Investigation of the Mechanism of CTP Synthetase Using Rapid Quench and Isotope Partitioning Methods†

Deborah A. Lewis and Joseph J. Villafranca*

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received April 12, 1989; Revised Manuscript Received June 28, 1989

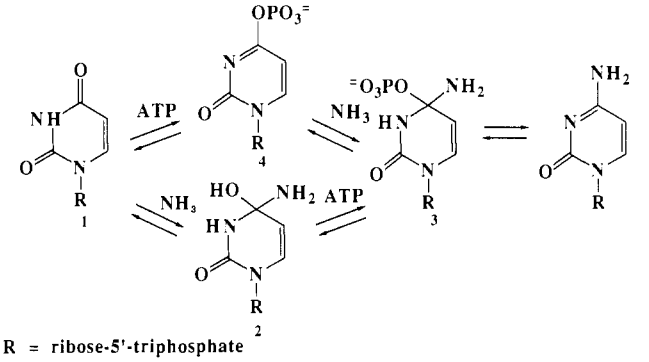
ABSTRACT: The UTP-dependent ATPase reaction and the glutamine-dependent overall reaction of *Escherichia coli* CTP synthetase have been studied by rapid quench and isotope partitioning kinetics. The effect of GTP, an allosteric effector, on the pre-steady-state kinetics of both reactions has also been examined. The time courses of the UTP-dependent ATPase reaction in the presence and absence of GTP are both characterized by a burst of acid-labile phosphate equivalent to 0.93 and 0.43 subunits, respectively. The time course of the glutamine-dependent reaction in the absence of GTP is also characterized by a burst of acid-labile phosphate corresponding to 0.8 subunit; however, in the presence of GTP, no burst was observed. These results along with positional isotope exchange experiments [von der Saal, W., Anderson, P. M., & Villafranca, J. J. (1985) *J. Biol. Chem. 260*, 14997] provide evidence that the mechanism of CTP formation involves phosphorylation of UTP followed by attack of NH_3, and finally release of phosphate, producing CTP, ADP, and P_i. A kinetic model for the first stages of the enzymatic reaction was developed from the rapid quench data, and the internal equilibrium constant for the formation of the phosphorylated UTP intermediate was determined. The internal equilibrium constants for the UTP-dependent reaction in the presence and absence of GTP were found to be 1.1 and 18, respectively. By contrast, the internal equilibrium constant for the reaction in the presence of glutamine was 50. Thus, the presence of glutamine shifts the internal equilibrium constant to favor formation of the phosphorylated UTP intermediate. Isotope partitioning experiments with [U-^{14}C]UTP and [γ-^{32}P]ATP demonstrated that none of the E–UTP could be trapped as CTP and 15% of the E–ATP could be trapped as P_i in the presence of GTP. This demonstrates that ATP and UTP dissociate from their respective binary complexes faster than products are formed.

CTP synthetase from *Escherichia coli* catalyzes the formation of CTP from ATP, UTP, and glutamine (eq 1); ammonia can also serve as the amino donor. In addition, the enzyme also has glutaminase activity (Levitzki & Koshland, 1971) and catalyzes a UTP-dependent ATPase reaction (von der Saal et al., 1985a).

$$\text{ATP} + \text{UTP} + \text{Gln} \rightarrow \text{ADP} + \text{CTP} + \text{Glu} + P_i \quad (1)$$

The mechanism of CTP synthetase was thought to initially proceed by attack of NH_3 (alone or generated from glutamine) on the 4-carbon atom of UTP to yield **2**, followed by phosphoryl transfer from ATP to the hydroxyl group of **2**, producing **3**; then phosphate is released, forming CTP (lower pathway, Scheme I) (Levitzki & Koshland, 1974). This mechanism was based on the lack of ATP–ADP exchange in the presence and absence of UTP (Levitzki & Koshland, 1971). Evidence for the formation of a phosphorylated intermediate was also provided by the work of Levitzki and Koshland (1971), in which the enzyme was found to catalyze the transfer of ^{18}O from [4-^{18}O]UTP to P_i. These two experiments taken together lead to the proposal of the mechanism given in the lower pathway of Scheme I. However, these experiments do not provide sufficient evidence to unequivocally prove the mechanism shown in the lower pathway of Scheme I, since exchange experiments require that substrates and products dissociate from the enzyme for the exchange to occur. Therefore, von der Saal et al. (1985a,b) carried out positional isotope exchange experiments to obtain more information on the mechanism of CTP formation. The results of positional isotope exchange experiments lead to the mechanism shown in the upper pathway of Scheme I: phosphorylation of UTP at the 4-oxygen atom by ATP, producing **4**, followed by attack of NH_3, producing **3**, and finally release of phosphate, producing CTP, P_i, and ADP. This mechanism was based on the observation of positional isotope exchange with ATP which was observed only in the absence of NH_3. Additional experiments in which the positional isotope exchange was found to be independent of the UTP concentration above saturating concentrations suggested that the order of addition of substrates is UTP followed by ATP and finally NH_3.

Scheme I



As previously indicated, both glutamine and ammonia can serve as the amino donor; therefore, CTP synthetase belongs to a group of enzymes known as glutamine amidotransferases. The glutamine amidotransferases share many common properties including the ability to use either glutamine or NH_3 as the amino donor, selective inactivation of glutamine-dependent activity by alkylation of an active-site cysteine with glutamine

† This work was supported by NSF Grant DMB-8943447.

* Correspondence should be addressed to this author.

0006-2960/89/0428-8454$01.50/0

affinity analogues, and glutaminase activity (Zalkin, 1985). The reaction of CTP synthetase (Levitzki & Koshland, 1971) and other glutamine amidotransferases has been shown to proceed through a covalent glutamyl-enzyme intermediate followed by hydrolysis of the intermediate to form glutamate and NH_3. The NH_3 is then used in the amination reaction. GTP is a positive allosteric effector of the glutamine-dependent reaction while having no effect on the NH_3-dependent reaction. The k_{cat} values of both the glutaminase reaction and the glutamine-dependent CTP formation reaction are increased by GTP, but this allosteric effector decreases K_{Gln} while having no effect on K_{NH_3}.

CTP synthetase was found to display several types of cooperativity. As an example, positive cooperativity was observed with respect to both ATP and UTP; however, Michaelis–Menten kinetics were observed for either nucleotide, if the other nucleotide is saturating (Long & Pardee, 1967). Michaelis–Menten kinetics were also observed with respect to glutamine and NH_3. The kinetic mechanism is not currently known since the cooperativity displayed by the enzyme makes it difficult to determine the kinetic mechanism by initial velocity methods.

This paper describes rapid quench and isotope partitioning experiments that were carried out to obtain information on the mechanism of action of CTP synthetase. Rapid quench experiments of the UTP-dependent ATPase reaction and glutamine-dependent reaction were performed to obtain more evidence for the existence of the phosphorylated UTP intermediate proposed as a result of positional isotope exchange experiments and to measure the rate of formation of the putative phosphorylated UTP intermediate. Isotope partitioning experiments were carried out to determine the order of addition of substrates. The effect of the allosteric effector GTP on both the rapid quench experiments and isotope partitioning experiments was also examined.

Experimental Procedures

General Materials. [γ-^{32}P]ATP (10–50 Ci/mmol) and [U-^{14}C]UTP (350 mCi/mmol) were obtained from New England Nuclear. 5′-Adenylyl imidodiphosphate (AMP-PNP)[1] was obtained from Boehringer Mannheim. All other biochemicals were from Sigma.

Enzyme. CTP synthetase was purified from *Escherichia coli* strain HB101 harboring the plasmid pMW5 by the following modification of the procedures of Long and Pardee (1967), Long and Koshland (1978), and Anderson (1983). The HB101/PMW5 strain was grown as described by Weng et al. (1985). All subsequent purification steps were carried out at 4 °C.

In a typical purification, 50 g of cells suspended in 250 mL of 0.2 M potassium phosphate buffer, pH 8.0, containing 1 mM EDTA and 2 mM glutamine was sonicated for five 3-min bursts using a Heat Systems–Ultrasonics Inc. sonicator at the highest setting. Cell debris was removed by centrifugation at 20000*g* for 30 min. Nucleic acids were precipitated by the slow addition of 40 mL of a 2% solution of protamine sulfate, followed by centrifugation at 20000*g* for 30 min. Protein was precipitated by the slow addition of ammonium sulfate (28.8 g/100 mL of supernatant) with stirring. The mixture was stirred for an additional 15 min and then centrifuged for 30 min at 20000*g*. The pellet was resuspended in a small volume (~125 mL) of 10 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA, 2 mM DTT, and 4 mM glutamine (buffer A). Ammonium sulfate was removed by dialysis against 4 L of buffer A with one change followed by ion-exchange chromatography on a 2.5 × 40 cm bed of DEAE-cellulose (Whatman DE 53) that had been equilibrated with buffer A. The column was washed with buffer A until the absorbance at 280 nm was 0 (~250 mL); the enzyme was then eluted with an 800-mL linear gradient of 0–0.15 M ammonium sulfate in buffer A. The sharp peak of enzyme activity which eluted near the end of the gradient was collected, and potassium phosphate was added to a final concentration of 100 mM. The protein was then precipitated by the slow addition of ammonium sulfate (40 g/100 mL) and collected by centrifugation (20000*g*, 30 min). The protein pellet was resuspended in ~20 mL of 100 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA, 2 mM DTT, 4 mM glutamine, 0.75 mM ATP, 0.75 mM UTP, and 10 mM $MgCl_2$ (buffer B). The concentrated protein solution was divided into 10-mL aliquots, and each was placed on a 2.5 × 40 cm bed of Bio-Gel A 0.5m that had been equilibrated with buffer B. The enzyme was eluted with buffer B, and the fractions containing maximum enzyme activity were pooled and concentrated using ammonium sulfate (40 g/100 mL), as described above. The resulting pellet was resuspended in 10 mL of buffer B minus the nucleotides and $MgCl_2$. This solution was then placed on the Bio-Gel column, and the column was eluted with buffer B minus nucleotides and $MgCl_2$. The fractions containing maximum activity were concentrated in the same manner as previously described, and the pure protein was resuspended in ~20 mL of 0.2 M Hepes buffer, pH 8.0, containing 1 mM EDTA, 2 mM DTT, 10 mM $MgCl_2$, and 20% glycerol (buffer C). The protein was dialyzed against 4 L of buffer C with one change. Then UTP and ATP were added to final concentrations of 2.5 mM each. The enzyme was frozen in liquid nitrogen and stored at −80 °C.

Properties of CTP Synthetase. CTP synthetase from *Escherichia coli* purified as described above gave a yield of 40% and a specific activity of 6.7 μmol of CTP min^{-1} mg^{-1} following the procedures described above. The k_{cat} value of 5.1 s^{-1} for the glutamine-dependent reaction in the presence of GTP (Table I) is comparable to the k_{cat} value calculated from the purification data of Anderson (1983). The enzyme was homogeneous as determined by SDS gel electrophoresis with a molecular weight of 60000. Protein concentration was determined by measuring the absorbance at 280 nm using $E^{280}_{0.1\%}$ = 0.89 that had been determined on the basis of a Lowry protein determination (Levitzki & Koshland, 1972b). Spectroscopic determination of the extinction coefficient by the method of Edelhoch (1967) confirmed the above extinction coefficient. Additionally, Manli et al. (1986) reported a comparable value for the extinction coefficient as determined by amino acid analysis.

Standard Assay. Enzyme activity was measured at 37 °C using a continuous spectrophotometric assay by following the increase in absorbance at 291 nm ($\Delta\epsilon$ = 1338 M^{-1} cm^{-1}) which is due to the conversion of UTP to CTP (Long & Pardee, 1967). The standard assay mixture consisted of 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 20 mM NH_4Cl, 1 mM ATP, 1 mM UTP, and 10 mM $MgCl_2$. The k_{cat} for all reactions was calculated on the basis of a molecular weight of 60300.

Steady-State Kinetics. The kinetic parameters for glutamine were determined at 25 °C by using the continuous

[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography.

spectrophotometric assay with reaction mixtures consisting of 70 mM Hepes buffer, pH 8.0, 0.5 mM EDTA, 10 mM $MgCl_2$, 1 mM ATP, 1 mM UTP, and 0.1–2 mM glutamine. When the effect of GTP was studied, 0.2 mM GTP was present in the assay mixture. The kinetic parameters for ATP and UTP were determined by using the following assay at 25 °C. When ATP was the variable substrate, reaction mixtures consisted of 70 mM Hepes buffer, pH 8.0, 0.5 mM EDTA, 10 mM $MgCl_2$, 1 mM UTP, 10 mM glutamine, 136 000 cpm of [γ-^{32}P]ATP, and 6–170 μM ATP. UTP concentration was varied between 6 and 198 μM when it was the variable substrate, and ATP concentration was held constant at 1 mM. GTP was present at 0.2 mM when it was present in the assay mixture. Reactions were incubated 1–4 min at 25 °C and quenched by the addition of 0.750 mL of 1.3 N HCl followed by neutralization with 0.150 mL of 1 M Tris buffer containing 4 N KOH. [^{32}P]P_i was isolated according to the method of Johnson (1986).

Rapid Quench Experiments. The rapid quench experiments were performed at 25 °C on an apparatus designed and built by Johnson (1986). The reactions were initiated by the simultaneous mixing of two solutions; one contained enzyme (0.045 mL), and the other contained substrates (0.045 mL). The final reaction mixture for glutamine-dependent reactions consisted of 70 mM Hepes buffer, pH 8.0, 0.5 mM EDTA, [γ-^{32}P]ATP (60 000 cpm), 1 mM ATP, 1 mM UTP, 10 mM glutamine, 10 mM $MgCl_2$, and 20 μM CTP synthetase. When GTP was included, it was present at a concentration of 0.2 mM. The reactions were quenched with 0.045 mL 0.5 N HCl and then neutralized with 0.050 mL of 1 M Tris containing 4 N KOH. [^{32}P]P_i was isolated as described by Johnson (1986). The UTP-dependent ATPase reactions were carried out in the same manner except glutamine was omitted. Manual rapid quench reactions were carried out in a similar manner except the reaction volume was 0.2 mL, and the reaction was quenched manually by the addition of 0.4 mL of 0.5 N HCl.

Isotope Partitioning Experiments with ATP. Isotope partitioning experiments with ATP were carried out at 25 °C. A 75-μL solution of Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 7 mM [γ-^{32}P]ATP (437 cpm/nmol), 10 mM $MgCl_2$, and 14.5 nmol of CTP synthetase was added to a rapidly stirred 1.5-mL chase solution of 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 14 mM ATP, 1 mM UTP, 10 mM glutamine, and 16 mM $MgCl_2$. The reaction was quenched after 3 s by the addition of 0.375 mL of 4 N HCl and then neutralized with 0.450 mL of 1 M Tris buffer containing 4 N KOH. A blank was run in which the [γ-^{32}P]ATP was added to the chase solution. A control to account for contaminating ATPase activity was also done in which the chase solution contained buffer only. When the isotope partitioning experiment was carried out in the presence of GTP, 0.2 mM GTP was present in the chase solution.

Isotope Partitioning Experiments Using [^{14}C]UTP. Isotope partitioning experiments with UTP were carried out at 25 °C. A 50-μL solution of 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 7 mM [U-^{14}C]UTP (1000 cpm/nmol), 10 mM $MgCl_2$, and 14.3 nmol of CTP synthetase was added to a rapidly stirred 1.5-mL chase solution of 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 14 mM UTP, 1 mM ATP, 16 mM $MgCl_2$, and 10 mM glutamine. The reaction was quenched after 3 s by the addition of 150 μL of 3 N HCl. [C-^{14}C]CTP was isolated by ion-exchange chromatography as described below. When the isotope partitioning experiment was done in the presence of GTP, it was added to the chase solution at a final concentration of 0.2 mM.

In experiments that included both UTP and ATP in the pulse solution, the following protocol was used: a 75-μL solution of 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 1 mM [U-^{14}C]UTP (4733 cpm/nmol), 1.5 mM ATP, 10 mM $MgCl_2$, and 3 nmol of CTP synthetase was incubated for 2 min at 25 °C and then added to a rapidly stirred 1.5-mL chase solution of 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA, 24 mM UTP, 1 mM ATP, 1 mM $MgCl_2$ and 10 mM glutamine. The reaction was quenched after 3 s by the addition of 150 μL of 3 N HCl. [C-^{14}C]CTP was isolated by ion-exchange chromatography as described below. A control to account for contaminating NH_3 was done in which glutamine was left out of the chase solution. As before, GTP was added to the chase solution at a final concentration of 0.2 mM, when its effect was studied. When AMP-PNP was present, it replaced ATP in the pulse solution at a concentration of 1.5 mM.

Purification of [γ-^{32}P]ATP. Commercial [γ-^{32}P]ATP was purified by using a Waters HPLC equipped with a Pharmacia Mono Q HR 5/5 column. A gradient of 0.1–0.5 M triethylammonium bicarbonate was used at a rate of 6.6% min^{-1} with a flow rate of 1.5 min^{-1}. The purified [γ-^{32}P]ATP which eluted at 18 min was diluted with 1 mL of deionized H_2O and then lyophilized. The lyophilized sample was dissolved in 200 μL of 70 mM Hepes buffer, pH 8.0, containing 0.5 mM EDTA and stored at −20 °C. The purification resulted in [γ-^{32}P]ATP which typically contained less than 1% P_i as determined by the method of Johnson (1986).

Isolation of [C-^{14}C]CTP. [C-^{14}C]CTP was isolated from quenched reaction mixtures by the following modification of the method of Savage and Weinfield (1970). The quenched reaction mixtures were boiled for 1 h to hydrolyze CTP to CMP. One-milliliter aliquots from each sample were diluted to 10 mL with water, and 800 nmol of carrier CMP was added. The samples were then placed on 1 × 5 cm beds of AG 50 W × 8 200–400 mesh that had been equilibrated with 0.1 M acetic acid. The columns were eluted with 3 mL of 0.1 M acetic acid to elute UMP, followed by 6 mL of 1 N NaOH to elute CMP. Three milliliters of the 1 N NaOH wash solution was placed in 15 mL of scintillation fluid and the radioactivity counted. Additional 200-μL aliquots of the boiled samples were placed in 15 mL of scintillation fluid plus 3 mL of water and were counted to obtain values for total radioactivity. CMP is stable throughout this procedure.

Data Analysis. Steady-state kinetic data were analyzed by using the computer program HYPER (Cleland, 1979) as modified for the Macintosh computer. Rapid quench data were fit to eq 2 by using the program RS 1 (BBN Software Products Corp.), in which P = nanomoles of inorganic phosphate, E = nanomoles of CTP synthetase, β = burst amplitide, k_{cat} = steady-state rate constant, λ = transient phase rate constant, and t = time.

$$P/E = \beta(1 - e^{-\lambda t}) + k_{cat}t \quad (2)$$

Results

Steady-State Kinetics. The steady-state kinetic parameters for CTP synthetase were determined with all substrates except one at saturating concentrations (Table I). The v versus [S] plots for all substrates except ATP in the presence of GTP approximated hyperbolas. The $[S]_{0.5}$ value for ATP in the presence of GTP was approximated by a plot of v versus $[ATP]^2$ (Segel, 1975). GTP was found to decrease K_{Gln} and to increase the k_{cat} of the glutamine-dependent reaction, while having no effect on the NH_3-dependent reaction. This is

Table I: Steady-State Kinetic Parameters[a]

	K_{NH_3} (mM)	K_{Gln} (μM)	K_{ATP} (μM)	K_{UTP} (μM)	k_{cat} (s^{-1})
-GTP	30 ± 6.6	520 ± 30	54 ± 8	27 ± 4	0.37 ± 0.01
+GTP	27 ± 3.2	170 ± 20	116 ± 11[b]	71 ± 3	5.1 ± 0.2

[a] Reactions were carried out at 25 °C and pH 8.0 with all substrates except one at saturating concentrations. K_{ATP} and K_{UTP} were determined for the glutamine-dependent reaction. Other conditions are given under Experimental Procedures. [b] The v vs [ATP] plot under these conditions was sigmoidal. $[S]_{0.5}$ for ATP was approximated from a plot of v vs $[ATP]^2$.

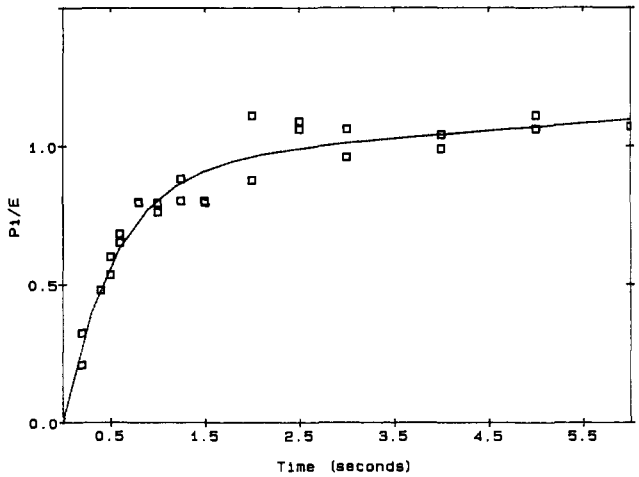


FIGURE 1: Time course of the UTP-dependent ATPase reaction between 0.2 and 6 s. Reaction mixtures consisted of 70 mM Hepes buffer, pH 8.0, 0.5 mM EDTA, 1 mM [γ-^{32}P]ATP, 1 mM UTP, 10 mM $MgCl_2$, and 25 μM CTP synthetase.

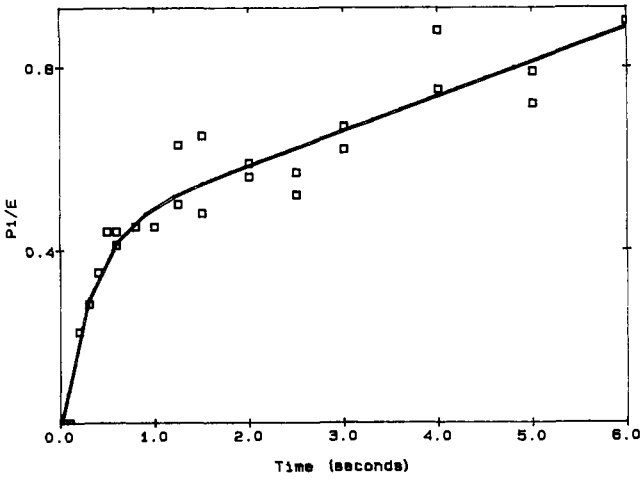


FIGURE 2: Time course of the UTP-dependent ATPase reaction in the presence of GTP. Reaction mixtures consisted of 70 mM Hepes buffer, pH 8.0, 0.5 mM EDTA, 1 mM [γ-^{32}P]ATP, 1 mM UTP, 10 mM $MgCl_2$, 0.2 mM GTP, and 31 μM CTP synthetase.

consistent with the earlier findings of Levitzki and Koshland (1972).

Rapid Quench Experiments. (A) UTP-Dependent ATPase Reaction. The results of the rapid quench experiments of the UTP-dependent ATPase reaction are shown in Figures 1 and 2. The time course for the manually quenched reaction in the absence of GTP between 5 and 30 s (data not shown) is characterized by a burst of acid-labile phosphate equivalent to 0.8 enzyme subunit and a k_{cat} of 0.01 ± 0.003 s^{-1}. In order to observe the transient phase, the rapid quench experiment was carried out using the rapid quench instrument described under Experimental Procedures. The transient phase of the UTP-dependent ATPase reaction (absence of GTP) is shown in Figure 1. The data were fit to a single exponential to obtain

Table II: Pre-Steady-State Kinetics of CTP Synthetase[a]

reaction	burst amplitude	λ (s^{-1})	k_{cat} (s^{-1})
UTP-dependent ATPase	0.93 ± 0.06	1.8 ± 0.2	0.027 ± 0.016
UTP-dependent ATPase + GTP	0.43 ± 0.03	3.2 ± 0.7	0.071 ± 0.01
glutamine-dependent CTP formation	0.80 ± 0.07	3.0 ± 0.5	0.38 ± 0.03

[a] Reactions were carried out at 25 °C and pH 8.0 at saturating concentrations of all substrates as described under Experimental Procedures.

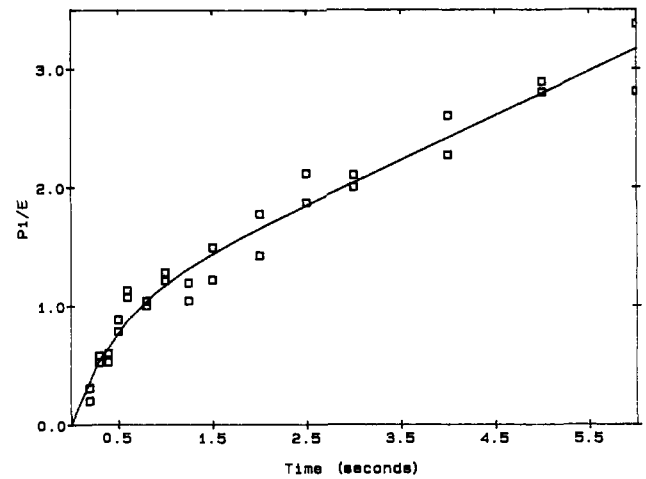


FIGURE 3: Time course of the glutamine-dependent reaction between 0.2 and 6 s. Reaction mixtures consisted of 70 mM Hepes buffer, pH 8.0, 0.5 mM EDTA, 1 mM [γ-^{32}P]ATP, 1 mM UTP, 10 mM $MgCl_2$, 10 mM glutamine, and 20 μM CTP synthetase.

values for the burst height (β), the transient phase rate constant (λ), and k_{cat} (Table II). The time course for the UTP-dependent ATPase reaction in the presence of GTP is also characterized by a burst of acid-labile phosphate followed by a slower steady-state phase (Figure 2). The parameters for this experiment are given in Table II.

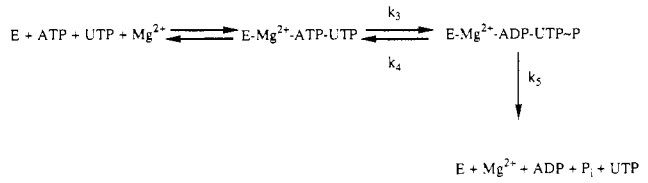
(B) Glutamine-Dependent Reaction. The time course for the manually quenched glutamine-dependent reaction was obtained (data not shown) and clearly showed a burst of acid-labile phosphate but no perceptible transient phase. The k_{cat} obtained was 0.42 ± 0.02 s^{-1}, which compares favorably with the rate constant obtained from steady-state kinetics (Table I).

Rapid quench experiments of the glutamine-dependent reaction were carried out in the presence and absence of GTP. The transient phase of the glutamine-dependent reaction in the absence of GTP is shown in Figure 3. These data were also fit to a single exponential, and as can be seen in Table II, the k_{cat} agrees with the values obtained from the manual quench experiment and steady-state kinetics (Table I).

The rapid quench experiment for the glutamine-dependent reaction in the absence of GTP was obtained between 0.1 and 1.5 s (data not shown) and was characterized by a k_{cat} of 1.6 ± 0.02 s^{-1}.

Isotope Partitioning Experiments. (A) Isotope Partitioning Experiments with Radioactive ATP (ATP).* A solution containing 13.2 nmol of E–ATP* [the concentration of E–ATP was determined by using the binding constant reported by Levitzki and Koshland (1972b)] and 10 mM $MgCl_2$ was added to a chase solution containing a 40-fold excess of unlabeled ATP and saturating concentrations of UTP, glutamine, and $MgCl_2$ (see Experimental Procedures). After termination of the reaction and product analysis, none of the E–ATP* was trapped as P_i*. When GTP was included in the chase solution,

Scheme II



15% of the initially bound MgATP was trapped as P_i*.

(B) Isotope Partitioning Experiments with Radioactitive UTP (UTP).* A solution containing 14.7 nmol of E–UTP* (the concentration of E–UTP* was determined in the same manner as described above for E–ATP*) and 10 mM $MgCl_2$ was added to a chase solution containing a 40-fold excess of unlabeled UTP and saturating concentrations of ATP, glutamine, and $MgCl_2$ (see Experimental Procedures). None of the E–UTP* was trapped as CTP* after product analysis. Addition of GTP to the chase solution did not result in any trapping of E–UTP* either.

The isotope partitioning experiments with UTP* were also carried out with ATP in the pulse solution since ATP lowers the binding constant for UTP (Levitzki & Koshland, 1972). A pulse solution containing 3 nmol of CTP synthetase, 1 mM UTP*, 1.5 mM ATP, and 10 mM $MgCl_2$ was incubated for 2 min and then added to a chase solution containing a 480-fold excess of unlabeled UTP and saturating concentrations of ATP, glutamine, and $MgCl_2$. After termination of the reaction and product analysis, none of the initially bound UTP* was trapped as CTP*. When the above experiment was modified to include GTP in the chase, 53% of the initially bound UTP* was trapped as CTP*.

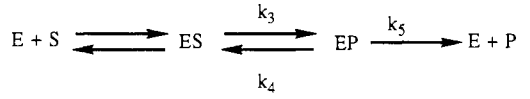
Control experiments were conducted to correct for contaminating NH_3 under conditions in which ATP was present in the pulse solution since contaminating NH_3 in the pulse solution would result in artifactural trapping of UTP*. In an attempt to eliminate the necessity for the control experiments, the ATP in the pulse solution was replaced by the nonhydrolyzable analogue AMP-PNP. It had been previously reported that AMP-PNP was an inhibitor of CTP synthetase with a K_i value comparable to the K_M for ATP (Levitzki & Koshland, 1971). However, when AMP-PNP was used in place of ATP in the isotope partitioning experiments with UTP*, no trapping of E–UTP* was observed.

Discussion

Two techniques, rapid quench and isotope partitioning kinetics, were used to investigate the mechanism of *E. coli* CTP synthetase. Previous positional isotope exchange experiments had suggested that the mechanism of CTP formation involved phosphorylation of UTP by ATP followed by attack of NH_3 (top pathway, Scheme I) (von der Saal et al., 1985). Therefore, rapid quench experiments of both the UTP-dependent ATPase reaction and the glutamine-dependent reaction were carried out to provide further evidence for the existence of a phosphorylated UTP intermediate. Isotope partitioning experiments were performed to determine the order of addition of substrates. The order of addition of substrates is not currently known because the cooperativity displayed by the enzyme makes it difficult to interpret initial velocity studies. However, positional isotope exchange experiments have suggested that the order of addition of substrates was UTP followed by ATP and then NH_3.

Rapid Quench Kinetics of the UTP-Dependent ATPase Reaction. The time courses for the UTP-dependent ATPase reaction with and without GTP are both characterized by a burst of acid-labile phosphate followed by a slower steady-state phase (Figure 3). A simple model for the UTP-dependent ATPase reaction is shown in Scheme II. Since all the substrates are saturating in the rapid quench experiments, eq 3–5

Scheme III



$$k_{cat} = \frac{k_3 k_5}{k_3 + k_4 + k_5} \quad (3)$$

$$\beta = \frac{k_3(k_3 + k_4)}{(k_3 + k_4 + k_5)^2} \quad (4)$$

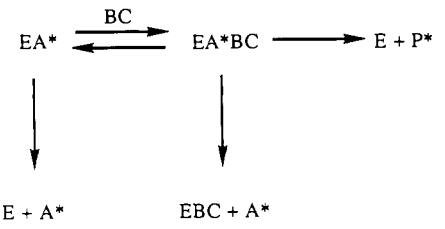
$$\lambda = k_3 + k_4 + k_5 \quad (5)$$

can be used to determine k_3, k_4, and k_5. Using experimental values for the UTP-dependent ATPase reaction in the absence of GTP (Table II), we calculated values for the three rate constants: $k_3 = 1.66 \pm 0.20\ s^{-1}$, $k_4 = 0.091 \pm 0.009\ s^{-1}$, and $k_5 = 0.029 \pm 0.003\ s^{-1}$. The internal equilibrium constant for the phosphorylation of UTP, k_3/k_4, is 18, demonstrating that the enzyme favors formation of the phosphorylated UTP intermediate.

Similar calculations can be done for the UTP-dependent ATPase reaction in the presence of GTP. By use of the values in Table II and eq 3–5, the following rate contants gave the best fit to the data: $k_3 = 1.6 \pm 0.2\ s^{-1}$, $k_4 = 1.5 \pm 0.02\ s^{-1}$, and $k_5 = 0.15 \pm 0.02\ s^{-1}$. The internal equilibrium constant, k_3/k_4, for the formation of the phosphorylated UTP intermediate in the presence of GTP is 1.1. The overall effect of GTP on the UTP-dependent ATPase reaction is an increase in the k_{cat} and a decrease in the internal equilibrium constant for the formation of the phosphorylated UTP intermediate. GTP decreases the internal equilibrium constant by increasing k_4, while having little effect on k_3. The observation that β has decreased in the presence of GTP is the result of the decrease in the internal equilibrium constant for the formation the phosphorylated UTP intermediate. Thus, GTP increases the rates of steps subsequent to intermediate formation.

Rapid Quench Kinetics of the Glutamine-Dependent Reaction. A burst of acid-labile phosphate was observed for the glutamine-dependent reaction only in the absence of GTP (Figure 2). A simplified model of the glutamine-dependent reaction is shown in Scheme III, where ES represents the E–MgATP–UTP–Gln complex, EP represents the intermediate that is responsible for the burst of acid-labile phosphate, and P represents all the products, CTP, ADP, P_i, and Glu. There is a minimum of three possibilities for the EP complex: E–MgADP–UTP~P, E–MgADP–UTP~P–NH_3, and E–MgADP–CTP~P–Glu. When eq 3–5 were used to determine the rate constants k_3, k_4, and k_5, the values that yielded the best estimates are $k_3 = 2.5 \pm 0.2\ s^{-1}$, $k_4 = 0.05 \pm 0.01\ s^{-1}$, and $k_5 = 0.45 \pm 0.04\ s^{-1}$. The internal equilibrium constant k_3/k_4 for formation of the intermediate (EP in Scheme III) is 50. A comparison of the internal equilibrium constants obtained in the presence (50) and absence (18) of glutamine indicates that the presence of glutamine shifts the internal equilibrium of the reaction toward formation of EP. The high equilibrium constant is in agreement with the failure to observe the reverse reaction of CTP synthetase (UTP formation) (Levitzki & Koshland, 1971) and the failure to observe positional isotope exchange in the presence of glutamine (von der Saal et al., 1985). The observation of a burst of acid-labile phosphate for the UTP-dependent ATPase reaction in the presence and absence of GTP and the glutamine-dependent

Scheme IV



reaction in the absence of GTP provides evidence that the formation of the phosphorylated UTP intermediate is kinetically competent. The lack of a burst of acid-labile phosphate for the glutamine-dependent reaction in the presence of GTP indicates that GTP increases the forward rate step (k_5) relative to the rate of reversal of formation of the intermediate (k_4) such that a burst is no longer observed. An additional factor is that k_5 most likely has increased relative to k_3 also suppressing the observation of a burst of acid-labile phosphate.

Isotope Partitioning Experiments. The general equation for an isotope partitioning experiment is shown in Scheme IV. The labeled substrate A has three fates; it can become incorporated into product, it can dissociate from the EA* complex, or it can dissociate from the EA*BC complex. The amount of label which it trapped as P* depends on the partition ratio for substrate dissociation and product formation. The ability to trap A* as P* indicates that E–A is formed in a catalytically competent manner and that A can bind first. There are three explanations for the inability to trap A* as P*: the EA complex may not be catalytically competent (i.e., A binds after B and C), A may dissociate from the E–A complex, or A may dissociate from the E–ABC complex (Rose, 1980). Therefore, the isotope partitioning experiments with CTP synthetase were designed to detect trapping of UTP* as CTP* since positional isotope exchange experiments indicated that UTP binds first. However, the results of these experiments demonstrated no trapping of UTP* as CTP* in the presence or absence of GTP. When the isotope partitioning experiments were done with UTP* and ATP in the pulse solution, 53% of the E–UTP* complex was trapped as CTP*. In addition, the isotope partitioning experiments with ATP* showed that 15% of the E–ATP* complex was trapped as P_i* in the presence of GTP. The ability to trap 15% of the E–ATP* complex as P_i* shows that the E–ATP* complex is formed in a catalytically competent manner; however, it does not imply that ATP binds first since a preferred order mechanism cannot be ruled out on the basis of the data in this paper or those of von der Saal et al. (1985).

The failure to trap any E–UTP* as CTP* in the presence or absence of GTP suggests that UTP* dissociates from the E–UTP* complex faster than it goes on to form products. In the isotope partitioning experiments with UTP*, where ATP was also included in the pulse solution, the main species should be E–UTP*~P–ADP since rapid quench experiments of the UTP-dependent ATPase reaction indicated that the internal equilibrium constant is 18. The failure to trap any E–UTP*–ATP as CTP* implies that UTP* dissociates from the E–UTP*~P–ADP or E–UTP*~P–ADP–Gln complexes faster than it goes on to form products. When GTP was added to the pulse solution, 53% of the E–UTP*~P–ADP complex was trapped as CTP*. The presence of GTP most likely increases the rate of product formation relative to the dissociation rate of UTP* from E–UTP*~P–ADP–Gln. This latter explanation is consistent with the rapid quench experiments of the glutamine-dependent reaction in the presence of GTP. Thus, the major conclusion derived from the kinetic experiments in this paper is that the initial chemical step catalyzed by CTP synthetase is the phosphorylation of UTP by ATP to form an enzyme-bound intermediate.

Acknowledgments

We thank Dr. Kenneth Johnson for help in designing the rapid quench experiments.

References

Anderson, P. M. (1983) *Biochemistry 22*, 3285.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
Edelhoch, H. (1967) *Biochemistry 6*, 1948.
Johnson, K. A. (1986) *Methods Enzymol. 134*, 677.
Koshland, D. E., Jr., & Levitzki, A. (1974) *Enzymes (3rd Ed.) 10*, 539.
Levitki, A., & Koshland, D. E., Jr. (1971) *Biochemistry 10*, 3365.
Levitzki, A., & Koshland, D. E., Jr. (1972a) *Biochemistry 11*, 241.
Levitzki, A., & Koshland, D. E., Jr. (1972b) *Biochemistry 11*, 247.
Long, C., & Pardee, A. B. (1967) *J. Biol. Chem. 242*, 4715.
Long, C., & Koshland, D. E., Jr. (1978) *Methods Enzymol. 51*, 79.
Meek, T. D., Johnson, K. A., & Villafranca, J. J. (1982) *Biochemistry 21*, 2158.
Rose, I. A. (1980) *Methods Enzymol. 64*, 47.
Savage, C. R., & Weinfield, H. (1970) *J. Biol. Chem. 245*, 2529.
Segel, I. H. (1975) in *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, p 365, Wiley-Interscience, New York.
von der Saal, W., Anderson, P. M., & Villafranca, J. J. (1985a) *J. Biol. Chem. 260*, 14993.
von der Saal, W., Villafranca, J. J., & Anderson, P. M. (1985b) *J. Am. Chem. Soc. 83*, 4755.
Weng, M., Makaroff, C. A., & Zalkin, H. (1986) *J. Biol. Chem. 261*, 5568.
Zalkin, H. (1985) *Methods Enzymol. 113*, 263.