Characterization of a Novel dUTP-Dependent Activity of CTP Synthetase from *Saccharomyces cerevisiae*†

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ABSTRACT: CTP synthetase [EC 6.3.4.2, UTP:ammonia ligase (ADP-forming)] from the yeast *Saccharomyces cerevisiae* catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to the C-4 position of UTP to form CTP. In this work, we demonstrated that CTP synthetase utilized dUTP as a substrate to synthesize dCTP. The dUTP-dependent activity was linear with time and with enzyme concentration. Maximum dUTP-dependent activity was dependent on $MgCl_2$ (4 mM) and GTP (K_a = 14 μM) at a pH optimum of 8.0. The apparent K_m values for dUTP, ATP, and glutamine were 0.18, 0.25, and 0.41 mM, respectively. dUTP promoted the tetramerization of CTP synthetase, and the extent of enzyme tetramerization correlated with dUTP-dependent activity. dCTP was a poor inhibitor of dUTP-dependent activity, whereas CTP was a potent inhibitor of this activity. The enzyme catalyzed the synthesis of dCTP and CTP when dUTP and UTP were used as substrates together. CTP was the major product synthesized when dUTP and UTP were present at saturating concentrations. When dUTP and UTP were present at concentrations near their K_m values, the synthesis of dCTP increased relative to that of CTP. The synthesis of dCTP was favored over the synthesis of CTP when UTP was present at a concentration near its K_m value and dUTP was varied from subsaturating to saturating concentrations. These data suggested that the dUTP-dependent synthesis of dCTP by CTP synthetase activity may be physiologically relevant.

The synthesis of deoxyribonucleotides plays an important role in the growth and metabolism of eukaryotic cells (*1*, *2*). The levels of deoxyribonucleotide triphosphates must be controlled for normal synthesis of DNA (*1*, *2*). In particular, the levels of dUTP must be controlled since uracil is not a normal component of DNA. Data indicate that elevated levels dUTP lead to its incorporation into DNA by the action of the DNA polymerase (*3*−*6*). DNA repair enzymes remove uracil from DNA; however, extensive incorporation of uracil into DNA ultimately leads to DNA fragmentation and cell death (*4*−*8*). The cellular levels of dUTP may be controlled through the action of dUTP pyrophosphatase (*5*, *9*). This enzyme is responsible for the conversion of dUTP to dUMP (Figure 1) (*1*). In light of the importance of controlling the levels of dUTP, we considered the possibility that another enzyme, namely, CTP synthetase, may also utilize dUTP as a substrate.

CTP synthetase has been studied extensively because of its role in the synthesis of CTP. This enzyme is a glutamine amidotransferase that catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to the C-4 position of UTP to form CTP (eq 1).

$$\text{UTP} + \text{ATP} + \text{glutamine} \xrightarrow[Mg^{2+}]{\text{GTP}} \text{CTP} + \text{ADP} + P_i + \text{glutamate} \quad (1)$$

GTP is an allosteric effector that accelerates the formation of a covalent glutaminyl enzyme catalytic intermediate (*10*−*12*). CTP synthetase is essential to the growth and metabolism of all organisms because CTP is required for the synthesis of nucleic acids, phospholipids, and sialoglycoproteins (*2*).

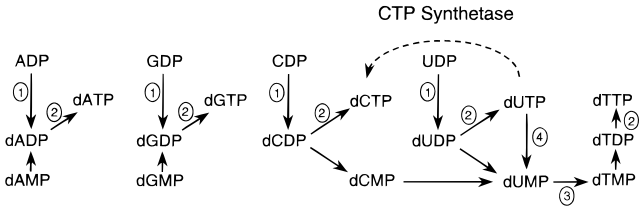

FIGURE 1: Proposed function of CTP synthetase in the deoxyribonucleotide pathway in *S. cerevisiae*. The pathways shown for synthesis of deoxyribonucleotides include the relevant steps discussed in the text. The following enzymes catalyze the indicated reactions: 1, ribonucleotide reductase; 2, nucleoside diphosphate kinase; 3, thymidylate synthase; and 4, dUTP pyrophosphatase. The dashed arrow indicates the proposed function of CTP synthetase in the pathway.

Proper regulation of CTP synthetase activity is of critical importance in all organisms. CTP synthetase is regulated by CTP product inhibition (*10*, *12*, *13*). Several mammalian mutant cell lines possess CTP synthetase activity that is insensitive to inhibition by CTP. As a consequence, these cell lines display complex phenotypes that include resistance to nucleotide analogue drugs used in cancer chemotherapy (*14*−*17*), an increased rate of spontaneous mutations (*14*, *15*, *18*), and increased cellular pools of CTP as well as dCTP (*18*−*20*). The source of dCTP in these mutant cells is unclear. The increased levels of dCTP may be synthesized from CTP via the reaction sequence CTP → CDP → dCDP → dCTP

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(*1*). Another source of dCTP may be the direct conversion of dUTP to dCTP through the action of CTP synthetase (Figure 1).

The hypothesis that CTP synthetase utilizes dUTP to synthesize dCTP was examined with purified *URA7*-encoded CTP synthetase from the yeast *Saccharomyces cerevisiae* (*12*). The *URA7*-encoded enzyme (*21*) contains a conserved glutamine amide transfer domain common to CTP synthetases from mammalian and bacterial organisms (*22*−*25*). The yeast enzyme has been studied extensively with respect to its kinetic and regulatory properties (*12, 26*−*30*). In addition to being regulated by CTP product inhibition (*12*), CTP synthetase activity is regulated by phosphorylation by protein kinases A (*28, 31*) and C (*26, 27*). Using this well-characterized enzyme, we demonstrated that CTP synthetase catalyzed the dUTP-dependent synthesis of dCTP. The enzymological properties of the dUTP-dependent reaction of CTP synthetase were characterized. In addition, we examined conditions in vitro where the dUTP-dependent synthesis of dCTP was favored relative to the UTP-dependent synthesis of CTP. These studies suggested that the dUTP-dependent activity of CTP synthetase may be physiologically relevant.

EXPERIMENTAL PROCEDURES

Materials. Growth medium supplies were from Difco. Nucleotides, glutamine, molecular mass standards for gel filtration chromatography, and bovine serum albumin were purchased from Sigma. Protein assay reagent and molecular mass standards for SDS−polyacrylamide gel electrophoresis were purchased from Bio-Rad. Superose 6, ultrapure deoxyribonucleotide triphosphate standards, and radiochemicals were purchased from Amersham Pharmacia Biotech. Reagents for electrophoresis and scintillation counting supplies were purchased from National Diagnostics. Microcon-30 filters were purchased from Millipore. The Partisil 10 SAX high-performance liquid chromatography (HPLC)[1] column and SAX guard column were purchased from Markson LabSales. HPLC-grade water and acetonitrile were purchased from Fisher Scientific.

Purification of CTP Synthetase. Cells overexpressing the wild-type *URA7*-encoded CTP synthetase were used for enzyme purification (*12*). CTP synthetase was purified to apparent homogeneity by ammonium sulfate fractionation of the cytosolic fraction followed by chromatography with Sephacryl 300 HR, Q-Sepharose, Affi-Gel Blue, and Superose 6 as described by Yang et al. (*12*). The specific activity of the CTP synthetase preparation used in this study was 1.3 units/mg.

Enzyme Assays and Analysis of Kinetic Data. dUTP-dependent CTP synthetase activity was determined by measuring the conversion of dUTP to dCTP by following the increase in absorbance at 291 nm on a recording spectrophotometer. Extinction coefficients of 137 and 1801 M^{-1} cm^{-1} were used for dUTP and dCTP, respectively. These values were determined by plots of the absorbance at 291 nm versus the concentration of dUTP and dCTP, respectively, at pH 8.0. The standard reaction mixture for dUTP-dependent CTP synthetase activity contained 50 mM Tris-HCl (pH 8.0), 2 mM dUTP, 2 mM ATP, 2 mM glutamine, 0.1 mM GTP, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and an appropriate dilution of enzyme protein in a total volume of 0.1 mL. UTP-dependent CTP synthetase activity was determined spectrophotometrically at 291 nm by measuring the rate of conversion of UTP to CTP (molar extinction coefficients of 182 and 1520 M^{-1} cm^{-1}, respectively) (*10*). The UTP-dependent CTP synthetase reaction mixture was the same as the dUTP-dependent reaction mixture except that 2 mM UTP was used instead of dUTP. Enzyme assays were performed in triplicate with an average standard deviation of ±3%. All assays were linear with time and protein concentration. A unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min. Protein was determined by the method of Bradford (*32*) using bovine serum albumin as the standard. Kinetic data were analyzed according to the Michaelis−Menten and Hill equations using the EZ-FIT Enzyme Kinetic Model Fitting Program (*33*). IC_{50} values were calculated from plots of the log of activity versus the inhibitor concentration.

HPLC Analysis of CTP Synthetase Reaction Products. CTP synthetase reaction products were determined by HPLC using the method of Mole et al. (*34*). Enzyme reactions were terminated by the addition of 0.2 mL of 0.3 M ammonium phosphate/acetonitrile (10:1, pH 5.55). The reaction mixtures were then filtered through Microcon-30 centrifuge filters. Samples (0.1 mL) were then subjected to analytical HPLC using a Partisil 10 SAX column (250 × 4.6 mm, inner diameter) with a SAX guard column. The HPLC column was equilibrated and eluted with 0.26 M ammonium phosphate/acetonitrile (10:1, pH 5.55) at a flow rate of 2 mL/min. The identity of the reaction products was determined by comparing elution profiles with those of authentic standards using an ultraviolet detector ($A_{265\ nm}$). The concentrations of dCTP and CTP were determined from standard curves using ultrapure standards.

Tetramerization of CTP Synthetase. The nucleotide-dependent tetramerization of the dimeric form of CTP synthetase was analyzed by Superose 6 gel filtration chromatography as described by Pappas et al. (*29*). A Superose 6 column (1 × 24 cm), attached to a Pharmacia fast protein liquid chromatography system, was equilibrated and eluted with 50 mM Tris-HCl (pH 8.0), 2 mM glutamine, 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 0.1 mM GTP in the presence of the indicated concentrations of nucleotides at 5 °C. The column was calibrated with Blue Dextran 2000 (for the void volume), thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). Purified CTP synthetase was incubated in the Superose 6 chromatography buffers (0.1 mL total volume) for 5 min and then applied and eluted from the Superose 6 column at a flow rate of 15 mL/h. Fractions (0.47 mL) were collected and analyzed for CTP synthetase protein by SDS−polyacrylamide gel electrophoresis (*35*) using 10% slab gels. Molecular mass standards were phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). CTP synthetase protein in column fractions was quantified by scanning densitometry of silver-stained (*36*) SDS−polyacrylamide gels.

[1] Abbreviation: HPLC, high-performance liquid chromatography.

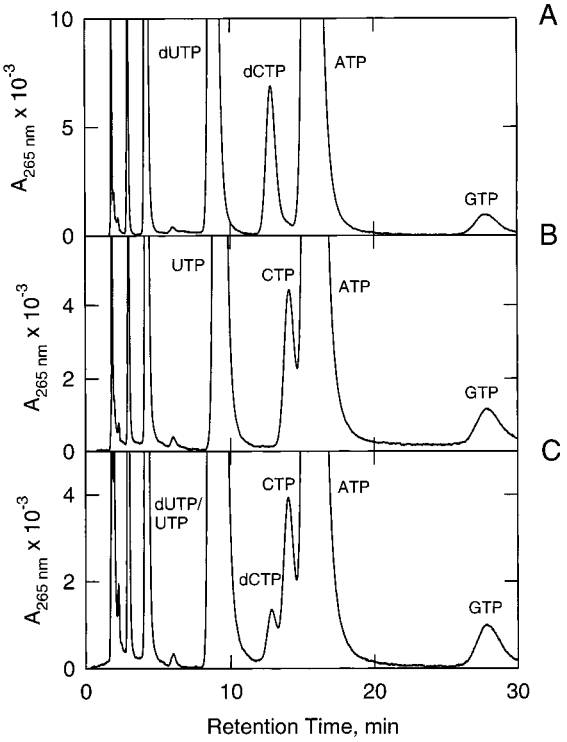

FIGURE 2: HPLC analysis of CTP synthetase reaction products. CTP synthetase activity was measured with 2 mM dUTP (panel A), 2 mM UTP (panel B), and 2 mM dUTP + 2 mM UTP (panel C). After incubation for 10 min, the reaction was terminated, and the mixture was analyzed by HPLC. The concentrations of ATP, glutamine, GTP, and $MgCl_2$ were maintained at 2, 2, 0.1, and 10 mM, respectively. The elution positions of dUTP, dCTP, UTP, CTP, ATP, and GTP are indicated in the figure. The elution positions of dUTP and UTP were too close to be identified separately when both substrates were present in the reaction (panel C). The elution profiles shown in the figure are representative of duplicate experiments.

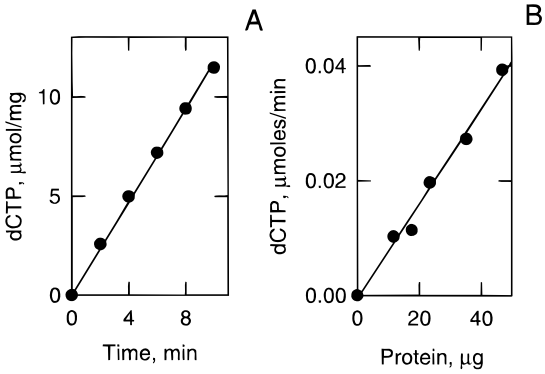

FIGURE 3: Time course and enzyme concentration dependence of the dUTP-dependent CTP synthetase reaction. The dependence of dUTP-dependent CTP synthetase activity on time (panel A) and enzyme concentration (panel B) was measured using the spectrophotometric assay under standard assay conditions. The data points were calculated from a continuous recorder tracing monitoring the conversion of dUTP to dCTP. The lines drawn were the result of a least-squares analysis of the data.

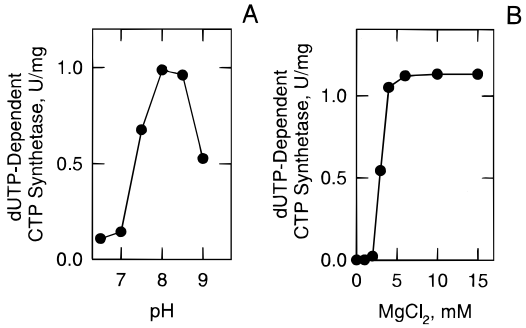

FIGURE 4: Effect of pH and $MgCl_2$ on dUTP-dependent CTP synthetase activity. dUTP-dependent CTP synthetase activity was measured using the spectrophotometric assay under standard assay conditions at the indicated pH values with 50 mM Tris−maleate−glycine buffer (panel A) and the indicated concentrations of $MgCl_2$ (panel B).

RESULTS

HPLC Analysis of CTP Synthetase Reaction Products. The purified *URA7*-encoded CTP synthetase from *S. cerevisiae* was examined for its ability to utilize dUTP as a substrate. The conversion of dUTP to dCTP was analyzed by HPLC. Indeed, the enzyme catalyzed the formation of dCTP from dUTP (Figure 2A). The HPLC assay was also used to analyze the CTP synthetase reaction products when UTP (Figure 2B) and dUTP + UTP (Figure 2C) were used as substrates in the reaction. The HPLC assay was primarily used to examine the CTP synthetase reaction products when dUTP + UTP were used as substrates together (see below).

Enzymological Properties of dUTP-Dependent CTP Synthetase Activity. The enzymological properties of dUTP-dependent CTP synthetase activity were examined with purified enzyme. The rate of conversion of dUTP to dCTP was routinely measured by following the increase in absorbance at 291 nm on a recording spectrophotometer. The spectrophotometric assay for dUTP-dependent CTP synthetase activity was convenient and was linear with time (Figure 3A) and with enzyme concentration (Figure 3B). Activity was measured with a Tris−maleate−glycine buffer from pH 6.5 to 9.0. The pH optimum for the reaction was 8.0 (Figure 4A). The addition of magnesium ions to the assay mixture resulted in a dose-dependent stimulation of dUTP-dependent CTP synthetase activity (Figure 4B). Maximum activity was obtained at a $MgCl_2$ concentration of 4 mM. At 4 mM $MgCl_2$ and pH 8.0, all of the nucleotides present in the assay are present as magnesium−nucleotide complexes (*37*). The sigmoidal shape of the curve in Figure 4B was likely due to the formation of magnesium−nucleotide complexes and the subsequent cooperative binding of magnesium−nucleotide to CTP synthetase (*12*). Manganese, calcium, or cobalt ions could not substitute for the magnesium ion requirement for dUTP-dependent activity.

A kinetic analysis was performed to examine the dependence of dUTP-dependent CTP synthetase activity on the substrates dUTP, ATP, and glutamine, and the activator GTP. The enzyme catalyzes a complex reaction where the concentration of one substrate affects the kinetic behavior of the enzyme with respect to another substrate (*12*). Furthermore, the dependence of activity on one substrate using a subsaturating concentration of another substrate results in cooperative kinetic behavior (*12*). To simplify our kinetic experiments, the dependence of activity on a specific reaction component was examined using saturating concentrations of the other reaction components. dUTP-dependent CTP synthetase activity exhibited positive cooperative (Hill number = 2.4) kinetics with respect to dUTP (Figure 5A). An analysis of the data according to the Hill equation yielded an apparent K_m value for dUTP of 0.18 mM and a V_{max} value

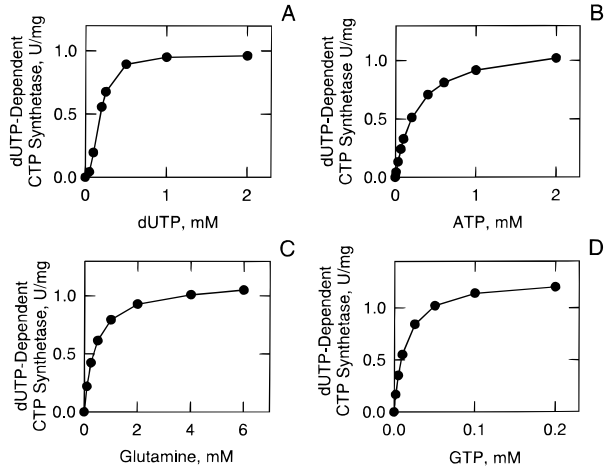

FIGURE 5: Dependence of dUTP-dependent CTP synthetase activity on the concentrations of dUTP, ATP, glutamine, and GTP. dUTP-dependent CTP synthetase activity was measured using the spectrophotometric assay as a function of the indicated concentrations of dUTP (panel A), ATP (panel B), glutamine (panel C), and GTP (panel D). For each experiment, the concentrations of the other reaction components were held constant at saturating concentrations.

of 1.0 unit/mg. The enzyme exhibited saturation kinetics with respect to ATP (Figure 5B). An analysis of the data according to the Michaelis−Menten equation yielded an apparent K_m value for ATP of 0.25 mM and a V_{max} value of 1.2 units/mg. dUTP-dependent CTP synthetase activity followed saturation kinetics with respect to glutamine (Figure 5C). The apparent K_m and V_{max} values were 0.41 mM and 1.1 units/mg, respectively. The addition of GTP to the assay system resulted in a dose-dependent stimulation of dUTP-dependent CTP synthetase activity (Figure 5D). The apparent K_a value for GTP was 14 μM, and the apparent V_{max} was 1.3 units/mg.

Effects of dCTP and CTP on dUTP-Dependent CTP Synthetase Activity. UTP-dependent CTP synthetase activity is potently inhibited by its product CTP (*12, 27, 28*). We examined whether dUTP-dependent CTP synthetase activity was inhibited by the product dCTP. dUTP-dependent CTP synthetase activity was measured in the absence and presence of dCTP. These experiments were performed using a subsaturating concentration of dUTP (0.2 mM). By using a concentration of dUTP near its K_m value, we could more readily observe inhibitory effects of dCTP on activity. dCTP was a weak inhibitor of the dUTP-dependent activity of the enzyme (Figure 6A). In addition, dCTP was a weak inhibitor of the UTP-dependent activity (Figure 6B). At a final concentration of 0.7 mM, only 25% and 22% of the dUTP-dependent and the UTP-dependent activities of CTP synthetase were inhibited, respectively. We examined the effect of CTP on dUTP-dependent CTP synthetase activity. CTP potently inhibited activity in a dose-dependent manner with an IC_{50} value of 0.16 mM (Figure 6A). The inhibitory effect of CTP on the dUTP-dependent activity was greater that the inhibitory effect of CTP (IC_{50} = 0.32 mM) on the UTP-dependent activity (Figure 6B) (*12*).

Effects of dUTP and dCTP on the Tetramerization of CTP Synthetase. In addition to serving as substrates for CTP synthetase, UTP and ATP are responsible for the tetramerization and activation of the inactive dimeric form of the enzyme (*12, 29*). UTP is absolutely required for the tetramerization of the enzyme when ATP is present at a saturating concentration (*29*). At 2 mM ATP, the maximum amount of CTP synthetase tetramerization occurs at a UTP concentration of between 0.2 and 0.3 mM (*29*). We examined the effect of 0.2 mM dUTP on the tetramerization of the enzyme in the presence of 2 mM ATP. The oligomeric forms of CTP synthetase were analyzed by Superose 6 gel filtration chromatography as described under Experimental Procedures. Under these conditions, 60% of the total enzyme protein existed as a tetramer (Figure 7A). As previously described (*29*), 84% of the total enzyme protein existed as a tetramer when 0.2 mM UTP was used to promote tetramerization (Figure 7A). These results correlated with the extent of dUTP-dependent activity (0.55 unit/mg) when compared with UTP-dependent activity (0.76 unit/mg) when 0.2 mM dUTP and 0.2 mM UTP, respectively, were used as substrates (Figure 7A).

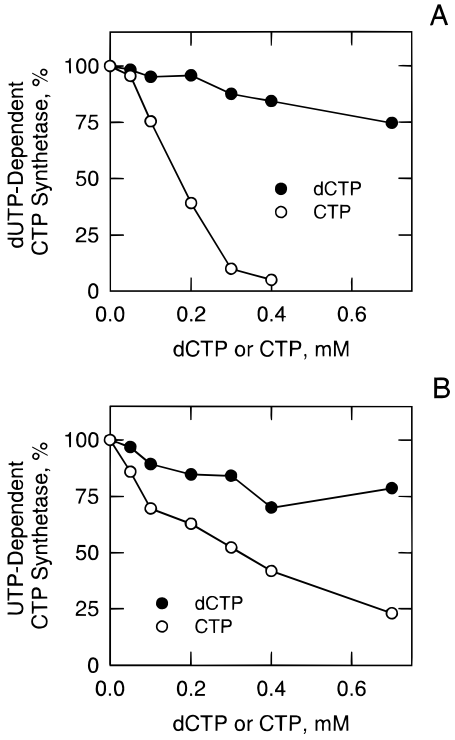

FIGURE 6: Effects of dCTP and CTP on dUTP-dependent CTP synthetase activity and on UTP-dependent CTP synthetase activity. (Panel A) dUTP-dependent CTP synthetase activity was measured using the spectrophotometric assay with 0.2 mM dUTP in the absence and presence of the indicated concentrations of dCTP or CTP. (Panel B) UTP-dependent CTP synthetase activity was measured using the spectrophotometric assay with 0.2 mM UTP in the absence and presence of the indicated concentrations of dCTP or CTP. The concentrations of ATP, glutamine, GTP, and $MgCl_2$ were maintained at 1, 2, 0.1, and 10 mM, respectively.

Although CTP is a potent inhibitor of UTP-dependent CTP synthetase activity (*12*), the mechanism of inhibition does not involve the inhibition of the UTP/ATP-dependent tetramerization of the enzyme (*12, 29*). In fact, CTP can substitute for UTP in promoting the tetramerization of CTP synthetase (*29*). This is likely due to the structural resemblance of CTP to UTP (*29*). dCTP could also substitute for UTP (and dUTP) in promoting the tetramerization of CTP synthetase (Figure 7B). However, the concentration of dCTP (1 mM) required to promote the tetramerization of about 70% of the total amount of CTP synthetase was 10-fold higher than the concentration of CTP required to promote about

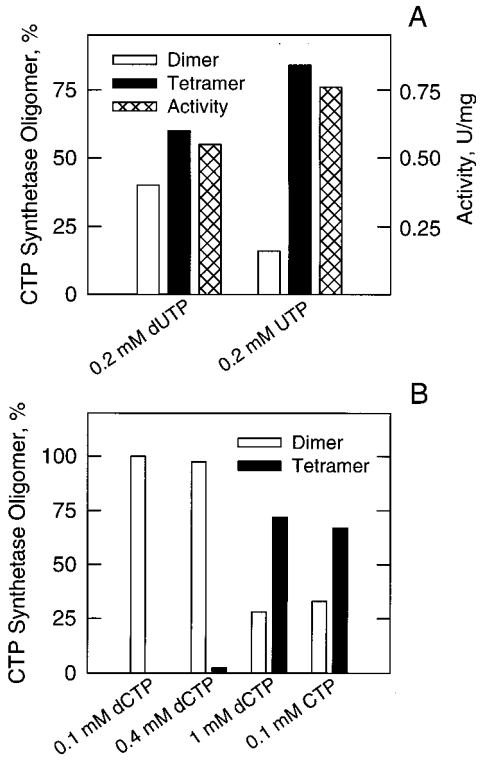

FIGURE 7: Effects of dUTP and dCTP on the tetramerization of CTP synthetase. Purified CTP synthetase (50 μg) was subjected to Superose 6 chromatography in the presence of dUTP or UTP (panel A) and in the presence of dCTP or CTP (panel B). The relative amounts of the dimeric and tetrameric forms of CTP synthetase were determined by quantifying the amount of protein in the column fractions by SDS−polyacrylamide gel electrophoresis and densitometry of silver-stained gels. CTP synthetase activity was measured using the spectrophotometric assay with 0.2 mM dUTP or with 0.2 mM UTP as a substrate. The concentrations of ATP, glutamine, GTP, and $MgCl_2$ were 2, 2, 0.1, and 10 mM, respectively. The data presented are an average of duplicate experiments.

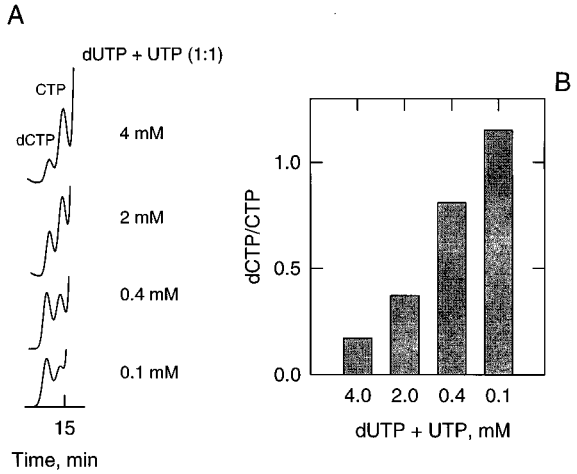

FIGURE 8: HPLC analysis of the CTP synthetase reaction products using dUTP + UTP as substrates. (Panel A) CTP synthetase reactions were performed using the indicated total concentrations of dUTP + UTP at a molar ratio of 1:1. The concentrations of ATP, glutamine, GTP, and $MgCl_2$ were 2, 2, 0.1, and 10 mM, respectively. After incubation for 10 min, the reactions were terminated, and the mixtures were analyzed by HPLC. A portion of each of the chromatograms is shown indicating the elution positions of dCTP and CTP. (Panel B) The molar concentrations of dCTP and CTP were calculated from each of the experiments described in panel A and were plotted as the ratio of dCTP to CTP versus the total concentration of dUTP + UTP.

the same amount of enzyme tetramerization (Figure 7B). These data correlated with the weak inhibitory effect that dCTP had on the dUTP-dependent and UTP-dependent activities of CTP synthetase. Taken together, this suggested that the enzyme has a weak affinity for dCTP when compared with CTP.

CTP Synthetase Reaction Products Using dUTP + UTP as Substrates. If the dUTP-dependent activity of CTP synthetase was to be relevant in vivo, the enzyme should be able to catalyze the synthesis of dCTP from dUTP in the presence of UTP. We examined the reaction products of CTP synthetase when dUTP + UTP were present in the reaction together. In the experiments shown in Figure 8, the sum of the molar concentrations of dUTP + UTP (1:1) was varied from saturating to subsaturating concentrations of each substrate. The apparent K_m values for dUTP and UTP with this enzyme preparation were 0.16 and 0.18 mM, respectively. Following incubation for 10 min, the products of the reaction were analyzed by HPLC (Figure 8A). CTP was the major product synthesized by the enzyme when dUTP + UTP were present at a final concentration of 4 mM. As the total concentration of dUTP + UTP was reduced to concentrations near the K_m values for both substrates, the amount of dCTP synthesized relative to CTP increased (Figure 8B). We also examined the synthesis of dCTP and CTP when the concentration of dUTP was varied from subsaturating to saturating concentrations and the concentration of UTP was held constant at a concentration (0.2 mM) near its apparent K_m value. Under these conditions, we could more readily observe the competition between dUTP and UTP as substrates for the enzyme. The addition of dUTP to the reaction mixture resulted in a dose-dependent increase in the amount of dCTP synthesized relative to CTP (Figure 9A). The enzyme synthesized about equal amounts of dCTP and CTP when the dUTP concentration was near its apparent K_m value (Figure 9B). dCTP was the major product synthesized by the enzyme when dUTP was added to the reaction mixtures at concentrations above 0.2 mM UTP (Figure 9B).

DISCUSSION

CTP synthetase is an essential enzyme in *S. cerevisiae* (*38*) because the product of the reaction, CTP, is required for the synthesis of nucleic acids, phospholipids, and sialoglycoproteins (*2*). The enzyme also plays an important role in the growth and metabolism of mammalian cells (*19*, *39*, *40*). Although the enzyme is generally considered to be responsible for the synthesis of CTP, we considered the hypothesis that CTP synthetase also catalyzed the synthesis of dCTP from dUTP. Using purified CTP synthetase from *S. cerevisiae*, we demonstrated that the enzyme exhibited a dUTP-dependent activity. To our knowledge, this is the first report of this activity for CTP synthetase from any organism. The enzymological properties of the dUTP-dependent activity were generally similar to those of the UTP-dependent activity of the enzyme (*12*). The UTP/ATP-dependent tetramerization of CTP synthetase is required for enzyme activity (*29*), and, indeed, dUTP promoted the tetramerization of the enzyme. The extent of tetramerization using dUTP as a substrate correlated with the dUTP-dependent activity of the enzyme.

The dUTP-dependent and UTP-dependent activities of CTP synthetase differed with respect to inhibition by the

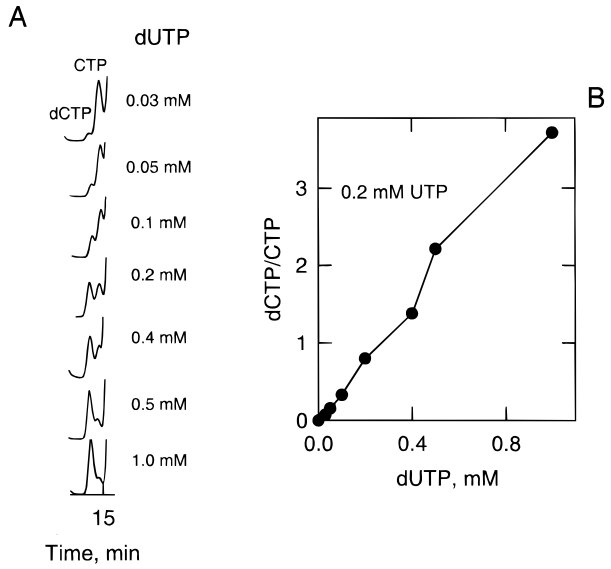

FIGURE 9: Effect of dUTP concentration on the CTP synthetase reaction products using dUTP + UTP as substrates. (Panel A) CTP synthetase reactions were performed using 0.2 mM UTP and the indicated concentrations of dUTP. The concentrations of ATP, glutamine, GTP, and $MgCl_2$ were 2, 2, 0.1, and 10 mM, respectively. After incubation for 10 min, the reactions were terminated, and the mixtures were analyzed by HPLC. A portion of each of the chromatograms is shown indicating the elution positions of dCTP and CTP. (Panel B) The molar concentrations of dCTP and CTP were calculated from the experiments described in panel A and were plotted as the ratio of dCTP to CTP versus the concentration of dUTP.

products of the respective reactions. Whereas dCTP was a poor inhibitor of the dUTP-dependent activity, CTP inhibits the UTP-dependent activity (*12*). CTP was also a potent inhibitor of the dUTP-dependent activity whereas dCTP was a poor inhibitor of the UTP-dependent activity. The differential effects of dCTP and CTP on the two activities of CTP synthetase may play a role in regulating the relative synthesis of dCTP and CTP from their respective substrates. When the concentrations of both dUTP + UTP were present at saturating concentrations, the synthesis of CTP was favored over that of dCTP. This was not due to differences in the apparent K_m values for dUTP and UTP. The K_m values for both substrates were about the same. Instead, the preferred utilization of UTP over dUTP at saturating concentrations may be attributed to the synthesis of CTP through the UTP-dependent reaction and the subsequent inhibition of the dUTP-dependent reaction by CTP. On the other hand, when dUTP + UTP were present at concentrations near their K_m values, the synthesis of dCTP increased relative to that of CTP. Under these conditions, the levels of CTP synthesized by the UTP-dependent reaction over the course of the reaction would be expected to be lower than the inhibitor constant for CTP of the dUTP-dependent reaction. This condition, coupled to the fact that dCTP was a poor inhibitor of dUTP-dependent activity, would favor the synthesis of dCTP. This hypothesis was further supported by the fact that the synthesis of dCTP was favored when UTP was present at a concentration near its K_m value and dUTP was varied from subsaturating to saturating concentrations.

We propose that the dUTP-dependent activity of CTP synthetase may be a mechanism by which dCTP is derived directly from dUTP in vivo. The K_m value for UTP is within the range of its cellular concentration (*12, 30, 41*). Thus, fluctuations in UTP concentration could affect both the dUTP-dependent and UTP-dependent activities of CTP synthetase as discussed above. A similar argument could be made for fluctuations in dUTP levels. Technical difficulties have precluded the determination of the cellular levels of dUTP in *S. cerevisiae* (*42*). However, based on the cellular concentrations of other deoxyribonucleotides (*42, 43*) relative to the cellular concentration of ribonucleotides (*12, 30, 41*), one would expect that dUTP levels in *S. cerevisiae* would be much lower than that of UTP. Yet, our in vitro studies showed that even when the concentration of UTP was much greater than that of dUTP, CTP synthetase still synthesized a small amount of dCTP.

Misregulation of enzymes in the deoxyribonucleotide pathway could lead to an increase in dUTP levels. For example, treatment of animal cells with the thymidylate synthase inhibitor methotrexate results in an accumulation of the cellular levels of dUTP (*8*). An increase in dUTP levels, especially if they were concentrated in a specific region of the cell, where CTP synthetase is localized, may regulate the dUTP-dependent activity of the enzyme. Interestingly, a *S. cerevisiae* mutant defective in thymidylate synthase accumulates elevated levels of dCTP (*42*). The elevated levels of dCTP in these cells could be derived from dUTP via the dUTP-dependent activity of CTP synthetase. It should be noted that the levels of other deoxyribonucleotides also increase due to the misregulation of thymidylate synthase (*8, 42*). The regulation of the pathway for the synthesis of deoxyribonucleotides in eukaryotic cells is complex and not fully understood (*1,2*).

The metabolism of dUTP plays a critical role in cell physiology. Elevated levels of dUTP lead to its incorporation into DNA, DNA fragmentation, and cell death (*3*−*6, 8*). Cellular dUTP levels in *S. cerevisiae* are controlled by dUTP pyrophosphatase (*9*). The utilization of dUTP by CTP synthetase may be another mechanism by which dUTP levels are controlled in the cell. The dUTP-dependent activity of CTP synthetase was weakly inhibited by the product dCTP. This would be an important feature if a function of the enzyme were to reduce the levels of dUTP in the cell.

In addition to being used for DNA synthesis, dCTP can be used for the synthesis of membrane phospholipids (*44*). The cytidylyltransferase enzymes responsible for the synthesis of CDP-diacylglycerol (*45*), CDP-choline (*46*−*49*), and CDP-ethanolamine (*46, 48, 49*) utilize dCTP as a substrate to form the deoxyribonucleotide derivatives of these phospholipid pathway intermediates. Moreover, purified phosphatidylserine synthase from *S. cerevisiae* utilizes dCDP-diacylglycerol as a substrate to synthesize phosphatidylserine (*50*). Data suggest that the pools of dCTP used for phospholipid synthesis are different from those used for DNA synthesis (*44, 49, 51, 52*). The dUTP-dependent CTP synthetase reaction may be involved in the synthesis of the different pools of dCTP used for the synthesis of phospholipids and DNA.

In summary, we identified a novel dUTP-dependent activity of the CTP synthetase from *S. cerevisiae*. We characterized the enzymological properties of dUTP-dependent activity and demonstrated conditions in vitro where the synthesis of dCTP was favored when dUTP and UTP were present together. The hypothesis that the dUTP-dependent activity of CTP synthetase has physiological relevance is

provocative. However, there are a number of questions that need to be addressed before the physiological relevance of the in vitro experiments reported in this work is established.

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