard to the relations between glutamine and ammonia. The first is whether the ammonia released from glutamine reacts directly in nascent form or equilibrates as free ammonia in solution. The second is whether the glutamine and ammonia sites are separate or overlapping.

One step in answering the first problem was to determine whether the release of ammonia in the glutamine reaction was sufficiently rapid to allow the overall synthesis of CTP. Thus, it became essential to separate the glutamine hydrolysis step from the overall CTP synthesis. The possibility of blocking CTP synthesis while allowing glutamylation, by replacing ATP with ATP analogs which cannot be hydrolyzed, was investigated.

Two compounds were tested for this role as shown in Table III—the nitrogen analog of ATP (ADPNP) prepared by Yount *et al.* (1971) and the CH_2 analog (ADPCH_2P) of Moos *et al.* (1960). It can be seen that the ADPNP had a K_I essentially equal to the K_M of ATP and led to no formation of CTP. The methylene analog also acted as an inhibitor but did not appear to be as analogous to ATP.

The nitrogen analog which Yount has shown to be isosteric with ATP (Yount *et al.*, 1971) was therefore used. The results are shown in Table IV. It is readily seen that in the presence of both substrates and allosteric effectors the overall rate of glutamine hydrolysis is very high and equal in rate to the synthesis of CTP. Apparently the ADPNP activates the protein so that glutamylation provides ammonia at a rate sufficient for the normal rate of the total reaction.

It remains to decide whether the ammonia mixes with the solution as a whole and then returns to the active site or if it

TABLE IV: Rates of NH_3 Production and CTP Formation by CTP-synthetase.[a]

ATP Compound Present	Product Measured	Moles of Product Formed/min per Mole of Enzyme Sites
ATP	CTP	150
ADPNP	NH_3	145

[a] Conditions: reaction mixture contained 0.01 M Mg^{2+}, 1×10^{-3} M GTP, 1×10^{-3} M UTP, 0.01 M glutamine, and 1×10^{-3} M ATP or ADPNP. The buffer was 0.02 M imidazole acetate at pH 7.2. The enzyme (0.75 unit) was added after the reaction mixture had been incubated for 10 min at 38°. CTP was measured by spectral absorption. NH_3 was measured by the enzyme assay procedure (Long and Pardee, 1967).

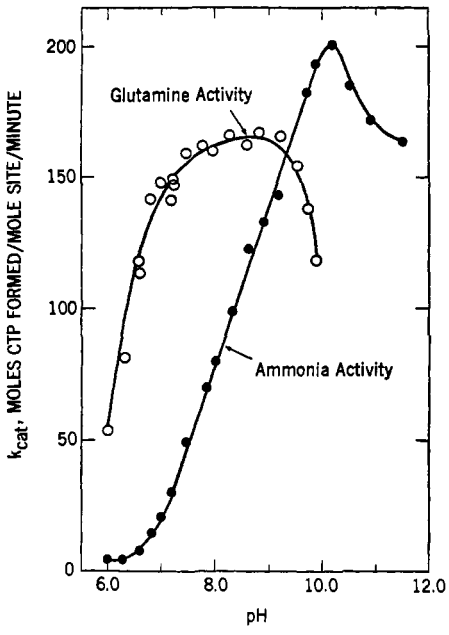


FIGURE 3: pH–activity profile of CTP-synthetase. The glutamine activity was measured in the presence of saturating concentrations of ligands: 3×10^{-4} M GTP, 0.01 M glutamine, 7.5×10^{-4} M ATP, 7.5×10^{-4} M UTP, and 0.01 M $MgCl_2$ in 0.02 M buffer. The ammonia activity was measured under the same conditions with 0.02 M $(NH_4)_2SO_4$ substituting for glutamine and in the absence of GTP. All assays were performed in a final volume of 1.0 ml at 38° in a Gilford 2000 spectrophotometer.

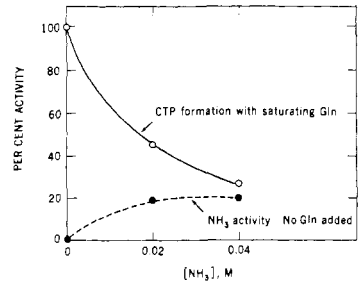


FIGURE 4: Inhibition of the glutamine activity by ammonia. The reaction mixture contained: 7.5×10^{-4} M ATP, 7.5×10^{-4} M UTP, 3×10^{-4} M GTP, 0.01 M $MgCl_2$ in 0.02 M imidazole acetate (pH 7.2), and increasing concentrations of $(NH_4)_2SO_4$. Assays were performed as usual at 38°.

reacts at the active site as it is released from glutamine. This was resolved by the help of the pH studies shown in Figure 2. The glutamine reaction in the presence of 3×10^{-4} M GTP shows activity rising to a maximum at approximately pH 7.5 and 9.3 and then falling largely due to inactivation of the enzyme. The pH dependence of the ammonia activity (Figure 3) rises steadily in the pH region 7–10. A maximum is observed (pH 10.3–10.4) but this is coincidental with rapid denaturation of the protein and therefore this maximum may be an artifact of this denaturation. These results lead to the conclusions: (a) that the pH profile for ammonia differs markedly from that of glutamine, (b) that the pH profile for the ammonia reaction is consistent with an argument that the main reactant is ammonia either because the free base NH_3 form is more reactive or is bound more readily to the active site, and (c) that the maximum activity with ammonia is greater than the maximal activity with glutamine but at low pH values the maximum reactivity with glutamine is far greater than that of ammonia.

If one now compares the velocity of reaction at pH 7.2 where 150 μmoles of CTP is formed per min in the glutamine reaction and 27 μmoles per min in the ammonia reaction, it is possible immediately to tell whether "nascent" or "free" ammonia is used in the glutamine reaction. The reactions were measured to the extent of only 5% in order to get initial velocities. Assuming that 5% of the glutamine reacts instantaneously, the concentration of ammonia in the solution would be 5×10^{-4} M. Since the NH_3 reaction is measured at a linear region of NH_3 concentration, one can calculate that the velocity to be expected for this NH_3 concentration would be $(27 \times 5 \times 10^{-4}/20 \times 10^{-3})$ 0.68 mole of CTP/min per mole of active sites.[2] Thus, if all ammonia were produced instantaneously from glutamine, and this reacted at the normal rate for the ammonia reaction, one would have a rate which is 0.68/150 of the observed rate of the glutamine reaction. This clearly shows that the ammonia could not be liberated into the solution, then reabsorbed on the active site prior to reaction. It must mean that the ammonia released during the glutamylation of the enzyme is delivered as *nascent ammonia* to the active site and there reacts with the UTP without equilibrating with the solution. This finding furthermore agrees with the observation that no traces of free ammonia were found in the solution during the glutamine reaction.

The above experiment led to the conclusion that glutamylation of the enzyme liberates NH_3 which immediately reacts with UTP. The next question is whether a single NH_3 site is common to both the NH_3 reacting from solution and the nascent NH_3 produced by glutamine hydrolysis. To throw light on this aspect the effect of ammonia on glutamine reaction and the effect of glutamine on the ammonia reaction were studied. If the two reactions were carried out at remote independent sites when ATP, UTP, and GTP were in abundance, the rate of CTP synthesis would be the sum of the NH_3 and the glutamine rates. At pH 9.25 where the turnover of each of the processes is 150 moles of CTP/mole of enzyme site per minute the rate of CTP synthesis in the presence of both substrates was found to be 150 moles of CTP/mole of enzyme site per minute, indicating the reactions were competitive, not additive.

In order to eliminate the possibility that an indirect interaction between separated ammonia sites and glutamine sites occurred, the effect of ammonia concentration on the glutamine reaction was studied at pH 7.2 where the ammonia reaction is only 18% of the glutamine reaction. The results are shown in Figure 4. It is seen that the glutamine reaction is inhibited by ammonia and the rate of CTP synthetase at high ammonia concentrations eventually equals the maximal rate for ammonia reaction at that particular pH. These data indicate that there is a single site for the external ammonia and the ammonia produced from glutamine. At this pH ammonia is exclusively in the form of NH_4^+. Thus, the external NH_4 competes with the nascent unprotonated ammonia released from glutamine. It seems therefore that the rate of reaction with NH_4^+ is slower than with unprotonated ammonia which is at low concentration at pH 7.2.

Discussion

The experiments reported above outline the covalent changes occurring during the conversion of UTP to CTP by

[2] 20×10^{-3} is the K_m value for ammonia at pH 7.2.

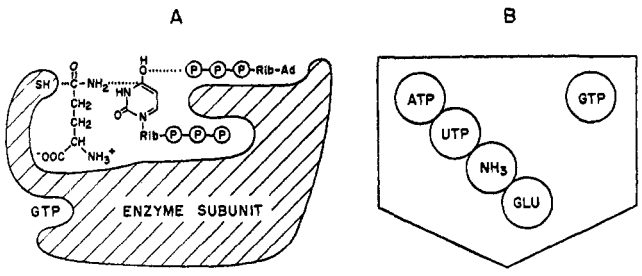
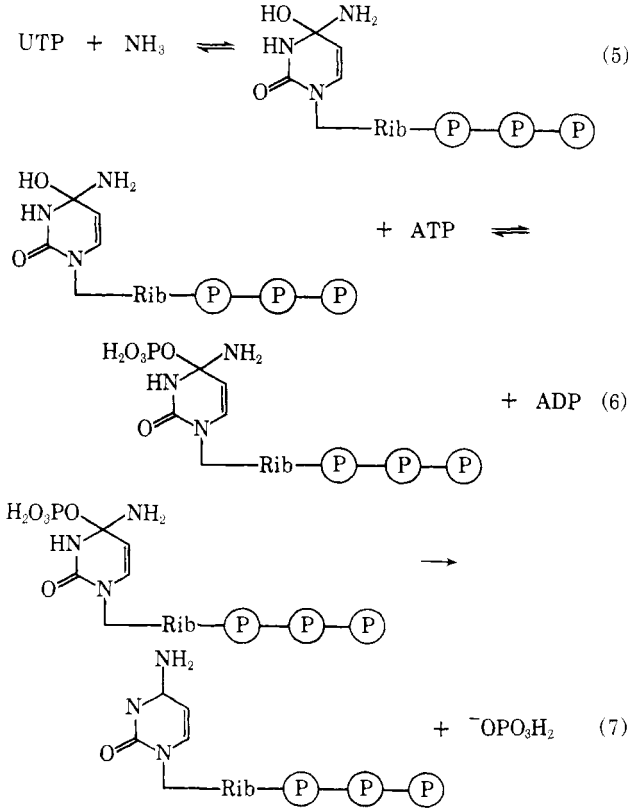


FIGURE 5: Schematic illustration of active and allosteric sites of CTP-synthetase. Arranged so that sequence of reactions of eq 3–7 can be executed with minimal rearrangement of substrates. The arrangement of substrates is necessitated by the activation relationship between ATP, UTP, the roles of glutamine and NH_3, and the absence of the ADP–ATP exchange (see text). The position of the GTP site in relation to the others is illustrative. There is no evidence to assign its distance from the glutamine site.

the enzyme CTP synthetase (*cf.* eq 3–7) and the geometric arrangement allowing these reactions is shown schematically in Figure 5.

$$\text{E} + \text{glutamine} \longrightarrow \text{glutamyl-enzyme} + \text{NH}_3 \qquad (3)$$

$$\text{glutamyl-enzyme} + \text{H}_2\text{O} \longrightarrow \text{glutamate} + \text{enzyme} \qquad (4)$$



In step 1 shown in eq 3 glutamine reacts with the enzyme to form a glutamyl-enzyme intermediate and liberates nascent ammonia. The evidence for step 1 is obtained from the formation of the covalent intermediate which can be observed in the absence of any substrates or in the presence of all the substrates. The use of ADPNP allowed one to observe the glutaminase stage of the reaction without completion of the overall reaction. In eq 5 UTP reacts with ammonia to form a tetrahedral adduct. Subsequent to the amination of UTP, the adduct reacts with ATP to form a phosphorylated tetrahedral intermediate (eq 6). The phosphorylated 4′-hydroxyl is an excellent leaving group and the phosphorylated intermediate is converted, practically in an irreversible fashion, to CTP and phosphate. The fact that the ADP–ATP-exchange rate is at most 1% of the overall rate and is not facilitated by UTP indicates that neither a phosphorylated enzyme intermediate nor a phosphorylated UTP species are formed in the absence of glutamine or ammonia. However, the incorporation of the 4′-hydroxyl oxygen of UTP into the P_i originating from the terminal phosphate of ATP proves the existence of a phosphorylated UTP species. The two observations taken together indicate that the phosphorylation of the 4′-hydroxyl in UTP occurs only in the presence of ammonia or glutamine. The tetrahedral adduct is a possible hypothesis but the evidence for it is largely the lesser probability of alternative mechanisms. A conformational change induced by NH_3 leading to the phosphorylation of UTP or a concerted process could also explain the lack of exchange. All three mechanisms involve a series of steps triggered by NH_3, but hard evidence for one alternative is still lacking.

The ammonia reacting with UTP on the enzyme surface may originate as "nascent ammonia" from glutamine hydrolysis or may originate from the solution when ammonia is used as a substrate. Evidence that glutamine reaction occurs though a nascent ammonia is obtained from the facts that (a) the glutamylation of the enzyme proceeds at a rate which is slightly faster than the overall reaction, (b) the glutaminase rate is higher than the NH_3 rate at pH 7.2, and (c) no free NH_3 is found in solution during the glutamine reaction. The pH dependence of the reaction suggests that NH_3, not NH_4^+, is the reactive agent in the NH_3 reaction. The precise species in the glutamine reaction is unknown but it is logical that it would be NH_3 in this case also as the sites are the same.

In eq 3–7 are the covalent changes which occur but the writing of these steps separately should not be taken to mean that they occur in a distinct stepwise manner. The high velocity with glutamine indicates that the nascent NH_3 immediately reacts with UTP. The lack of ADP–ATP exchange indicates an almost instantaneous reaction of the H_2N–UTP adduct to the phosphorylated compound. The finding that the maximal turnover for CTP formation is higher for the ammonia reaction than the glutamine reaction indicates that the rate-limiting step in the glutamine reaction may be the hydrolysis of the glutamyl-enzyme intermediate.

This enzyme is of some interest because it is similar to a large number of enzymes (Mizobuchi *et al.*, 1968; Hartman, 1963; Khedouri *et al.*, 1966), which use either glutamine or NH_3 as a nitrogen source. The other enzymes show great similarity to CTP-synthetase in their chemistry, specificity, and reaction with DON. It would seem therefore that the conclusions reached here in regard to the glutamyl-enzyme intermediate and the common site of NH_3 and glutamine are likely to be general. One might question why organisms would preserve the capacity to use two nitrogen sources for so many enzymes in so many organisms. The fact of a common NH_3 site for both substrates explains this observation.

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Half-of-the-Sites Reactivity and the Conformational States of Cytidine Triphosphate Synthetase*

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ABSTRACT: The affinity label 6-diazo-5-oxonorleucine (DON) reacts with only half of the total glutamine sites of CTP-synthetase, even though the subunits are identical. The binding of DON to half of the subunits abolishes the glutamine activity of the enzyme; thus, vacant active sites as well as occupied sites are turned off. However, the DON-labeled enzyme retains its overall activity with ammonia and its characteristic binding of other ligands such as ATP, UTP, and GTP. It is postulated that DON labeling is an example of extreme or absolute negative cooperativity in which the conformational change induced by DON in the covalently labeled subunit is transmitted to the neighboring subunit so that no DON reaction can occur there. This model provides a general explanation for other enzymes in which half-of-the-sites reactivity has been observed. The fact that the DON-induced change can abolish glutamine activity while leaving ammonia activity and nucleotide binding unaffected indicates that the protein is designed to allow specific transmission of conformational changes.

An increasing number of enzymes have been shown to have "half-of-the-sites reactivity." By this we mean that these proteins are composed of identical subunits with n potential sites and yet they react with a substrate or an inhibitor so that only $n/2$ sites are occupied when the enzyme is saturated with that ligand. A summary of enzymes which exhibit this phenomenon is shown in Table I. The phenomenon seems to originate from the architecture of the multisubunit enzymes exhibiting the property and therefore its analysis may reveal clues to protein design.

CTP-synthetase[1] is a particularly attractive example with which to pursue this problem, since the chemistry of its interactions has been elucidated (Levitzki and Koshland, 1971) and since it was shown to exhibit an apparent half-of-the-sites reactivity with the affinity label, 6-diazo-5-oxonorleucine (DON) (Long *et al.*, 1970). Accordingly, the reactivity of DON with CTP-synthetase in the dimer and tetramer forms and the reactivity of this affinity label with the protein containing varying amounts of substrates and activators were studied. The results not only clarify the nature of half-of-the-sites reactivity, but reveal an interesting mosaic of interactions between the different ligand sites within a single subunit and between different subunits.

Materials and Methods

CTP-synthetase was prepared and assayed as described earlier (Long *et al.*, 1970; Levitzki and Koshland, 1970). DON was obtained from Parke-Davis. Chemicals were of the

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[1] Abbreviations used are: CTP, cytidine 5'-triphosphate; DON, 6-diazo-5-oxonorleucine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); CTPS, CTP-synthetase; F_2DPS, *p,p'*-difluoro-*m,m'*-dinitrophenylsulfone.