Purification and Characterization of CTP Synthetase, the Product of the *URA7* Gene in *Saccharomyces cerevisiae*†

Weng-Lang Yang,‡ Virginia M. McDonough,‡ Odile Ozier-Kalogeropoulos,§ Marie-Thérèse Adeline,‖ Mark T. Flocco,⊥ and George M. Carman*,‡

Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey 08903, Centre de Génétique Moléculaire du CNRS, Laboratoire propre associé à l'Université Pierre et Marie Curie, 91198 Gif-sur-Yvette Cedex, France, Institut de Chimie des Substances Naturelles, 91198 Gil-sur-Yvette Cedex, France, and Bioanalytical Systems Group, Beckman Instruments, Somerset, New Jersey 08875

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ABSTRACT: In the yeast *Saccharomyces cerevisiae*, CTP synthetase [EC 6.3.4.2; UTP:ammonia ligase (ADP-forming)] is the product of the *URA7* gene. CTP synthetase was purified 503-fold to apparent homogeneity from cells bearing the *URA7* gene on a multicopy plasmid that directed a 10-fold overproduction of the enzyme. The purification procedure included ammonium sulfate fractionation of the cytosolic fraction followed by chromatography with Sephacryl 300 HR, Q-Sepharose, Affi-Gel Blue, and Superose 6. The N-terminal amino acid sequence of purified CTP synthetase was identified and aligned perfectly with the deduced sequence of the *URA7* gene. The minimum subunit molecular mass (68 kDa) of purified CTP synthetase was in good agreement with the size (64.7 kDa) of the *URA7* gene product. Antibodies were raised against a maltose-binding protein–CTP synthetase fusion protein which immunoprecipitated CTP synthetase from wild-type cells. Immunoblot analysis was used to identify CTP synthetase in wild-type cells and cells bearing the *URA7* gene on a multicopy plasmid. The results of gel filtration chromatography indicated that the size of native CTP synthetase was consistent with a dimeric structure for the enzyme. CTP synthetase oligomerized to a tetramer in the presence of its substrates UTP and ATP. Maximum CTP synthetase activity was dependent on magnesium ions (4 mM) and 2-mercaptoethanol at the pH optimum of 8.0. CTP synthetase exhibited positive cooperative kinetics with respect to UTP and ATP and negative cooperative kinetics with respect to glutamine and GTP. CTP synthetase was potently inhibited by the product CTP which also increased the positive cooperativity of the enzyme toward UTP. In wild-type cells, the cellular concentration of CTP was 2-fold higher than the apparent inhibitor constant determined using pure enzyme. The cellular CTP concentration in cells that overexpressed CTP synthetase was only 2.8-fold higher than the CTP concentration found in wild-type cells. These results raised the suggestion that CTP synthetase activity in *S. cerevisiae* may be regulated by CTP *in vivo*.

The nucleotide CTP plays an essential role in the growth and metabolism of all organisms. CTP is used in the synthesis of nucleic acids (Traut, 1988) as well as membrane phospholipids (Kennedy, 1986). The enzyme responsible for the *de novo* synthesis of CTP is CTP synthetase [EC 6.3.4.2; UTP:ammonia ligase (ADP-forming)] (Liberman, 1956; Long & Pardee, 1967). CTP synthetase is a glutamine amidotransferase that catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to C-4 of UTP to form CTP (reaction 1). GTP is an allosteric effector which accelerates

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

the formation of a covalent glutaminyl enzyme catalytic intermediate (Levitzki & Koshland, 1972a; Long & Pardee, 1967). Ammonia can substitute for glutamine and GTP, but at nonphysiological concentrations (Levitzki & Koshland, 1972a; Long & Pardee, 1967). Genes encoding for CTP synthetase have been cloned and sequenced from *Escherichia coli* (Weng et al., 1986), *Bacillus subtilis* (Trach et al., 1988), *Saccharomyces cerevisiae* (Ozier-Kalogeropoulos et al., 1991, 1994), and human (Yamauchi et al., 1990). The deduced protein product of these genes (Ozier-Kalogeropoulos et al., 1991, 1994; Trach et al., 1988; Weng et al., 1986; Yamauchi et al., 1990) contains conserved glutamine amide transfer domains characteristic of glutamine amidotransferases.

Regulation of CTP synthetase activity should play a major role in nucleotide and phospholipid metablism and cellular growth in general. Detailed studies on the mode of action and regulation of CTP synthetase activity are dependent on the availability of highly purified preparations of the enzyme. CTP synthetase has been purified to apparent homogeneity from *Escherichia coli* (Anderson, 1983; Long & Pardee, 1967). The *E. coli* enzyme has been extensively characterized with respect to its physical, enzymological, and kinetic properties (Levitzki & Koshland, 1969, 1971, 1972a,b; Lewis & Villafranca, 1989; Long & Pardee, 1967; Robertson & Villafranca, 1993; von der Saal et al., 1985). These studies have shown that CTP synthetase exhibits complex kinetic behavior characteristic of allosteric enzymes. Whereas CTP synthetase has been purified to apparent homogeneity from

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* To whom correspondence and reprint requests should be addressed.
‡ Rutgers University.
§ Centre de Genetique Moleculaire du CNRS and Institut de Chimie des Substances Naturelles.
‖ Institut de Chimie des Substances Naturelles.
⊥ Beckman Instruments.

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rat liver (Thomas et al., 1988), little is known about the kinetic properties of this eucaryotic enzyme.

Owing to its amenable molecular genetic system, we are using the yeast *S. cerevisiae* as a model eucaryote to study the regulation of CTP synthetase. As an initial step toward understanding the regulation of eucaryotic CTP synthetase activity, we report in this paper the purification and characterization of the enzyme from *S. cerevisiae*. In *S. cerevisiae*, CTP synthetase is encoded by the *URA7* gene (Ozier-Kalogeropoulos et al., 1991). The purification of CTP synthetase was facilitated by using a strain bearing the *URA7* gene on a multicopy plasmid that directed a 10-fold overproduction of the enzyme. The N-terminal amino acid sequence of the purified CTP synthetase was identified and aligned perfectly with the deduced sequence of the *URA7* gene. Antibodies raised against a fusion protein translated from coding sequences of the *URA7* gene recognized pure CTP synthetase, which further substantiated this gene–product relationship. The enzymological and kinetic properties of CTP synthetase are reported. The pure enzyme was potently inhibited by the product CTP. The apparent inhibitor constant determined for CTP using pure enzyme was below the cellular concentration of CTP in wild-type cells. While the cellular concentration of CTP was elevated in cells which overexpressed CTP synthetase, the increase in CTP concentration was reduced relative to the overexpression of the enzyme. These results raised the suggestion that the inhibition of CTP synthetase activity by CTP may be physiologically relevant in *S. cerevisiae*.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were reagent grade. Growth medium supplies were purchased from Difco Laboratories. Nucleotides, L-glutamine, phenylmethanesulfonyl fluoride, benzamide, aprotinin, leupeptin, peptstatin, molecular mass standards for gel filtration chromatography, poly(ethylenimine)–cellulose plates, and bovine serum albumin were purchased from Sigma. Poly(vinylidene difluoride) paper was purchased from Millipore. Centricon-10 concentration filters were purchased from Amicon. Radiochemicals and EN^{3}HANCE spray were purchased from Du Pont–New England Nuclear. Protein assay reagent, Affi-Gel Blue, DEAE-Affi-Gel Blue, molecular mass standards for SDS–polyacrylamide gel electrophoresis, electrophoresis reagents, and immunochemical reagents were purchased from Bio-Rad. Protein A–Sepharose CL-4B, Sephacryl 300 HR, Q-Sepharose, Superose 6, and Superdex 200 HR were purchased from Pharmacia LKB Biotechnology, Inc. Restriction enzymes and the protein fusion (Maina et al., 1988) kit used to make maltose-binding protein–CTP synthetase fusion protein were purchased from New England Biolabs.

Strains and Growth Conditions. CTP synthetase was purified from *S cerevisiae* mutant strain OK8 (*MATα leu2 trp1 ura3 ura7Δ::TRP1 ura8*) bearing the plasmid pFL44S-*URA7* (Ozier-Kalogeropoulos et al., 1991, 1994). This plasmid directs the overexpression of CTP synthetase activity (Ozier-Kalogeropoulos et al., 1991). Mutant strain OK8 bears a mutation in the *URA8* gene which is a duplicate gene encoding for CTP synthetase (Ozier-Kalogeropoulos et al., 1994). Strain OK8 and wild-type strain FL100 (*MAT***a**, ATCC 28383) were grown in complete synthetic medium (Culbertson & Henry, 1975) without uracil at 30 °C. For enzyme purification, cells were grown in 1.5-L batches of growth medium at 30 °C on a rotary shaker at 250 rpm. Cells were grown to the exponential phase of growth and harvested by centrifugation. Cell numbers were determined by microscopic examination with a hemacytometer.

Escherichia coli strain DH5α was used for the propagation of plasmids and the production of maltose-binding protein–CTP synthetase fusion protein directed by the inducible pMAL-c2-*URA7* plasmid. For the production of fusion protein, *E. coli* cells were grown at 37 °C in 1-L batches of 0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.2% glucose, and 100 μg/mL ampicillin. When the cell density reached 2×10^8 CFU/mL, the expression of the fusion protein was induced by the addition of 0.3 mM isopropyl β-D-thiogalactoside to the growth medium. After incubation for 2 h, the induced cells were harvested by centrifugation and frozen at −80 °C.

Enzyme Assays and Product Identification. CTP synthetase activity was determined at 30 °C by measuring the conversion of UTP to CTP (molar extinction coefficients of 182 and 1520 M^{-1} cm^{-1}, respectively) by following the increase in absorbance at 291 nm on a recording spectrophotometer (Long & Pardee, 1967). The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.1 mM GTP, 2 mM ATP, 2 mM UTP, and an appropriate dilution of enzyme protein in a total volume of 0.2 mL. Alternatively, CTP synthetase activity was measured by following the formation of ^{3}H-labeled CTP from [5,6-^{3}H]UTP (6 μCi/μmol) (Williams et al., 1978) under the assay conditions described above. CTP was analyzed by thin-layer chromatography on poly(ethylenimine)–cellulose plates using the solvent system 2.0 N acetic acid/2.0 M lithium chloride (1:1) (Ozier-Kalogeropoulos et al., 1991). The positions of the labeled substrate and product on the chromatograms were determined by fluorography using EN^{3}HANCE and compared with standard UTP and CTP. 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 CTP per minute under the assay conditions described above. Specific activity was defined as units per milligram of protein.

Protein Determination. Protein was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Buffers which were identical to those containing the protein samples were used as blanks. Protein was monitored during purification on columns by measuring the absorbance at 280 nm.

Purification of CTP Synthetase. All steps were performed at 5 °C.

Step 1: *Preparation of Cytosol.* Freshly harvested cells were disrupted with glass beads with a Bead–Beater (Biospec Products) in buffer A [50 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA, 20 mM L-glutamine, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamide, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 6 μg/mL pepstatin] as described previously (Fischl & Carman, 1983). Glass beads and unbroken cells were removed by centrifugation at 1500*g* for 5 min. The supernatant was then centrifuged at 100000*g* for 1.5 h to obtain the cytosolic fraction.

Step 2: *Ammonium Sulfate Fractionation.* The cytosolic fraction was adjusted to a protein concentration of 4.5 mg/mL by the addition of buffer A. Enzyme-grade ammonium sulfate was added to the cytosol to 45% saturation (27.7 g/100 mL) with slow stirring. After all of the ammonium sulfate was dissolved, the solution was stirred slowly for an additional

20 min. The precipitate was removed by centrifugation at 12000*g* for 20 min, dissolved in a minimum volume of buffer A, and stored at −20 °C until used for further purification. This fractionation step yielded a 50% recovery of CTP synthetase activity. The yield of activity after ammonium sulfate precipitation could be increased by using a higher percentage of ammonium sulfate for the fractionation, however, at the expense of a lower enrichment in specific activity.

Step 3: *Sephacryl 300 HR Chromatography.* A gel filtration Sephacryl 300 HR column (2.5 × 40 cm) was equilibrated with buffer B [50 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA, 4 mM L-glutamine, 10% glycerol, and 10 mM 2-mercaptoethanol]. The ammonium sulfate fraction from the previous step was applied to the column at a flow rate of 40 mL/h followed by elution of the enzyme from the column with buffer B. Fractions containing CTP synthetase activity were pooled and used directly for the next step in the procedure.

Step 4: *Q-Sepharose Chromatography.* A Q-Sepharose column (1.5 × 12 cm) was equilibrated with buffer B. Enzyme from the previous step was applied to the column at a flow rate of 30 mL/h. The column was washed with buffer B until all of the unbound protein was removed from the column. CTP synthetase was then eluted from the column with 10 column volumes of a linear NaCl gradient (0–1.0 M) in buffer B. The peak of CTP synthetase activity eluted from the column at a NaCl concentration of about 0.2 M (Figure 1A). The most active fractions were pooled and diluted with buffer B to a NaCl concentration of 0.1 M.

Step 5: *Affi-Gel Blue Chromatography.* An Affi-Gel Blue column (1.5 × 12 cm) was equilibrated with buffer B containing 0.1 M NaCl. The equilibration of the column with buffer containing 0.1 M NaCl eliminated the need to desalt the enzyme preparation from the previous step. The enzyme from the previous step was applied to the column at a flow rate of 30 mL/h. The column was washed with buffer B containing 0.1 M NaCl until all of the unbound protein was removed from the column. CTP synthetase was then eluted from the column wth 10 column volumes of a linear NaCl gradient (0.1–0.7 M) in buffer B. The peak of CTP synthetase activity eluted from the column at about 0.2 M NaCl (Figure 1B). The most active fractions were pooled and concentrated to about 0.4 mL using an Amicon Centricon-10 filter.

Step 6: *Superose 6 Chromatography.* A gel filtration Superose 6 column (1 × 24 cm) was equilibrated with buffer B. The concentrated enzyme from the previous Affi-Gel Blue step was applied to the column at a flow rate of 15 mL/h followed by elution of CTP synthetase from the column with buffer B. CTP synthetase activity and protein eluted from the column as a single peak (Figure 1C). Fractions containing activity were pooled and stored −20 °C. The purified enzyme was completely stable to storage at −20 °C for 2 months.

Superdex 200 HR Chromatography. A Superdex 200 HR column (1 × 24) was equilibrated and eluted with buffer B containing 10 mM $MgCl_2$ in the absence and presence of 2 mM UTP and 2 mM ATP at 5 °C. Purified CTP synthetase in buffer B containing 10 mM $MgCl_2$ in the absence and presence of 2 mM UTP and 2 mM ATP was applied and eluted from the column at a flow rate of 30 mL/h.

Electrophoresis and N-Terminal Amino Acid Sequencing. SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) was performed with 10% slab gels. Proteins on polyacrylamide gels were visualized with Coomassie blue. CTP synthetase on an SDS–polyacrylamide gel was transferred to poly-

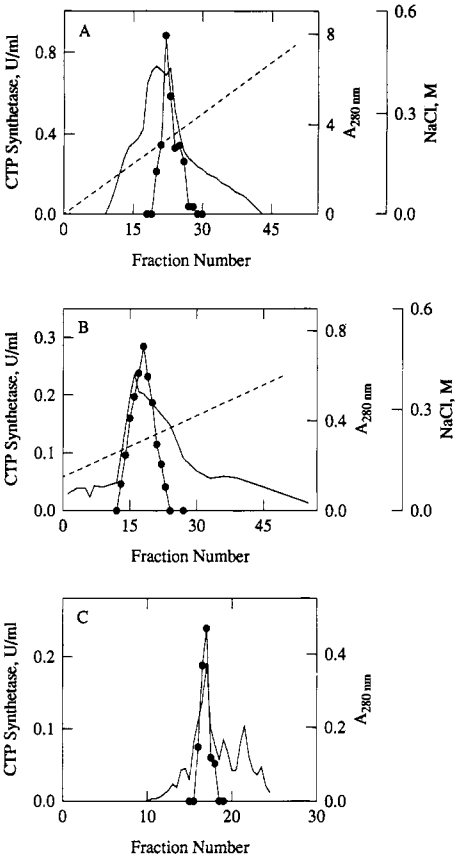

FIGURE 1: Elution profiles of CTP synthetase activity after chromatography with Q-Sepharose, Affi-Gel Blue, and Superose 6. CTP synthetase was subjected to chromatography using Q-Sepharose (*panel A*), Affi-Gel Blue (*panel B*), and Superose 6 (*panel C*) as described under Experimental Procedures. Fractions (2 mL for Q-Sepharose and Affi-Gel Blue and 1 mL for Superose 6) were collected and assayed for CTP synthetase activity (●) and protein (—). NaCl gradient profiles are indicated by a *dashed line*.

(vinylidene difluoride) paper and subjected to N-terminal amino acid sequence analysis (Matsudaira, 1987) using a Beckman LF-3400 sequencer.

Analysis of Kinetic Data. Kinetic data were analyzed according to the Michaelis–Menten and Hill equations using the EZ-FIT Enzyme Kinetic Model Fitting Program developed by Perrella (1988). This computer program uses the Nelder–Mead Simplex and Marquardt/Nash nonlinear regression algorithms sequentially and tests for the best fit of the data among different kinetic models.

Construction of Inducible pMAL-c2-URA7 Plasmid. A 1.6 kb fragment was isolated from plasmid pFL44S-*URA7* (Ozier-Kalogeropoulos et al., 1994) by *Hin*dIII and partial *Dra*I digestion by standard recombinant DNA manipulations (Maniatis et al., 1982). This fragment contained the *URA7* open reading frame starting at codon 33, through the stop codon, and ending within the multiple cloning site of plasmid pFL44S (Bronneaud et al., 1991). The fragment was introduced into plasmid YEp352 (Hill et al., 1986) at the *Sma*I and *Hin*dIII sites. In order to place the *URA7* sequence in-frame with the *malE* gene, YEp352-*URA7* was digested with *Eco*RI, and a blunt end was generated by treatment with the Klenow fragment. A 1.6 kb fragment containing *URA7* was then liberated from the linearized plasmid by digestion with *Hin*dIII and isolated. The pMAL-c2 vector was digested with *Eco*RI and treated by Klenow fragment and then digested with *Hin*dIII. This was ligated to the 1.6 kb fragment containing *URA7* to generate the inducible pMAL-c2-*URA7* plasmid.

Table 1: Purification of CTP Synthetase[a]

purification step	total units (μmol/min)	protein (mg)	sp act. (units/mg)	yield (%)	purification (*x*-fold)
(1) cytosol	17.00	3333	0.005	100	1
(2) ammonium sulfate	8.50	385	0.022	50	4.4
(3) Sephacryl 300 HR	8.16	192	0.043	48	8.6
(4) Q-Sepharose	7.91	60	0.132	46.5	26.4
(5) Affi-Gel Blue	6.32	9.84	0.643	37.2	128.6
(6) Superose 6	4.61	1.83	2.515	27.1	503

[a] CTP synthetase was purified from *S. cerevisiae* as described under Experimental Procedures. The data are based on starting with 130 g (wet weight) of cells.

Purification of Maltose-Binding Protein–CTP Synthetase Fusion Protein. All steps were performed at 5 °C. Frozen *E. coli* cells containing the induced maltose-binding protein–CTP synthetase fusion protein were washed once in 50 mM Tris-HCl (pH 8.0) buffer and suspended (0.5 g of cells/mL of buffer) in buffer A. Cells were disrupted by sonic oscillation and centrifuged at 12000*g* for 10 min to remove unbroken cells and cell debris. The supernatant was then centrifuged at 100000*g* for 1 h to obtain the cytosolic fraction. Samples of the cytosolic fraction were subjected to SDS–polyacrylamide gel electrophoresis. The maltose-binding protein–CTP synthetase fusion protein migrated with a molecular mass of about 100 kDa. Cells that were not induced with isopropyl β-D-thiogalactoside did not contain the 100-kDa fusion protein. The 100-kDa fusion protein was excised from SDS–polyacrylamide gels and used for the preparation of antibodies.

Preparation of Antibodies, Immunoprecipitation, and Immunoblotting. Antibodies to the maltose-binding protein–CTP synthetase fusion protein were raised in New Zealand White rabbits by standard procedures (Harlow & Lane, 1988) at the Pocono Rabbit Farm (Canadensis, PA). The IgG fraction was isolated from antisera by DEAE-Affi-Gel Blue chromatography as described by the manufacturer. The IgG antibodies were precipitated with 50% ammonium sulfate and resuspended and dialyzed against 20 mM K_2HPO_4 (pH 8.0) buffer. Immunoprecipitation (Kinney & Carman, 1988) and immunoblot (Fischl et al., 1986; Haid & Suissa, 1983) analyses of yeast cytosol and purified CTP synthetase were performed as described previously. The density of the CTP synthetase bands on immunoblots was quantitated by scanning densitometry. Immunoblot signals were optimized by analyzing a number of antigen and antibody concentrations and were in the linear range of detectability.

Extraction and Analysis of Nucleotides. Wild-type cells and mutant OK8 cells bearing plasmid pFL44S-*URA7* were grown to the exponential phase of growth. Cellular nucleotides were extracted (Ozier-Kalogeropoulos et al., 1991) and analyzed by high-performance liquid chromatography (Ozier-Kalogeropoulos et al., 1994) as described previously.

RESULTS

Purification of CTP Synthetase. CTP synthetase was purified from strain OK8 bearing the *URA7* gene on a multicopy plasmid which directed the overexpression of CTP synthetase activity (Ozier-Kalogeropoulos et al., 1991). The availability of the CTP synthetase overproducing cells facilitated the purification of the enzyme. CTP synthetase activity was routinely measured spectrophotometrically. The spectrophotometric assay was convenient, measured initial reaction rates, and was linear with protein concentration. The more cumbersome radioactive assay, which required thin-layer chromatography to separate radioactive substrate from product, was primarily used to confirm the product (CTP) of the reaction. Under the conditions used to extract CTP synthetase from yeast cells, the enzyme activity was 100% stable for 3–5 days at 5 °C and for 2 months at −20 °C. The stability of the enzyme was dependent on using freshly grown cells. CTP synthetase was labile if the cells were frozen prior to the preparation of cell extracts. The reason for this was unclear. The enzyme was purified by conventional protein purification techniques. A summary of the purification of CTP synthetase from the cytosol is shown in Table 1. Overall, CTP synthetase was purified 503-fold over the cytosolic fraction with an activity yield of 27% to a final specific activity of 2.5 μmol min^{-1} mg^{-1}.

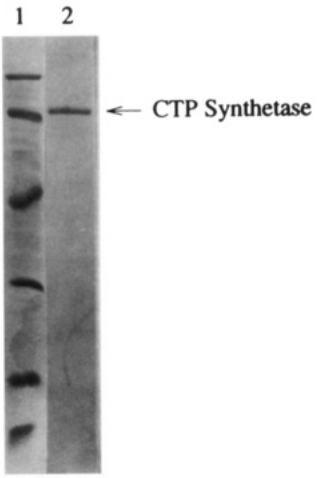

FIGURE 2: SDS–polyacrylamide gel electrophoresis of purified CTP synthetase. Purified CTP synthetase was subjected to SDS–polyacrylamide gel electrophoresis as described under Experimental Procedures. Lane 1 contains the protein molecular mass standards. The standards from top to bottom are phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa). Lane 2 contains the purified CTP synthetase. The position of CTP synthetase is indicated in the figure.

The purification scheme resulted in the isolation of a single peptide as shown by SDS–polyacrylamide gel electrophoresis (Figure 2). The purified enzyme migrated on the SDS–polyacrylamide gel with a minimum subunit molecular mass of 68 kDa (Figure 2). The deduced protein sequence of the open reading frame of *URA7* contains 580 amino acids corresponding to a protein of 64.7 kDa (Ozier-Kalogeropoulos et al., 1991, 1994). The result of SDS–polyacrylamide gel electrophoresis analyses of the purified CTP synthetase was consistent with this size for the enzyme subunit. The purified enzyme on a duplicate SDS-polyacrylamide gel was electrophoretically transferred to poly(vinylidene difluoride) paper and subjected to N-terminal amino acid sequence analysis. The amino acid sequence of the N-terminus of the peptide was unambiguously identified as Met-Lys-Tyr-Val-Val-Val-Ser-Gly-Gly-Val-Ile-Ser-Gly. This sequence aligned perfectly with the deduced N-terminal amino acid sequence of the open reading frame of the *URA7* gene (Ozier-Kalogeropoulos et

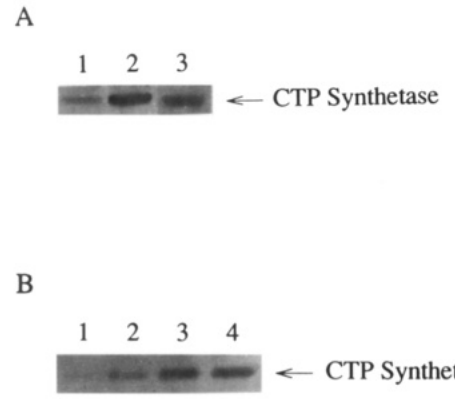

FIGURE 3: Immunoblot analysis and immunoprecipitation of CTP synthethase. (*Panel A*) Wild-type cells and cells overexpressing CTP synthetase were grown to the exponential phase of growth, and the cytosolic fraction was prepared. Samples (25 μg) from wild-type (lane 1) and overexpressing (lane 2) cells were subjected to immunoblot analysis using an IgG anti-CTP synthetase antibodies as described under Experimental Procedures. Lane 3 is an immunoblot of purified CTP synthetase. A portion of the immunoblot is shown, and the position of CTP synthetase is indicated. The antibodies did not cross-react with other proteins in the cytosolic fractions. The immunoblot signals were in the linear range of detectability. (*Panel B*) Wild-type cells were grown to the exponential phase of growth, and the cytosolic fraction was prepared. Samples (90 μg) were precleared with 10 μL of protein A–Sepharose CL-4B for 30 min. The samples were then treated with preimmune IgG (lane 1) and 1.1 μg (lane 2), 5.6 μg (lane 3), and 11.3 μg (lane 4) of IgG anti-CTP synthetase antibodies for 5 h followed by incubation with 10 μL of protein A–Sepharose CL-4B for 1 h. The precipitates were collected by centrifugation, the enzyme–antibody complexes were dissociated, and the samples were subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. CTP synthetase proteins were stained with Ponceau S, and their identification was verified by immunoblot analysis as described under Experimenal Procedures. A portion of the blot is shown, and the position of CTP synthetase is indicated.

al., 1991). This information unequivocally identified the purified CTP synthetase as the product of the *URA7* gene.

Polyclonal antibodies were raised against maltose-binding protein–CTP synthetase fusion protein expressed in *E. coli*. These antibodies reacted with pure CTP synthetase and CTP synthetase in cell extracts from wild-type cells and cells overexpressing the enzyme (Figure 3A). Scanning densitometry of the CTP synthetase bands on the immunoblot shown in Figure 3A indicated that the level of CTP synthetase protein was expressed 10-fold higher in cells bearing the *URA7* gene on the multicopy plasmid when compared with wild-type cells. This correlated with overexpression of CTP synthetase activity found in cells bearing the *URA7* gene on the multicopy plasmid (Ozier-Kalogeropoulos et al., 1991). In addition, the anti-CTP synthetase antibodies immunoprecipitated CTP synthetase from the cytosolic fraction of wild-type cells (Figure 3B). The immunospecificity of these antibodies raised against the fusion protein directed from the coding sequences of the *URA7* gene further substantiated that the product of the *URA7* gene was indeed CTP synthetase.

Oligomerization of CTP Synthetase. The native size of CTP synthetase was analyzed by gel filtration chromatography using Superdex 200 HR. Figure 4 shows the elution patterns of CTP synthetase when the enzyme was subjected to chromatography in the absence and presence of saturating concentrations of UTP and ATP. In the absence of UTP and ATP, native CTP synthetase eluted from the Superdex 200 HR column at a position consistent with a dimeric form of the enzyme. When CTP synthetase was chromatographed in the presence of UTP and ATP, most of the enzyme eluted at

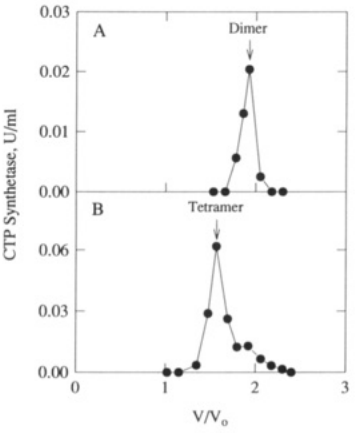

FIGURE 4: Elution profiles of CTP synthetase activity after chromatography with Superdex 200 HR in the absence and presence of ATP and UTP. CTP synthetase was subjected to Superdex 200 HR chromatography in the absence (*panel A*) and presence (*panel B*) of ATP and UTP as described under Experimental Procedures. The column was calibrated with Blue Dextran 2000 (for the void volume), thyroglobulin (669 kDa), apoferritin (443 kDa), and alcohol dehydrogenase (150 kDa). Fractions (1 mL) were collected and assayed for CTP synthetase activity. The positions of the dimeric and tetrameric forms of CTP synthetase are indicated in the figure.

a position consistent with a tetrametic form of the enzyme. About a 50/50 mixture of the dimeric and tetrameric forms of CTP synthetase was present when the enzyme was chromatographed with subsaturating concentrations of UTP and ATP (data not shown). Thus, CTP synthetase from *S. cerevisiae* formed a tetramer under optimal assay conditions.

Identification of Reaction Product. A standard CTP synthetase reaction was carried out using [5,6-^{3}H]UTP as labeled substrate. The product of the reaction was analyzed by thin-layer chromatography as described under Experimental Procedures. The ^{3}H-labeled product of the reaction comigrated with authentic CTP.

Effect of pH, Magnesium, and Thioreactive Agents on CTP Synthetase Activity. CTP synthetase activity was measured with a Tris–maleate–glycine buffer from pH 5.5 to 9.5. The pH optimum for the reaction was 8.0 (Figure 5A). Enzyme activity was measured with saturating concentrations of ATP (2 mM), UTP (2 mM), and GTP (0.1 mM) in the absence and presence of magnesium ions (Figure 5B). CTP synthetase was dependent on 4 mM magnesium ions for maximal activity. At 4 mM magnesium ions and pH 8.0, all of the nucleosides present in the enzyme assay would be present as magnesium–nucleotide complexes (Robertson & Villafranca, 1993). The sigmoidal shape of the curve in Figure 5B was likely due to the formation of magnesium–nucleotide complexes and the subsequent cooperative binding of magnesium–nucleotide to the enzyme. The magnesium requirement could not be substituted by manganese ions. CTP synthetase activity was measured in the presence of thioreactive agents. At a concentration of 0.8 mM, $HgCl_2$, *p*-(chloromercuri)benzenesulfonic acid, and *N*-ethylmaleimide totally inhibited activity. The addition of 10 mM 2-mercaptoethanol to the assay system prevented this inhibition. CTP synthetase activity was stimulated 40% by the addition of 2-mercaptoethanol to the assay system. These results implied that a sulfhydryl group was required for CTP synthetase activity.

Dependence of CTP Synthetase Activity on UTP, ATP, Glutamine, and GTP. The dependence of CTP synthetase activity on UTP and ATP was examined using saturating concentrations of glutamine and GTP. The magnesium ion concentration used in these experiments was also saturating,

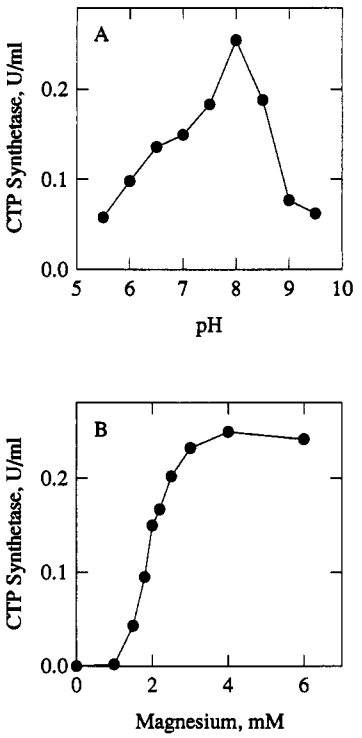

FIGURE 5: Effect of pH and magnesium on CTP synthetase activity. CTP synthetase activity was measured at the indicated pH values with 60 mM Tris–maleate–glycine buffer (*panel A*) and the indicated concentrations of $MgCl_2$ (*panel B*).

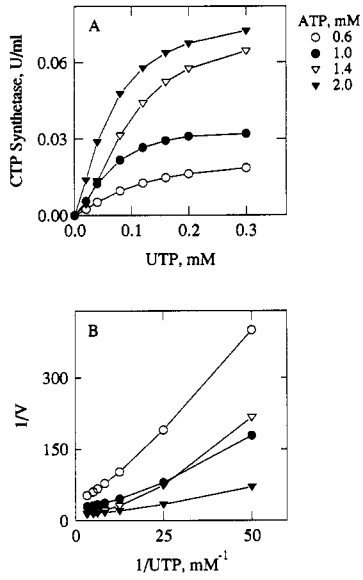

FIGURE 6: Dependence of CTP synthetase activity on the concentration of UTP at fixed concentrations of ATP. (*Panel A*) CTP synthetase activity was measured as a function of the concentration of UTP at the indicated set concentrations of ATP. The concentrations of glutamine, GTP, and $MgCl_2$ were 2 mM, 0.1 mM, and 10 mM, respectively. (*Panel B*) Reciprocal plot of the data in *panel A*.

and all three nucleotides in the assay system existed in the form of magnesium–nucleotide complexes (Robertson & Villafranca, 1993). CTP synthetase activity did not follow typical saturation kinetics when the UTP concentration was varied at various fixed concentrations of ATP (Figure 6A) or when the ATP concentration was varied at several fixed concentrations of UTP (Figure 7A). Instead, the enzyme showed positive cooperative kinetic patterns (Levitzki & Koshland, 1969; Segel, 1975). These positive cooperative kinetic patterns were also demonstrated by double-reciprocal plots of these data (Figure 6B and Figure 7B). The curves

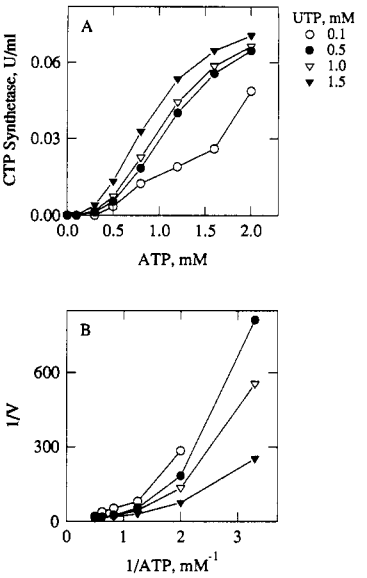

FIGURE 7: Dependence of CTP synthetase activity on the concentration of ATP at fixed concentrations of UTP. (*Panel A*) CTP synthetase activity was measured as a function of the concentration of ATP at the indicated set concentrations of UTP. The concentrations of glutamine, GTP, and $MgCl_2$ were 2 mM, 0.1 mM, and 10 mM, respectively. (*Panel B*) Reciprocal plot of the data in *panel A*.

in the double-reciprocal plots were not linear but were concave-upward. An analysis of these data according to the Hill equation yielded Hill numbers for UTP of 1.4–2.0 and Hill numbers for ATP of 2.5–3.0. Accurate determinations of the true K_m values for UTP and ATP could not be made because of this kinetic behavior.

The dependence of CTP synthetase activity on glutamine and GTP was examined using saturating concentrations of UTP, ATP, and magnesium ions. GTP was not an absolute requirement for CTP synthetase activity. However, the addition of GTP to the assay system stimulated activity 4-fold (data not shown). CTP synthetase activity was measured as a function of the glutamine concentration at set concentrations of GTP. Although the enzyme appeared to follow saturation kinetics (Figure 8A), the curves from double-reciprocal plots of the data were not linear but were concave-downward (Figure 8B). A double-reciprocal plot of the CTP synthetase activity *versus* the GTP concentration at set concentrations of glutamine also showed nonlinear curves that were concave-downward (data not shown). The nonlinear concave-downward curves were most evident when the concentration of the fixed substrate was the lowest. These results were indicative of the enzyme exhibiting negative cooperative kinetics with respect to glutamine and GTP (Levitzki & Koshland, 1969; Segel, 1975). The Hill numbers for glutamine and GTP ranged from 0.8 to 1.0. This kinetic behavior prevented the accurate determination of the true kinetic constants for glutamine and GTP. The glutamine and GTP requirements for maximum CTP synthetase activity could be substituted by 50 mM NH_4Cl (data not shown).

Effect of Cytidine Nucleotides on CTP Synthetase Activity. The effect of CTP and other cytidine nucleotides on CTP synthetase activity was examined. CTP synthetase activity was potently inhibited by CTP in a dose-dependent manner with an IC_{50} value of 0.3 mM (Figure 9). In addition to being the product of the CTP synthetase reaction, CTP plays a central role in the synthesis of membrane phospholipids (Carman & Henry, 1989). CTP is the precursor of the activated, energy-rich phospholipid pathway intermediates CDP-choline (Kennedy & Weiss, 1956), CDP-ethanolamine

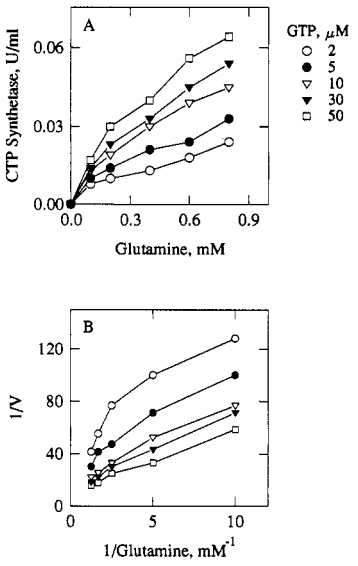

FIGURE 8: Dependence of CTP synthetase activity on glutamine and GTP. (*Panel A*) CTP synthetase activity was measured as a function of the concentration of glutamine at the indicated set concentrations of GTP. The concentrations of UTP, ATP, and $MgCl_2$ were 2 mM, 2 mM, and 10 mM, respectively. (*Panel B*) Reciprocal plot of the data in *panel A*.

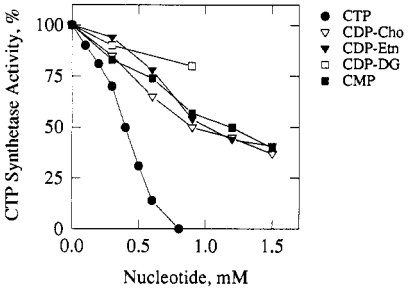

FIGURE 9: Effect of cytidine nucleotides on CTP synthetase activity. CTP synthetase activity was measured under standard assay conditions with 0.1 mM UTP in the absence and presence of the indicated concentrations of cytidine nucleotides. CDP-diacylglycerol was added to the enzyme assay mixture as a uniform Triton X-100 mixed micelle (Carman & Dowhan, 1979). Abbreviations: CDP-Cho, CDP-choline; CDP-Etn, CDP-ethanolamine; CDP-DG, CDP-diacylglycerol.

(Kennedy & Weiss, 1956), and CDP-diacylglycerol (Carter & Kennedy, 1966). CDP-choline (IC_{50} value of 1.03 mM) and CDP-ethanolamine (IC_{50} value of 1.14 mM) also inhibited CTP synthetase activity in dose-dependent manners (Figure 9). CDP-diacylglycerol did not have a significant inhibitory effect on CTP synthetase activity (Figure 9). Under the assay conditions used in these experiments, CDP-diacylglycerol existed as part of a Triton X-100/phospholipid mixed-micelle (Carman & Dowhan, 1979). Thus, this liponucleotide may not be in the proper (soluble) environment to affect enzyme activity. Enzymatic reactions using CDP-choline, CDP-ethanolamine, and CDP-diacylglycerol as substrates produce CMP as a product (Carman & Henry, 1989). CMP also inhibited CTP synthetase activity in a dose-dependent manner (IC_{50} value of 1.18 mM). Since the IC_{50} values for CDP-choline, CDP-ethanolamine, and CMP were relatively high, it is unlikely that they play a physiological role in the regulation of CTP synthetase. Accordingly, they were not examined further.

Effect of CTP on the Kinetics of CTP Synthetase Activity. A kinetic analysis was performed on CTP synthetase to examine the mechanism of CTP inhibition on enzyme activity. As indicated above, CTP synthetase catalyzes a complex

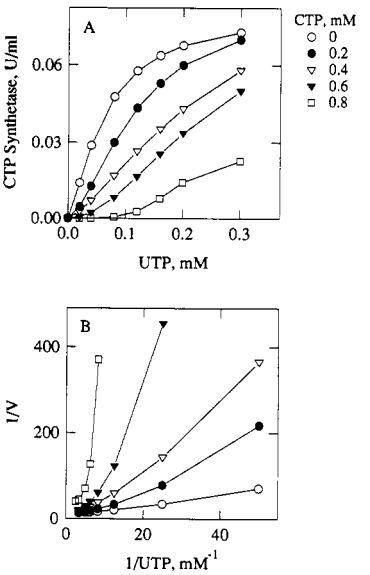

FIGURE 10: Effect of CTP on the kinetics of CTP synthetase activity with respect to UTP. (*Panel A*) CTP synthetase activity was measured as a function of the concentration of UTP at the indicated set concentrations of CTP. The concentrations of ATP, glutamine, GTP, and $MgCl_2$ were 2 mM, 2 mM, 0.1 mM, and 10 mM, respectively. (*Panel B*) Reciprocal plot of the data in *panel A*.

reaction where the concentration of one substrate affected the kinetic behavior of the enzyme with respect to the other substrates. Thus, a kinetic analysis of the effect of various inhibitor concentrations on activity would be very complex. To simply our kinetic studies when examining the effect of CTP on the kinetics of CTP synthetase with respect to UTP, the other reaction components were kept at saturating concentrations. As indicated above, in the absence of CTP the dependence of CTP synthetase activity on UTP was cooperative with a Hill number of 1.4 (Figure 10A). CTP synthetase activity was inhibited by CTP in a dose-dependent manner at each UTP concentration (Figure 10A). The presence of CTP in the reaction caused an increase in the cooperativity of the enzyme with respect to UTP. The increase in the positive cooperativity displayed by the enzyme was also demonstrated by a double-reciprocal plot of the data where the curves were concave-upward (Figure 10B). The Hill number for UTP in the presence of 0.8 mM CTP increased to 4.6. CTP also caused a decrease in the apparent V_{max} values and an increase in the apparent K_m values with respect to UTP. These results were consistent with CTP being an allosteric inhibitor (Segel, 1975). Because of the cooperative kinetic behavior of the enzyme, we could not accurately determine a true inhibitor constant for CTP.

CTP synthetase existed as a tetramer in the presence of the substrates UTP and ATP (see above). We considered whether the effect of CTP on CTP synthetase activity was due to inhibition of the tetramerization of the enzyme. Under the conditions shown to promote tetramerization, CTP synthetase was subjected to gel filtration chromatography in the absence and presence of 0.8 mM CTP. CTP did not affect the tetramerization of the enzyme (data not shown). Thus, the CTP-dependent increase in the positive cooperative kinetic behavior of CTP synthetase toward UTP was not due to CTP preventing the tetramerization of the enzyme.

Cellular Concentrations of CTP in Wild-Type Cells and Cells Overexpressing CTP Synthetase. The cellular concentrations of CTP were determined in wild-type cells and cells that overexpressed CTP synthetase 10-fold (Table 2). This analysis showed that the CTP concentration in wild-type

Table 2: Cellular Concentrations of UTP and CTP in Wild-Type Cells and Cells Overexpressing CTP Synthetase

strain	cellular concn (mM)	
	UTP	CTP
FL100 (wild-type)	0.75	0.59
OK8 + pFL44S-*URA7*	0.72	1.66

cells was 2-fold higher than the IC_{50} value (0.3 mM) determined for CTP using pure enzyme. The CTP concentration in cells that overexpressed CTP synthetase was 2.8-fold higher than the CTP concentration found in wild-type cells. The cellular concentrations of UTP, the substrate for the reaction, were not affected by the overexpression of CTP synthetase (Table 2).

DISCUSSION

We undertook the purification of CTP synthetase to facilitate well-defined studies on the regulation of CTP synthetase activity. CTP synthetase was purified from *S. cerevisiae* cells bearing the *URA7* gene on a multicopy plasmid which directed the 10-fold overproduction of the enzyme. The six-step purification scheme reported here yielded an apparently homogeneous preparation of CTP synthetase. The enzyme was purified 503-fold relative to the specific activity in the cytosolic fraction to a final specific activity of 2.5 μmol min^{-1} mg^{-1}. Since CTP synthetase was overproduced 10-fold in cells overexpressing the *URA7* gene, the enzyme would have been purified 5030-fold relative to wild-type cells. The minimum subunit molecular mass of the purified enzyme (68 kDa) was in good agreement with the predicted size (64.7 kDa) of the protein deduced from the open reading frame of the *URA7* gene (Ozier-Kalogeropoulos et al., 1991, 1994). The N-terminal amino acid sequence of purified CTP synthetase aligned precisely with the amino acid sequence of the protein deduced from the *URA7* gene (Ozier-Kalogeropoulos et al., 1991). Anti-CTP synthetase antibodies were prepared against a maltose-binding protein–CTP synthetase fusion protein expressed in *E. coli* which was translated from the coding sequences of the URA7 gene. These antibodies recognized pure CTP synthetase and CTP synthetase from the cytosolic fraction of yeast cells. Thus, we have unequivocally identified *URA7* as the structural gene for CTP synthetase in *S. cerevisiae*.

The purified CTP synthetases from *E. coli* (Anderson, 1983; Long & Pardee, 1967) and rat liver (Thomas et al., 1988) have similar subunit molecular masses and assay requirements. The purified enzyme from *S. cerevisiae* shared these similar properties. On the other hand, previous studies have raised the suggestion that the procaryotic and eucaryotic forms of CTP synthetase differ with respect to their kinetic properties. Kinetic studies using pure CTP synthetase from *E. coli* have shown that the enzyme exhibits both positive and negative cooperative behavior toward its substrates (Levitzki & Koshland, 1969, 1971, 1972a; Lewis & Villafranca, 1989; Long & Pardee, 1967; Robertson & Villafranca, 1993; von der Saal et al., 1985). These cooperative kinetic properties are characteristic of allosteric enzymes (Segel, 1975). However, kinetics studies using an impure preparation of CTP synthetase from bovine liver have indicated that the enzyme exhibits typical Michaelis–Menten kinetics with respect to its substrates (McPartland & Weinfeld, 1979; Savage & Weinfeld, 1970). Detailed kinetic experiments were not performed on the pure CTP synthetase from rat liver (Thomas et al., 1988). The kinetic studies reported here using purified CTP synthetase from *S. cerevisiae* clearly showed that this eucaryotic CTP synthetase behaved in a kinetic manner consistent with an allosteric enzyme. Allosteric enzymes typically form oligomeric structures in the presence of their substrates (Segel, 1975), and, indeed, pure CTP synthetases from *E. coli* (Levitzki & Koshland, 1972a,b) and *S. cerevisiae* show nucleotide-dependent oligomerization. Pure CTP synthetase from rat liver (Thomas et al., 1988) and partially purified CTP synthetase from bovine liver (McPartland & Weinfeld, 1979) also exhibit nucleotide-dependent oligomerization (McPartland & Weinfeld, 1979; Thomas et al., 1988). If allosteric enzymes typically display complex cooperative kinetic behavior (Segel, 1975), it is unclear why the higher eucaryotic forms of CTP synthetase fail to show cooperative kinetic patterns with respect to their substrates. Perhaps better defined kinetic studies using pure enzymes would resolve this question. It should be noted that the pure rat liver (Thomas et al., 1988) and partially pure bovine liver (McPartland & Weinfeld, 1979) CTP synthetases do show cooperative kinetic patterns toward UTP in the presence of CTP. The complex cooperative kinetic behavior of the yeast CTP synthetase precluded the determination of true kinetic constants for the enzyme. In addition, the dependence of CTP synthetase activity for one substrate varied with respect to the concentration of another substrate. Thus, estimates of K_m values for one substrate at saturating concentrations of the other substrates would not have meaning.

A characteristic common to the CTP synthetases purified from *E. coli* (Long & Pardee, 1967) and rat liver (Thomas et al., 1988) is the inhibition of their activities by the product CTP. Indeed, CTP was an inhibitor of CTP synthetase purified from *S. cerevisiae*. Since CTP synthetase activity was inhibited by CTP *in vitro*, we questioned whether this inhibition was physiologically relevant. If CTP was to have a role in the regulation of CTP synthetase activity *in vivo*, the cellular concentration of CTP should be within the range of the apparent inhibitor constant determined for CTP using purified enzyme. The cellular concentration of CTP (0.59 mM) was 2-fold greater than the IC_{50} value (0.3 mM) determined with pure enzyme. Although this IC_{50} value for CTP was below the cellular concentration of CTP, which could result in enzyme inhibition, the cellular concentration of UTP (0.72 mM) was a saturaing concentration for the enzyme *in vitro* (Figure 6). On the basis of kinetic patterns of CTP synthetase inhibition by CTP with respect to UTP (Figure 10), the activity of the enzyme *in vivo* would be sensitive to changes in the cellular concentrations of CTP and UTP. CTP synthetase would be very sensitive to CTP inhibition at low UTP concentrations because CTP increased the positive cooperative behavior of the enzyme with respect to UTP (Figure 10). This would especially be the case if the enzyme was localized in close proximity to the major UTP and CTP pools *in vivo*. The notion that CTP synthetase activity may be regulated by CTP *in vivo* was also supported by the analysis of the CTP levels in wild-type cells and cells which overexpressed CTP synthetase. The discrepancy between the 10-fold overexpression of CTP synthetase and only a 2.8-fold increase in the cellular CTP concentration was consistent with the inhibition of CTP synthetase activity by CTP in these cells.

In mammalian cells, inhibition of CTP synthetase by CTP appears to play an important role in the balance of the pyrimidine nucleotide triphosphate pools (Aronow & Ullman, 1987). A number of mammalian mutant cell lines possess CTP synthetase activity which is insensitive to inhibition by

CTP. As a result, these cell lines display complex phenotypes which include the following: increased intracellular pools of CTP and dCTP (Robert de Saint Vincent & Buttin, 1980; Trudel et al., 1984); resistance to nucleotide analog drugs used in cancer chemotherapy (Aronow et al., 1984; Chu et al., 1984; Kaufman, 1986; Meuth et al., 1982); and an increased rate of spontaneous mutations (Aronow et al., 1984; Chu et al., 1984; Meuth et al., 1979). In addition, elevated levels of CTP synthetase activity have been reported in rapidly growing tumors of liver (Kizaki et al., 1980), colon (Weber et al., 1980), and lung (Weber et al., 1979). These studies suggest an important role for the feedback inhibition of CTP synthetase activity by CTP *in vivo*.

In summary, we have purified CTP synthetase from *S. cerevisiae* to homogeneity and have unequivocally identified the enzyme as the product of the *URA7* gene. This report has been the first detailed study on the enzymological properties of a homogeneous preparation of CTP synthetase from a eucaryotic organism. Moreover, we have provided evidence that the inhibition of CTP synthetase by CTP appears to be physiologically relevant. Clearly, more studies are warranted to fully understand the regulation of CTP synthetase activity in eucaryotic cells. The *URA7* gene encoding for CTP synthetase from *S. cerevisiae* shows a high degree of homology with the human gene for the enzyme. Thus, the availability of pure CTP synthetase from *S. cerevisiae*, its antibodies, and the tractable molecular genetics of this organism will permit defined studies on the regulation of this important enzyme.

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