CTP Synthetase from *Escherichia coli*: An Improved Purification Procedure and Characterization of Hysteretic and Enzyme Concentration Effects on Kinetic Properties†

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ABSTRACT: Previous studies have shown that CTP synthetase exists as a dimer which aggregates to a tetramer in the presence of the substrates ATP and UTP [Long, C. W., Levitzki, A., & Koshland, D. E., Jr. (1970) *J. Biol. Chem. 245*, 80]. A new, relatively simple purification procedure resulting in enzyme of high purity and in good yield has been established by using two successive hydrophobic chromatography steps, the first in the absence of ATP and UTP (dimer binds) and the second in the presence of ATP and UTP (tetramer does not bind). Several previously unreported properties of CTP synthetase are described which suggest that alterations in the state of association and dissociation of the enzyme have a controlling influence on the observed kinetic properties of the enzyme. The specific activity of CTP synthetase decreases with decreasing enzyme concentration, particularly when the concentrations of ATP and UTP in the reaction mixture are nonsaturating. The concentration of ATP or UTP required for half-maximal activity is significantly increased as the concentration of enzyme in the reaction mixture is decreased. CTP synthetase displays reversible cold lability and hysteretic properties (lags or bursts in the time course of product formation), both of which are influenced by the concentration of enzyme and/or the presence of ATP and UTP in the preincubation mixture and/or assay mixture. Gel filtration studies have shown that CTP synthetase can dissociate to an apparently inactive monomer. The dissociation is reversible, and the rate of association is slow.

CTP synthetase from *Escherichia coli* catalyzes the following amidotransferase reaction:

$$\text{UTP} + \text{ATP} + \text{L-glutamine} \xrightarrow[Mg^{2+}]{\text{GTP}} \text{L-glutamate} + \text{ADP} + P_i + \text{CTP}$$

GTP functions as a positive allosteric effector and is required for glutamine-dependent activity but not for the ammonia-dependent activity which is also catalyzed by the enzyme. The enzyme has been highly purified from *Escherichia coli*, and the physical and kinetic properties of the purified enzyme have been described [for reviews, see Koshland & Levitzki (1974) and Long & Koshland (1978)]. These studies have provided evidence that in the absence of substrates, the enzyme exists as a dimer ($M_r \simeq 105\,000$) composed of two identical subunits. In the presence of UTP and ATP (in the presence of Mg^{2+}), the enzyme aggregates to a tetramer ($M_r \simeq 210\,000$). Either ATP or UTP alone, at higher concentrations, can induce tetramer formation from dimer, but the two substrates together are more effective than either alone. The enzyme exhibits negative cooperativity for the effector GTP and the substrate glutamine. Pronounced positive cooperativity is observed for the substrates ATP and UTP if the one nucleotide which is held constant (as the other is varied) is not saturating (if one is saturating, normal Michaelis–Menten kinetics are observed for the other). These effects of ATP and UTP have been interpreted to mean that the binding of these substrates induces conformational changes which facilitate the binding of subsequent molecules (site–site interaction) and that these conformational changes are accompanied by polymerization of the dimer to the tetramer.

The present study with this CTP synthetase originated with our interest in the mechanism of ammonia transfer catalyzed by amidotransferases and from evidence that cyanate may act as a rather specific inhibitor of amidotransferases in general (Anderson & Carlson, 1975). Considerable frustration was experienced in our initial studies by our inability to consistently isolate the enzyme in reasonable yield when following the published purification procedure (Long & Pardee, 1967; Long & Koshland, 1978). The new and much simpler isolation procedure reported here was developed as a result of these difficulties. Subsequent studies revealed apparent inconsistencies in our results which were ultimately traced to unusual and previously unreported kinetic properties of the enzyme, including hysteresis, cold lability, variations in specific activity with enzyme concentration, and alteration of these properties by ATP and UTP. These properties are described in this paper. The results suggest that the association–dissociation behavior and/or the hysteretic properties of CTP synthetase may play a significant role in generating the positive cooperativity exhibited by ATP and UTP.

Materials and Methods

Escherichia coli B cell paste (unwashed, 3/4 log phase, grown on minimal media) was obtained from Grain Processing Corp. Protamine sulfate was purchased from Calbiochem. DEAE-Sephadex A-50,[1] Sephadex G-25, Sephadex G-200, and phenyl-Sepharose (CL-4B) were purchased from Pharmacia Fine Chemicals, Inc. Ammonium sulfate was ultrapure (enzyme grade) from Schwarz/Mann. Other biochemicals were purchased from Sigma Chemical Co.

CTP synthetase activity was determined by following the rate of increase in absorbance at 291 nm at 38 °C due to the conversion of UTP to CTP; the molar extinction coefficients for UTP and CTP at 291 nm are 182 and 1520, respectively (Long & Pardee, 1967; Long & Koshland, 1978). The standard assay mixture contained 0.06 M Hepes buffer, pH 8.0, 0.5 mM EDTA, 0.01 M L-glutamine, 0.01 M $MgCl_2$, 1 mM ATP, 1 mM UTP, and 0.2 mM GTP in a final volume

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DEAE-Sephadex, diethylaminoethyl-Sephadex; $NaDodSO_4$, sodium dodecyl sulfate.

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of 2 mL. Reaction was initiated by the addition of enzyme to the assay mixture equilibrated at 38 °C. Assay mixtures without enzyme or without UTP served as blanks; with highly purified enzyme the background rate was negligible. The absorbance change with time was monitored with a Beckman DU-8 computing spectrophotometer equipped with a six-sample temperature-controlled enzyme kinetic analysis system and digital printer–plotter.

Specific activity is expressed as micromoles of CTP formed per minute per milligram of protein. The concentration of the highly purified enzyme used in the kinetic studies was determined by measuring the absorbance at 280 nm (Levitzki & Koshland, 1972). The molar concentrations of the enzyme were determined on the basis of the molecular weight of the dimer (≃105 000). The protein concentration after each of the purification steps except the last was measured by the dye-binding procedure described by Bradford (1976) with reagents obtained from Bio-Rad Laboratories.

Results

Enzyme Isolation. The following purification procedure describes the isolation of CTP synthetase from 200 g of *Escherichia coli* B cell paste (3/4 log, minimal media). All steps were carried out at 4 °C.

(*A*) *Cell Extract.* The cell paste, suspended in 600 mL of 0.2 M potassium phosphate buffer, pH 7.8, containing 1 mM EDTA and 20 mM L-glutamine, was divided into three equal fractions, and each fraction was subjected to ultrasonic treatment for 20 min at full power (Branson Model W185). The sonicated fractions were combined, and cell debris was removed by centrifugation at 10000*g* for 30 min. A 2% solution of protamine sulfate was added dropwise over a 15-min period to the supernatant with stirring; 1 mL was added for each 4 mL of supernatant. The resulting precipitate was removed by centrifugation at 10000*g* for 30 min and discarded.

(*B*) *Ammonium Sulfate Fractionation.* Ammonium sulfate (28.5 g/100 mL of supernatant) was added with slow stirring over a 10-min period. Slow stirring was continued for an additional 15 min after all of the $(NH_4)_2SO_4$ had dissolved, and the resulting precipitate was removed by centrifugation at 10000*g* for 30 min. The supernatant was discarded, and the precipitate was dissolved in a small volume (≃140 mL) of 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and 4 mM L-glutamine. The $(NH_4)_2SO_4$ was removed by gel filtration chromatography on a Sephadex G-25 column (5 × 45 cm) equilibrated with the same buffer used to dissolve the precipitate.

(*C*) *DEAE-Sephadex Ion-Exchange Chromatography.* The solution containing enzyme was added to a column (5 × 28 cm) of DEAE-Sephadex A-50 equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 4 mM L-glutamine, and 70 mM mercaptoethanol. The enzyme and other proteins were eluted from the column by increasing the potassium phosphate concentration from 0.1 to 0.29 M; the mixing chamber and the reservoir of the linear gradient system each contained 1400 mL of 0.1 and 0.29 M potassium phosphate buffer, pH 7.5, respectively, and both solutions contained 1 mM EDTA, 4 mM L-glutamine, and 70 mM mercaptoethanol. The elution rate was about 80 mL/h. The enzyme activity was eluted midway through the gradient and the fractions containing most of the enzyme activity were combined.

(*D*) *Hydrophobic Column Chromatography.* The protein (including the enzyme) in the combined fractions was precipitated by adding $(NH_4)_2SO_4$ (40 g/100 mL). After the $(NH_4)_2SO_4$ had dissolved, the protein precipitate was removed by centrifugation at 10000*g* for 30 min. The precipitate was dissolved in ≃15 mL of 20 mM potassium phosphate buffer, pH 7.6, containing 1 mM EDTA, 2 mM L-glutamine, and 2 mM dithiothreitol. This solution was added directly to a column (4 × 18 cm) of phenyl-Sepharose equilibrated with the same buffer solution used to dissolve the precipitate. The column was washed with this same buffer until the large peak of protein which did not adsorb to the column had eluted. The enzyme and other proteins were then eluted from the column at a rate of ≃50 mL/h by a linear gradient of decreasing potassium phosphate concentration and increasing ethylene glycol concentration. The mixing chamber and the reservoir of the linear gradient system each contained 300 mL of eluting buffer. The buffer solution in the mixing chamber was the same as that used to wash the column. The buffer solution in the reservoir contained 0.5 mM potassium phosphate buffer, pH 7.9, 0.5 mM EDTA, 1 mM L-glutamine, 2 mM dithiothreitol, and 30% ethylene glycol. The enzyme eluted as a broad peak toward the end of the gradient, and in most cases elution with the buffer reservoir had to be continued after the gradient was complete in order to remove all of the enzyme from the column. The fractions containing most of the enzyme activity were pooled.

(*E*) *Hydrophobic Column Chromatography in the Presence of ATP, UTP, and* Mg^{2+}. In order to increase the ionic strength and reduce the concentration of ethylene glycol in the pooled fractions, an equal volume of 0.2 M potassium phosphate buffer, pH 7.7, containing 20 mM L-glutamine, 1 mM EDTA, and 2 mM dithiothreitol was added to the pooled fractions. The enzyme and other proteins in the pooled and diluted fractions were precipitated by adding $(NH_4)_2SO_4$ (40 g/100 mL). After the $(NH_4)_2SO_4$ had dissolved, the protein precipitate was removed by centrifugation at 10000*g* for 60 min. The precipitate was dissolved in ≃15 mL of 20 mM potassium phosphate buffer, pH 7.7, containing 2 mM L-glutamine, 1 mM EDTA, 0.7 mM UTP, 2 mM ATP, 10 mM $MgCl_2$, and 2 mM dithiothreitol. This solution was then added directly to a column (4 × 11 cm) of phenyl-Sepharose equilibrated with the same buffer solution used to dissolve the precipitate, and the enzyme was eluted by washing the column with ≃400 mL of this same buffer solution at a rate of ≃70 mL/h. All fractions containing enzyme activity were combined.

(*F*) *Gel-Filtration Chromatography on Sephadex G-200.* The protein in the pooled fractions was precipitated by adding $(NH_4)_2SO_4$ (40 g/100 mL) after increasing the potassium phosphate buffer concentration to 0.1 M (pH 7.6). After the $(NH_4)_2SO_4$ had dissolved, the protein precipitate was removed by centrifugation at 10000*g* for 30 min. The precipitate was dissolved in ≃4 mL of 60 mM Hepes buffer, pH 8.0, containing 1 mM EDTA, 2 mM ATP, 0.7 mM UTP, and 10 mM $MgCl_2$ and applied to a column (2.5 × 95 cm) of Sephadex G-200 equilibrated with this same buffer solution. The enzyme was eluted at a rate of ≃18 mL/h. The fractions containing most of the enzyme activity were pooled, and the volume was reduced to ≃5 mL by ultrafiltration using a YM30 Amicon filter. The enzyme (≃10 mg) was stored at −20 °C in a solution containing 0.2 M Hepes buffer, pH 8.0, 70 mM mercaptoethanol, 2.5 mM ATP, 2.5 mM UTP, 10 mM $MgCl_2$, 1 mM EDTA, and 20% glycerol.

The results of a typical enzyme isolation are summarized in Table I. The total units present after the first two steps were somewhat variable. This is probably due to difficulty in accurately assaying CTP synthetase activity in crude extracts by the method used because of a significant background

Table I: Purification of CTP Synthetase[a]

purification step	vol (mL)	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)
extract	812	22680	148	0.007	
$(NH_4)_2SO_4$ fractionation	265	13250	153	0.012	
DEAE-Sephadex chromatography	385	1463	163	0.11	100
first phenyl-Sepharose chromatography	400	340	132	0.39	81
second phenyl-Sepharose chromatography	200	18	117	6.5	72
Sephadex G-200 chromatography	41	11	96	8.7	59

[a] Procedures are described in the text. A unit is defined as that amount of enzyme which catalyzes the formation of 1 μmol of CTP in 1 min at 38 °C.

rate in the absence of UTP, even after removing low molecular weight compounds by gel-filtration chromatography. Nevertheless, it is clear that little or no activity is lost during these steps. The specific activity of the purified enzyme is comparable to the values of 5.8–6.1 μmol h^{-1} mg^{-1} reported previously for highly purified CTP synthetase; however, the yield of 50–60% is about double that obtained by the method previously described (Long & Koshland, 1978), and the new method described above involves fewer steps and considerably less time. Polyacrylamide gel electrophoresis of the enzyme obtained after the last step in the presence of $NaDodSO_4$ as described by Weber et al. (1972) gives a single protein band corresponding to a molecular weight of ≃50 000. In many cases, the last step (Sephadex G-200) may not be necessary, since the enzyme is often quite pure after the second phenyl-Sepharose step, as judged by acrylamide gel electrophoresis in the presence of $NaDodSO_4$. The purified enzyme is stable for months when stored at −20 °C as indicated above.

Kinetic and Other Properties of the Purified Enzyme. (*A*) *Hysteretic Properties, Cold Lability, and Effects of Dilution.* Our initial studies revealed inconsistencies in CTP synthetase activity which were ultimately related to the preincubation conditions under which the stock enzyme solution used for assay existed. The standard preincubation procedure used in this study involved placing the enzyme in 0.06 M Hepes buffer, pH 8.0, containing 1 mM EDTA by passing enzyme present in the storage buffer solution through a small Sephadex G-25 column equilibrated with the Hepes–EDTA buffer at 26 °C. Small volumes were then removed from this stock enzyme solution, which was maintained at 26 °C, for assay. The enzyme remains fully active over a period of up to 2 days under these conditions if the enzyme concentration is approximately 0.05 mg/mL or higher. As shown in Figures 1 and 3, enzyme concentration, temperature, and the additional presence of ATP and UTP in the preincubation mixture significantly affect the time course of product formation when equivalent amounts of enzyme are assayed under identical saturating conditions. When the CTP synthetase concentration in the preincubation solution is about 0.05 mg/mL or higher, a significant lag in the time course of product formation is observed, but the final steady-state rate representing a maximum specific activity is the same regardless of the concentration of enzyme in the preincubation solution. However, as the concentration of enzyme in the preincubation solution is decreased below 0.05 mg/mL, the lag period becomes more pronounced and the final steady-state rate decreases. This effect of dilution is time dependent (Figure 2), is prevented by the presence of ATP and UTP in the preincubation mixture (Figures 1 and 2), and can be reversed by the addition of ATP and UTP (Figure 2). A small burst in the rate of product formation is observed when the enzyme is preincubated in the presence of ATP and UTP (Figure 1).

The effect of dilution described above can be magnified by decreasing the temperature. As shown in Figure 3, when the

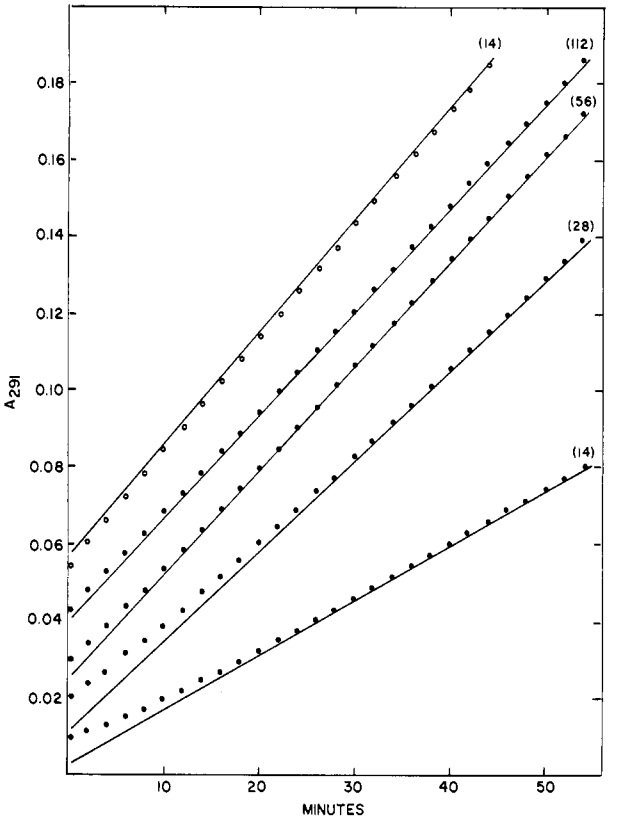

FIGURE 1: Effect of preincubation conditions on the time course of CTP formation. CTP synthetase at four different concentrations (112, 56, 28, and 14 μg/mL) was preincubated at 26 °C for 9 h in 0.06 M Hepes buffer, pH 8.0, containing 1 mM EDTA (closed circles) or in this same solution containing, in addition, 2 mM ATP, 2 mM UTP, and 10 mM $MgCl_2$ (open circles). An appropriate volume corresponding to 0.5 μg of enzyme was then removed from each preincubation solution and added to the standard assay mixture. The formation of CTP with time was followed by measurement of the increase in A_{291} as described in the text. The different time courses are identified by the numbers in parenthesis which indicate the concentration of enzyme (μg/mL) in the preincubation solution from which enzyme was removed.

temperature in the preincubation mixture is decreased to 4 °C, the lag period in the time course of product formation observed when the enzyme is assayed becomes very large and the apparent final rate of product formation is significantly reduced. This pronounced lag and reduced activity is reversible, and the cold lability is prevented by the presence of ATP and UTP in the preincubation mixture; the effect is also greatly reduced if the enzyme concentration in the preincubation solution is greater than about 0.05 mg/mL.

As noted above, a burst phase in the time course of product formation is observed when the enzyme is preincubated in the presence of ATP and UTP before assaying under saturating conditions. A burst also occurs when ATP and UTP are absent from the preincubation mixture if the assay is carried out under

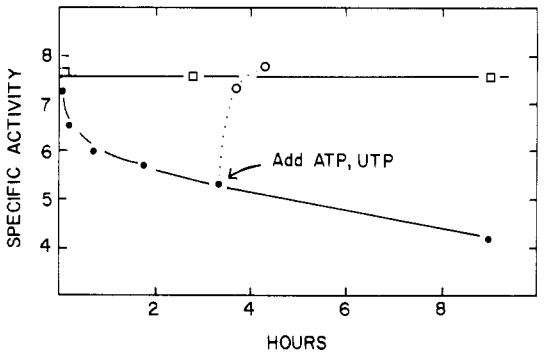

FIGURE 2: Changes in specific activity of CTP synthetase with time after dilution. CTP synthetase (120 μg/mL) in 0.06 M Hepes buffer, pH 8.0, containing 1 mM EDTA at 26 °C was diluted in this same buffer (closed circles) or in this same buffer containing, in addition, 2 mM ATP, 2 mM UTP, and 10 mM $MgCl_2$ (open squares) to give a final concentration of 14 μg/mL. At the indicated times, a volume of 40 μL (containing 0.56 μg of enzyme) was removed and added to the standard assay mixture, and the specific activity was determined after measuring the rate of CTP formation as described in the text. A small volume of a solution containing ATP, UTP, and $MgCl_2$ was added to a portion of the diluted preincubation mixture which did not contain these substrates (closed circles) after 3.3 h, as indicated in the graph, to give a preincubation solution containing these substrates at the same concentration as that represented by the open squares, and the specific activity of the enzyme in this solution was determined at the indicated times (open circles).

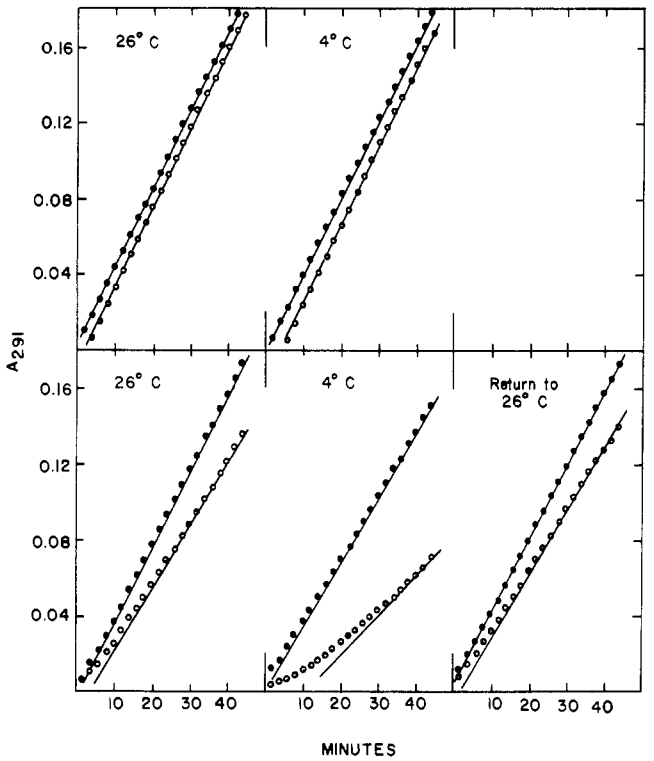

FIGURE 3: Effect of temperature and enzyme concentration during preincubation on the time course of CTP formation. The preincubation solutions contained CTP synthetase at two different concentrations [140 (closed circles) and 17 μg/mL (open circles)] in either 0.06 M Hepes buffer, pH 8.0, containing 1 mM EDTA (lower panel) or this same solution containing, in addition, 2 mM ATP, 2 mM UTP, and 10 mM $MgCl_2$ (upper panel). These solutions were preincubated for 2 h at 26 °C followed by 2 h at 4 °C, and then a final 2 h at 26 °C (left to right, respectively). At the end of each 2-h incubation period, appropriate volumes corresponding to 0.7 μg of enzyme were removed from each of the four preincubation solutions and added to the standard assay mixture. The formation of CTP with time was following by measurement of the increase in A_{291} as described in the text.

conditions which are nonsaturating with respect to ATP and UTP. As shown in Figure 4, the magnitude of this burst is

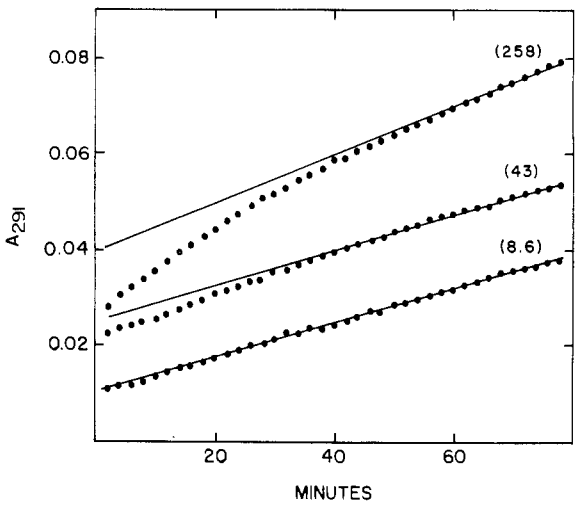

FIGURE 4: Effect of enzyme concentration during preincubation on the time course of CTP formation when ATP and UTP are nonsaturating. CTP synthetase at three different concentrations (8.6, 43, and 258 μg/mL) was preincubated at 26 °C for 1 h after dilution in 0.06 M Hepes buffer, pH 8.0, containing 1 mM EDTA. Appropriate volumes corresponding to 0.50 μg of enzyme were then removed from each of the three different preincubation solutions and added to the 2-mL assay mixtures which contained the same components as the standard assay mixture except the concentrations of ATP and UTP were 0.3 and 0.2 mM, respectively. The formation of CTP with time was followed by measurement of the increase in A_{291} as described in the text. The different time courses are identified by the numbers in parentheses which indicate the concentration of enzyme (μg/mL) in the preincubation solution from which enzyme was removed.

dependent upon the concentration of CTP synthetase in the preincubation mixture (identical amounts of enzyme were present in the assay mixtures); the higher the concentration, the greater the burst. The large burst observed when the concentration of enzyme in the preincubation solution is high (and assay conditions are nonsaturating) is retained when the concentration of enzyme in the preincubation mixture is low if ATP and UTP are present in the preincubation mixture (data not shown).

It would seem reasonable to conclude (along with the results described below) that the effects of dilution and temperature (cold lability) described above reflect dissociation of the enzyme to an inactive species and that the observed lag periods represent slow conversion (under saturating assay conditions but considerably lower enzyme concentration) back to an active associated species. The reduced final rate of product formation observed under these conditions suggests that the process is not fully reversible under the assay conditions. It is possible that the apparent reduced rate of product formation in these circumstances may be due to irreversible inactivation of some of the inactive species before it is converted back to an active form in the assay mixture (which is at 38 °C). A number of control studies have indicated, however, that the enzyme is fully stable at very low concentrations over an assay period of 60 min, even under conditions which are nonsaturating with respect to ATP and UTP. Alternatively, it may be that the return to apparent full activity in the presence of all substrates (which, as indicated by the long lags, is very slow) would be observed if the assay could be carried out for a longer period of time witout significant accumulation of products.

The effects of ATP and UTP described above require the presence of both nucleotides. The presence of GTP, glutamine, or Mg^{2+} alone or of GTP and/or glutamine in the presence of ATP or UTP alone had little effect.

As shown in Figure 5, the length of the lag period is reduced when the concentration of enzyme in the assay mixture is

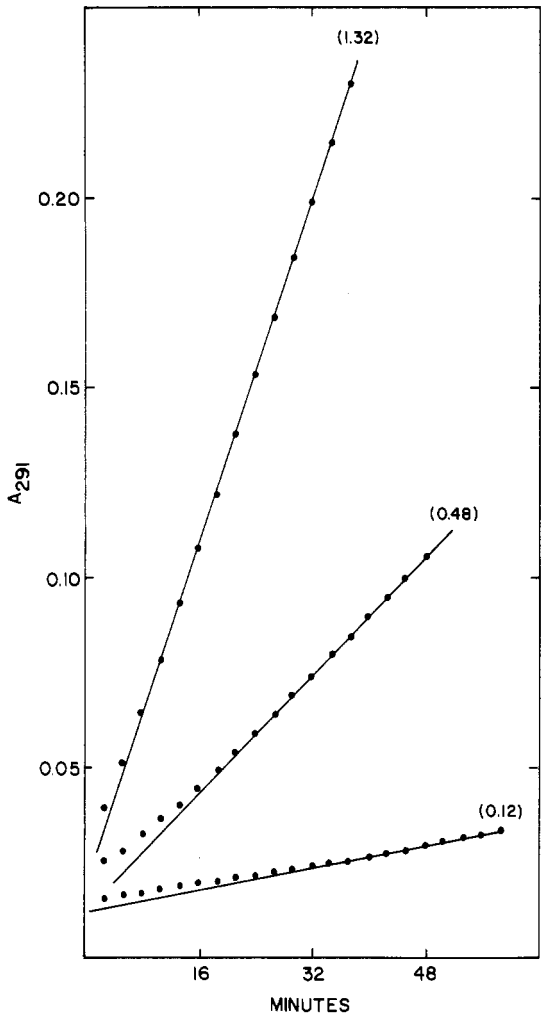

FIGURE 5: Effect of enzyme concentration in the assay mixture on the lag period in the time course of CTP formation. CTP synthetase (6 μg/mL) in 0.06 M Hepes buffer, pH 8.0, containing 1 mM EDTA was preincubated at 26 °C for 3 h. Volumes of 20, 80, and 220 μL (corresponding to 0.12, 0.48, and 1.32 μg of enzyme) were then removed and added to standard assay mixtures, and the formation of CTP with time was followed by measuring the increase in A_{291} as described in the text. The different time courses are identified by the numbers in parentheses which indicate the micrograms of enzyme in the 2-mL assay mixture.

increased. In this experiment, the concentration of enzyme in the preincubation mixture was constant, and different volumes were removed and added to the assay mixture.

(*B*) *Effect of CTP Synthetase Concentration on Specific Activity and Apparent Cooperativity.* Another variable which was found to contribute to apparent inconsistencies in CTP synthetase activity in our initial studies was the effect of enzyme concentration in the assay mixture on specific activity, particularly when the assay system was not saturated with respect to ATP and/or UTP. The effect of enzyme concentration on specific activity is shown in Figure 6. The data in Figures 6–8 represent the observed steady-state rates measured after the lag period if a lag period was observed. When both ATP and UTP are present at nonsaturating concentrations, the observed specific activity decreases to zero with decreasing enzyme concentration. Similar results are obtained when only one of the nucleotides is nonsaturating, although lower concentrations of enzyme are required before the decrease in specific activity is observed. Even when both nucleotides are present in the assay mixture at saturating concentrations, a small decrease in specific activity is observed at very low enzyme concentrations. These results are characteristic of an associating–dissociating system in which only an associated form of the enzyme is active (Frieden, 1981; Kurganov, 1973, 1974; Kurganov et al., 1974). Such self-associating systems can give rise to nonhyperbolic kinetics such as the positive cooperativity observed when the activity of CTP synthetase is measured as a function of either ATP or UTP concentration while the concentration of the other is nonsaturating. If this kind of associating–dissociating system is a contributing factor to the nonhyperbolic kinetics exhibited by CTP synthetase, then it would be expected that the nonhyperbolic kinetic properties might be dependent on the enzyme concentration. The results shown in Figure 7 show that this is, indeed, the case. The curves are all sigmoid, but in each case the concentration of nucleotide substrate required to give half-maximal activity is markedly affected by the enzyme concentration, increasing as the concentration of enzyme in the assay mixture is decreased. The Hill coefficients determined from Hill plots of these data are also affected by enzyme concentration; the values at 30 and 2 nM CTP synthetase are 2.3 and 3.5 for ATP and 1.3 and 1.8 for UTP, respectively. It has been reported previously that normal hyperbolic kinetics are observed when the concentration of the nonvarying nucleotide substrate is saturating. The results in Figure 8 show, however, that even under these circumstances nonhyperbolic kinetics are observed if the enzyme concentration is very low, at least when UTP is saturating and ATP is the variable substrate; the Hill coefficients determined from Hill plots of these data are 1.2 and 1.4 for ATP and 1.0 and 1.2 for UTP at 14 and 1.4 nM CTP synthetase, respectively.

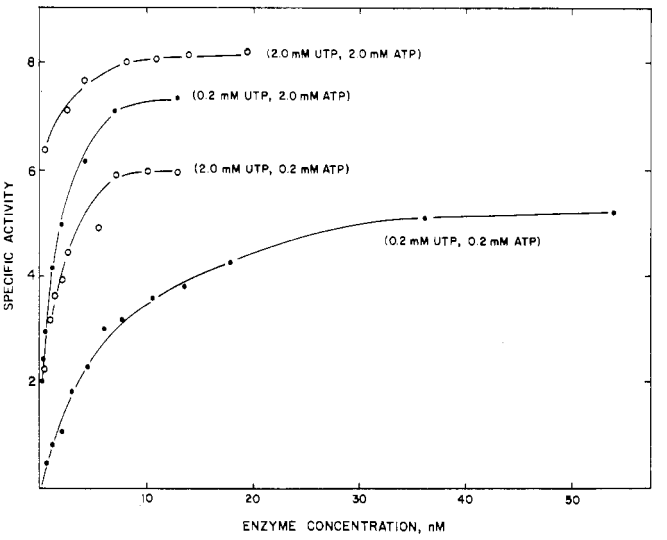

FIGURE 6: Effect of enzyme concentration on specific activity. The enzyme (0.063 mg/mL) used for these assays was in 0.06 M Hepes, pH 8.0, containing 1 mM EDTA at 26 °C, and appropriate volumes (1.5–180 μL) containing an amount of enzyme corresponding to the indicated nanomolar concentration of enzyme in the assay mixtures were removed and added to the assay mixture. The activity was determined under standard assay conditions, except that different concentrations of ATP and UTP were present as indicated in the graph. Division of the nanomolar concentration by 10 yields an approximate value for the micrograms of CTP synthetase per milliliter of assay mixture.

(*C*) *Apparent Molecular Size.* Previous studies by Long et al. (1970) and Levitzki & Koshland (1972) provided evidence that in the absence of the nucleotide substrates (ATP and UTP) CTP synthetase exists only as a dimer which does not associate to tetramer or dissociate to monomer over a wide range of enzyme concentrations and that in the presence of ATP and UTP the enzyme associates to tetramer even at very low concentrations of enzyme. The effects of dilution and

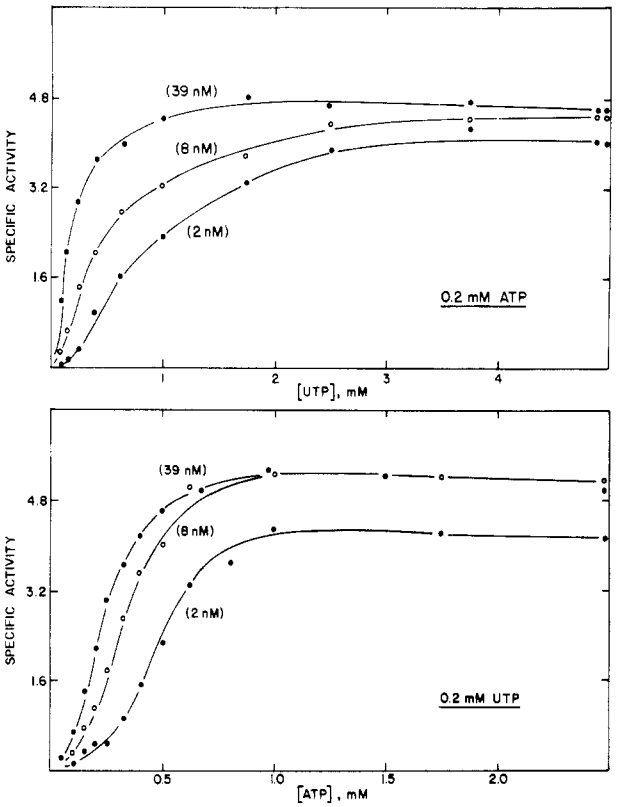

FIGURE 7: Effect of enzyme concentration on the activity vs. substrate concentration curve for UTP (upper panel) and ATP (lower panel) when the concentration of the nonvarying nucleotide substrate is nonsaturating. The enzyme was assayed essentially as described in Figure 6. Assays were carried out in the standard assay mixture, except ATP and UTP were varied or maintained at a fixed nonsaturating concentration as indicated in the graphs. The concentration of CTP synthetase in the assay mixtures is indicated by the number in parentheses in the graphs for each of the substrate saturation curves.

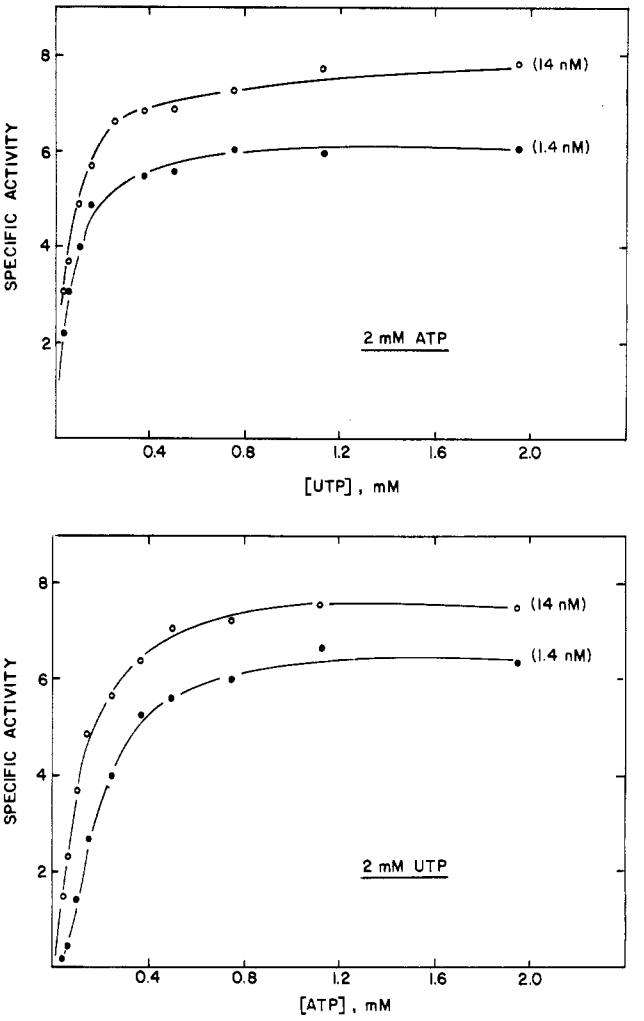

FIGURE 8: Effect of enzyme concentration on the activity vs. substrate saturation curve for UTP (upper panel) and ATP (lower panel) when the concentration of the nonvarying nucleotide substrate is saturating. Assay conditions are the same as described in Figure 8, except that the concentration of the nonvarying nucleotide substrate was saturating (2 mM).

temperature described above, however, are most simply interpreted in terms of alterations in the state of association of an oligomeric form of a reversibly associating–dissociating enzyme system (at least in the the absence of ATP and UTP). This possibility was investigated by gel filtration chromatography on Sephadex G-200 equilibrated with the standard preincubation buffer (0.06 M Hepes, pH 8.0, 1 mM EDTA) at 26 °C. The elution profiles were measured as a function of enzyme concentration in the sample added to the column. As shown in Figure 9, the elution position of the peak of enzyme activity occurs at increasingly later positions with decreasing enzyme concentration, indicating a lower degree of association when the enzyme concentration is decreased, as would be expected if the enzyme was capable of reversible dissociation. Although it cannot be determined from these data whether some tetramer exists at the higher enzyme concentrations, it seems quite likely on the basis of the elution volumes and profiles that dissociation of dimer does occur under these conditions, particularly at low enzyme concentration. Sedimentation data have suggested that the dimer has an elongated shape (Long et al., 1970); if this is correct, elution of dimer from Sephadex G-200 might occur at a somewhat earlier position than would be expected on the basis of its molecular weight. Thus, the rather broad elution profile observed at the highest enzyme concentration probably represents dimer and increasing amounts of monomer on the trailing side; that is, very little, if any, tetramer exists.

When the experiment involving a lower enzyme concentration was carried out at 4 °C instead of 26 °C, the peak of elution occurred at an even later position (later than bovine serum albumin), providing additional evidence that the dimer can dissociate to monomer under these conditions and that cold lability is related to dissociation.

When gel filtration was carried out in the presence of ATP and UTP, the enzyme eluted at a much earlier position, presumably as a tetramer, and the position and elution profile was the same whether the concentration of enzyme added to the column was very high or very low. Thus, it would appear that in the presence of saturating concentrations of ATP and UTP the formation of tetramer is virtually irreversible.

Discussion

The relatively simple procedure established for the purification of CTP synthetase in good yield takes advantage of the differences in affinity of the associated and dissociated forms of the enzyme for phenyl-Sepharose during two successive hydrophobic chromatography steps. Under the conditions employed, the enzyme binds tightly to the hydrophobic support when ATP and UTP are absent (enzyme exists as a dimer) and is eluted only by changing to conditions which weaken hydrophobic interactions (very low ionic strength, presence of ethylene glycol). When ATP and UTP are present, however, the enzyme (which exists as a tetramer under these conditions) does not bind to the hydrophobic support and is

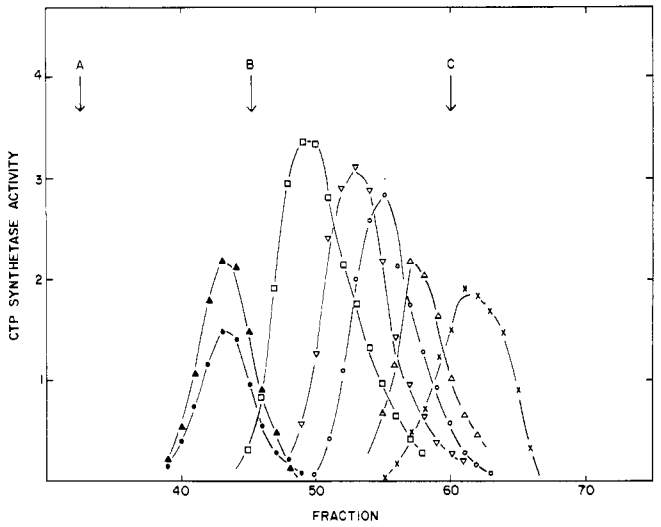

FIGURE 9: Gel filtration chromatography of CTP synthetase on Sephadex G-200. A 4-mL sample containing enzyme and bovine serum albumin (8 mg) in the same buffer used to equilibrate the column was added to a column of Sephadex G-200 (2.5 × 94 cm) equilibrated either with 0.06 M Hepes, pH 8.0, containing 1 mM EDTA [open symbols and (×)] or in the presence of this same buffer containing, in addition, 2 mM ATP, 2 mM UTP, and 10 mM $MgCl_2$ (closed symbols). Elution was carried out at 26 °C (open and closed symbols) or at 4 °C (×) by using a peristaltic pump at a constant rate of 18 mL/h, and 6-mL fractions were collected. The elution volumes (peak of the elution profile) of Blue Dextran 2000 (arrow A; void volume of the column), human γ-globulin (arrow B; M_r 150000), carbonic anhydrase (fraction 74; M_r 30000), and ammonium chloride (fraction 94) were also determined in a separate experiment. The elution volume of bovine serum albumin (M_r 68000) is indicated by arrow C. CTP synthetase activity in the eluted fractions was determined with the standard assay mixture. When the enzyme was eluted in the absence of substrates, 0.1 mL of a solution containing 0.01 M ATP, 0.01 M UTP, and 0.1 M $MgCl_2$ was added to 1-mL aliquots of each eluted fraction, and the enzyme activity was measured after 10–15 h at 26 °C. The micrograms of enzyme in the 4-mL sample added to the column in each of the elution experiments were as follows: (▲) 3400, (●) 7, (□) 4200, (▽) 300, (○) 60, (△) 9, and (×) 26 μg. CTP synthetase activity in each fraction is expressed in relative units for each elution profile.

eluted in the second hydrophobic chromatography step as a highly purified enzyme. Addition of ATP and UTP to the eluant after the enzyme is adsorbed in the absence of these ligands does not result in release of the enzyme (P. M. Anderson, unpublished observations). These observations suggest that tetramer formation may involve interaction between the same hydrophobic sites on the dimer as those that are responsible for binding of the dimer to the hydrophobic support. Thus, when the enzyme is added as tetramer the hydrophobic sites are masked and the enzyme does not bind. Accordingly, when the enzyme is bound as the dimer, subsequent addition of ATP and UTP would then not result in release of the enzyme because the hydrophobic sites on the dimer are, if anything, made more accessible and tetramer formation is, therefore, prevented because the dimer remains tightly bound to the hydrophobic support. Similar substrate-induced differences in affinity for a hydrophobic chromatography support have been used in the purification of phenylalanine hydroxylase (Shiman et al., 1979). In this case, however, the hydrophobic sites responsible for interacting with the hydrophobic support are available only when the substrate phenylalanine is present. Thus, enzyme bound to the support in the presence of phenylalanine is release when phenylalanine is removed from the eluant.

The effects of enzyme concentration on the kinetic properties of CTP synthetase and the hysteretic properties of CTP synthetase described in this paper represent previously unreported properties of this enzyme which also need to be taken into consideration as a basis for explaining the cooperativity exhibited by the enzyme for ATP and UTP, as well as, perhaps, other properties of CTP synthetase such as the negative cooperativity exhibited for the substrate glutamine and for GTP, an allosteric effector. It has been well established that the properties of association–dissociation and of hysteresis in an enzyme can give rise to cooperativity and may play a significant role in the regulatory properties of an enzyme (Neet, 1980a,b; Frieden, 1981; Nichol et al., 1967; Nichol & Winzor, 1976; Kurganov, 1973, 1974, 1977; Kurganov et al., 1974).

It is apparent from these studies that CTP synthetase exhibits properties of an associating–dissociating enzyme system in which the association–dissociation equilibrium has a controlling effect on the observed properties of the enzyme. As noted above, association–dissociation equilibria in a polymerizing enzyme system can give rise to cooperativity in ligand binding and catalytic activity. This can arise if an enzyme exists as an equilibrium mixture of polymeric forms which differ in their affinity for ligand, the addition of which results in a shift in the preexisting equilibrium (Nichol et al., 1967). Alternatively, polymerization may be ligand induced; i.e., binding of ligand may induce a conformational change which subsequently results in a polymerization which does not occur in the absence of ligand (Nichol & Winzor, 1976). In such systems, the nonhyperbolic kinetic data which are obtained when enzyme activity is measured as a function of ligand concentration will be dependent upon the enzyme concentration (Frieden, 1981). The dependence of the observed apparent cooperativity for ATP and UTP on CTP synthetase concentration, therefore, suggests that the positive cooperativity exhibited by these substrates might be explained on the basis of the association–dissociation behavior of the enzyme, rather than by subunit (site–site) interactions.

Since previous studies had indicated that the dimeric form of CTP synthetase does not dissociate under any nondenaturing conditions and that association of dimer to tetramer does not occur at all unless ATP and/or UTP are present, CTP synthetase was considered to be a ligand-induced polymerizing system (Levitzki & Koshland, 1972). The results described in this paper support the view that tetramer formation probably does not occur in the absence of ATP and/or UTP and that formation of tetramer is virtually irreversible when ATP and UTP are saturating. However, the data indicate that dimer can dissociate reversibly. The presence of dissociated dimer was probably not observed in earlier studies because the monomer is apparently inactive and reassociation of the monomer in the presence of all substrates is very slow. When the total enzyme concentration is very low, the presence of monomer (e.g., in fractions obtained after gel filtration chromatography) is not readily detected unless preincubation with ATP and UTP is carried out prior to assaying for catalytic activity. The possibility of a preexisting monomer–dimer equilibrium must, therefore, be taken into account in addition to an induced dimer–tetramer equilibrium when the role of association–dissociation in the kinetic properties of this enzyme is considered.

The catalytic activity of CTP synthetase is also directly influenced by the state of polymerization of the enzyme. The plots of specific activity vs. enzyme concentration indicate that a dissociated form of the enzyme is inactive and that the equilibrium is displaced toward an active, associated form of the enzyme by ATP and/or UTP and also by increasing enzyme concentration.

The observed hysteretic properties of CTP synthetase also appear to be related to the association–dissociation behavior of the enzyme. Thus, the lag in catalytic activity which is observed when a small volume of enzyme, present at low concentrations in the preincubation solution, is added to a saturating assay mixture and the cold lability of CTP synthetase can both be eliminated by either (1) the presence of ATP and UTP in the preincubation mixture or (2) increasing the concentration of CTP synthetase in the preincubation mixture. The effects of ATP and UTP can be explained on the basis that the active tetrameric form of the enzyme would prevail under these conditions. Since dimer does not associate to tetramer in the absence of ATP and UTP, even at higher enzyme concentration, the effects of enzyme concentration would suggest that dimer can dissociate reversibly to an inactive monomer and that formation of dimer from monomer under standard assay conditions is slow, thus giving rise to the observed lag. The gel filtration studies show that in the absence of ligands the enzyme can, in fact, dissociate to monomer and that dissociation is favored by dilution and lowered temperature. The decrease in the duration of the lag with increasing enzyme concentration in the saturated assay mixture is also characteristic of an associating enzyme system (Frieden, 1981).

The magnitude of the burst in catalytic activity observed when enzyme preincubated in the absence of ATP and UTP is assayed under nonsaturating conditions is also a function of the concentration of CTP synthetase in the preincubation mixture. A possible explanation consistent with the above discussion is that addition of enzyme in an associated (dimeric) state to a nonsaturated assay mixture is followed by a slow dissociation (as a result of dilution) to an equilibrium mixture of inactive dissociated and active associated species of the enzyme, the position of the equilibrium being dependent upon the concentrations of ATP and UTP in the reaction mixture. The observations that the burst can be eliminated if the enzyme in the preincubation mixture is diluted (shifting the equilibrium toward inactive monomer) whereas the burst is observed at any enzyme concentration if ATP and UTP are present in the preincubation mixture (the enzyme would exist as the fully associated tetramer) support this explanation.

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