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PURIFICATION AND PROPERTIES OF CTP SYNTHETASE FROM EHRLICH ASCITES TUMOR CELLS

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CTP synthetase (UTP: ammonia ligase (ADP-forming), EC 6.3.4.2) was purified 200-fold from Ehrlich ascites tumor cells with about 50% purity as judged by polyacrylamide gel electrophoresis. The molecular weight was estimated to be 118 000 by gel filtration. The optimal pH was 8.6. 2-Mercaptoethanol was required for optimal activity and stabilization of the enzyme. Magnesium was essential for the reaction and other divalent cations were ineffective. Ammonia could replace glutamine as the amino donor. When other substrates were at saturating concentrations, Michaelis-Menten kinetics were observed yielding K_m values for UTP, ATP and glutamine of 0.18, 0.8 and 0.13 mM, respectively. With ATP at subsaturating concentration, the double-reciprocal plot for UTP saturation was sinusoidal and the Hill plot showed an n value of 1.3. The double-reciprocal plot for ATP saturation, when UTP was at subsaturating concentration, departed from Michaelis-Menten kinetics with an n value of 1.4. These data suggest the existence of cooperative interactions between enzyme and substrates at subsaturating concentrations of ATP or UTP. GTP was not essential, but it acted as an activator on the glutamine reaction; optimal activation was observed at 1 mM GTP. The affinity for glutamine was not affected by GTP.

Introduction

CTP synthetase (UTP: ammonia ligase (ADP-forming), EC 6.3.4.2) catalyzes the formation of CTP from UTP in the presence of ATP, glutamine, Mg^{2+} and GTP. The enzyme was first characterized from *Escherichia coli* [1–3] and intensively studied [4]. A similar enzyme was later demonstrated in rat and calf liver [5,6].

The markedly different kinetic properties of the bacterial and liver enzymes have been reported. The bacterial enzyme exhibited a high degree of cooperativity in substrate binding at subsaturating concentration of other substrates [3,4], whereas the liver enzyme followed Michaelis-Menten kinetics [6]. To compare the properties and regulatory mechanism of the enzyme activity in rapidly proliferating cells to that of the bacterial and liver enzymes, CTP synthetase was partially purified from Ehrlich ascites tumor cells, and some properties of the enzyme were investigated.

Materials and Methods

Materials

[2-^{14}C]UTP tetrasodium salt (46.8 Ci/mol) was purchased from New England Nuclear Corp., Boston, MA. Calcium phosphate gel and blue dextran agarose. Affigel Blue, were obtained from BioRad Laboratories, Richmond, CA; Matrex Gel Red A from Amicon Corp., Lexington, MA; Sephacryl S-300 and Sephadex G-200 from Pharmacia Inc., Piscataway, NJ. All other chemicals were of reagent grade and purchased from Nakarai Chemical Corp., Tokyo; Polyethyleneimine (PEI)-cellulose plates (Polygram Cel 300 PEI) were from Brinkmann Instruments Inc., Westbury, NY.

The radioactive nucleotide solution (supplied in

50% ethanol solution) was evaporated to dryness in the N_2 gas stream and redissolved in water adjusted to 50 mCi/l.

Ehrlich ascites cells

Ehrlich ascites cells were maintained by weekly transplantation via intraperitoneal injection of ascites fluid in male ddY strain No. 28 mice weighing about 20 g. Animals were given standard laboratory chow (Oriental MF) and water ad libitum.

Enzyme assay

CTP synthetase activity was determined by measuring the formation of labeled CTP from [^{14}C]UTP using a modification of the method of Williams et al. [7]. During purification, the activity was measured in a 50 μl reaction mixture which contained 35 mM Tris-HCl buffer (pH 8.6)/12 mM ATP/1 mM GTP/10 mM glutamine/0.5 mM UTP/50 mM 2-mercaptoethanol/8 mM phospho*enol*pyruvate/18 mM $MgCl_2$/10 mM NaF/0.0625 μCi [^{14}C]UTP. For kinetic studies with the highly purified enzyme, assay conditions were the same except that NaF and phospho*enol*pyruvate were omitted and 1 mM UTP was used instead of 0.5 mM. Reaction was carried out at 37°C and terminated at 3, 5 or 10 min intervals by placing the tubes in boiling water for 2 min. The tubes were centrifuged for 2 min in a Microfuge B centrifuge (Beckman Instruments Inc., Palo Alto, CA). A 10 μl aliquot of the clear supernatant was spotted on PEI-cellulose plates together with 10 μl of the carrier solution containing CTP and UTP, each at 2.5 mM. Chromatography was performed at 4°C using 0.8 M $(NH_4)_2SO_4$ solution as a solvent [7]. The radioactivity of the CTP spot was measured in 7 ml scintillation fluid (4 g 2,5-diphenyloxazole and 40 mg 2,2'-*p*-phenylene-bis-(5-phenyloxazole) in 1 l toluene) using a Horiba liquid scintillation counter.

The specific activity is given in units/mg protein; one unit catalyzes the formation of 1 μmol CTP/h under the assay conditions.

Protein assay

Protein was determined by the method of Bradford [8] using bovine serum γ-globulin as standard.

Purification of CTP synthetase

All procedures were carried at 0–4°C.

Step 1. Preparation of crude extract. Mice were killed 8–10 days after implantation of about 10^8 Ehrlich ascites tumor cells. The tumor cells were collected by centrifugation of ascites fluid and washed twice with cold 0.07 M NaCl solution and once with 0.25 M sucrose solution. The washed cells were resuspended in 4 vol. 35 mM Tris-HCl buffer (pH 7.4)/100 mM 2-mercaptoethanol and homogenized for 1 min at 12 000 rev./min in a Waring blendor. The homogenate was centrifuged at 8000 × *g* for 20 min, then at 100 000 × *g* for 60 min, and the supernatant was used in the next step (Fraction I).

Step 2. Streptomycin sulfate treatment. To Fraction I, solid streptomycin sulfate was added to yield a final concentration of 0.5%. After stirring for 20 min, the solution was centrifuged at 10 000 × *g* for 20 min and the supernatant was collected (Fraction II).

Step 3. $(NH_4)_2SO_4$ fractionation. To fraction II, solid $(NH_4)_2SO_4$ was added to bring it to 40% saturation. After being stirred for 40 min, the pellet was collected by centrifugation and dissolved in 10 mM Tris-HCl buffer (pH 7.4)/50 mM 2-mercaptoethanol/20% glycerol (Buffer A), (Fraction III).

Step 4. Calcium phosphate adsorption. Fraction III was adjusted to a protein concentration of about 10 mg/ml with Buffer A. Calcium phosphate gel, one tenth of the amount of the protein, was added with stirring. After 20 min stirring, the pellet collected by centrifugation at 800 × *g* for 10 min was suspended in 35 mM Tris-HCl buffer (pH 7.4)/100 mM 2-mercaptoethanol/20% glycerol/50 mM EDTA. This solution was dialyzed overnight against 2 l of the same buffer. After dialysis, insoluble material was removed by centrifugation and $(NH_4)_2SO_4$ was added to this fraction to bring it to 50% saturation. The pellet was collected by centrifugation at 10 000 × *g* for 20 min and dissolved in Buffer A (Fraction IV).

Step 5. Affigel Blue chromatography. Fraction IV was applied to an Affigel-Blue column (2.2 × 10 cm) which had been equilibrated previously with Buffer A. The column was washed with Buffer A until the absorbance of the eluate at 280 nm was less than 0.05. Then the column was eluted with 2 bed vol. 1 M KCl in Buffer A. The eluate was concentrated by the addition of $(NH_4)_2SO_4$ (50% saturation). The pellet collected was dissolved in Buffer A and dialyzed overnight against 1 l Buffer A (Fraction V).

Step 6. Matrex Gel Red A chromatography. Fraction V was applied to a Matrex Gel Red A column

(2.2 X 10 cm) equilibrated with Buffer A. The column was washed with Buffer A until the absorbance at 280 nm was near 0. The column was eluted with 300 ml gradient of 0–1.0 M KCl in Buffer A at a flow rate of 70 ml/h and 3.5-ml fractions were collected. The active fractions were pooled and concentrated by the addition of $(NH_4)_2SO_4$ (50% saturation). The precipitate collected was dissolved in a minimum amount of Buffer A (Fraction VI).

Step 7. Sephacryl S-300 gel filtration. Fraction VI was applied on a Sephacryl S-300 column (1.5 X 70 cm) which had been equilibrated with Buffer A. Elution was performed with Buffer A at a flow rate of 7 ml/h and 1.3 ml-fractions were collected. The active fractions were pooled and precipitated by $(NH_4)_2SO_4$ of 50% saturation. The pellet collected was dissolved in a minimum amount of 35 mM Tris-HCl buffer, pH 7.4/100 mM 2-mercaptoethanol/30% glycerol, and dialyzed for 16 h against 200 vol. of the above buffer with one change of the buffer (Fraction VII).

Molecular weight determination

The column (12 X 90 cm) of Sephadex G-200 was prepared and equilibrated with 35 mM Tris-HCl buffer (pH 7.4)/100 mM 2-mercaptoethanol. The final enzyme sample was applied on the column and the column was eluted with the above buffer at a flow rate of 6 ml/h collecting 1.5-ml fractions. Aldolase (158 000), bovine serum albumin (67 000), and ovalbumin (43 000) were used as calibration proteins.

Polyacrylamide gel electrophoresis

Electrophoresis in polyacrylamide gels (10%) was carried out by the method of Davis [9] except that the sample gel was omitted. For the measurement of the activity of the bands, the gel was cut into 2-mm discs after electrophoresis and each disc was extracted overnight with Buffer A at 4°C. The activity was determined by the method described above except that 0.1 mM UTP was used instead of 0.5 mM, and the incubation time was 60 min.

Results

Purification of CTP synthetase. The results of a typical purification are given in Table I. This procedure routinely yields 200- to 260-fold purification o of CTP synthetase with 6–12% recovery. Electrophoresis on 10% acrylamide gel of the final fraction showed one major and three to four minor protein bands. The major band corresponded to the peak of CTP synthetase activity and it constituted about 50% of the total protein as determined by densitometric analysis.

The enzyme was unstable even in the presence of 30% glycerol and 100 mM 2-mercaptoethanol. 65% of the activity was lost at 4°C and 40% at –20°C in 7 days. The enzyme was relatively stable at 80°C with 65% of the activity retained after 4 weeks.

Molecular weight. The molecular weight of the native enzyme, in the absence of ATP and UTP, was determined by Sephadex G-200 gel filtration and estimated to be 118 000 ± 10 000.

Effect of pH. The effect of pH on the activity was evaluated over pH ranges of 6.2–9.0 in 35 mM Tris-HCl buffer and 8.6–10.0 in 35 mM glycine-KOH buffer (Fig. 1). Maximum activity was observed at pH

TABLE I

PURIFICATION OF CTP SYNTHETASE FROM EHRLICH ASCITES TUMOR CELLS

Purification step	Protein (mg)	Activity (10^3 X unit)	Specific activity (10^3 X units/mg protein)	Yield (%)
100 000 X *g* supernatant	3 440	58 480	17	100
Streptomycin sulfate treatment	2 159	58 293	27	100
40% $(NH_4)_2SO_4$	709	62 392	88	107
Calcium phosphate adsorption	247	48 165	195	82
Affigel Blue chromatography	50	33 800	676	58
Matrex Gel Red A chromatography	5	9 600	1 920	17
Sephacryl S-300 gel filtration	1.9	6 878	3 620	12

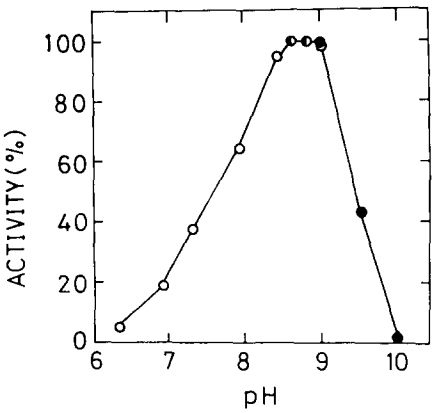

Fig. 1. The pH-activity profile of CTP synthetase. The activity was measured under standard conditions except that the buffers were varied; from pH 6.2 to 9.0, 35 mM Tris-HCl buffer (○), and from pH 8.6 to 10.0, 35 mM glycine-KOH buffer (●). The activity was expressed as percent of the maximum activity.

between 8.6 and 8.9. The activity decreased sharply below pH 8.3 and above pH9.0. At the physiological pH, 7.4, it was about 30% of the maximum activity. The pH-activity profile with ammonia as an amino donor was similar to the glutamine reaction and maximum activity was observed at pH 8.6.

Effect of sulfhydryl reagents on enzyme activity (Table II). To examine the requirement of a sulfhydryl reagent for the activity, the enzyme precipitated with $(NH_4)_2SO_4$ after gel filtration was dissolved in 35 mM Tris-HCl buffer (pH 7.4)/30% glycerol and dialyzed against the above buffer for 16 h with one change of the buffer after 8 h. During this manipulation, 30% of the activity was lost. The addition of 2-mercaptoethanol or dithiothreitol above 50 mM increased the activity to 230% of the control value. *p*-Chloromercuribenzoate inhibited the activity by 50% at 10 μM and nearly complete inhibition was observed at 50 μM. The preincubation of *p*-chloromercuribenzoate-treated (50 μM) enzyme with 100 mM 2-mercaptoethanol restored the activity to about 90% of maximum activity. The sulfhydryl reagent was not absolutely required, but it increased the activity and was essential for the stabilization of the activity.

Effect of divalent cations. The effect of divalent cations, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} at a concentration of 18 mM was examined. Of all the divalent ions tested, Mg^{2+} was the only effective cation and no activity was observed in the absence of Mg^{2+}.

Effect of amino donors. $(NH_4)_2SO_4$, glycine, glutamic acid, arginine, asparagine and aspartic acid were examined at a concentration of 10 mM. $(NH_4)_2SO_4$ could replace glutamine, but with other amino acids, activity was not detected. The activities with glutamine and ammonia obtained under standard conditions were 3.23 and 3.86 units/mg protein, respectively.

Kinetic characteristics. Kinetic studies of the gluta-

TABLE II

EFFECT OF SULFHYDRYL REAGENT

Additions		Activity	
		Units/mg protein	%
None		1.04	100
2-Mercaptoethanol	5 mM	1.55	149
	50 mM	2.29	221
	100 mM	2.45	236
Dithiothreitol	1 mM	1.53	147
	10 mM	1.88	181
	50 mM	2.32	223
p-Chloromercuribenzoate	5 μM	0.69	66
	10 μM	0.49	48
	50 μM	0.04	4
p-Chloromercuribenzoate + 100 mM 2-mercaptoethanol	50 μM	2.20	212

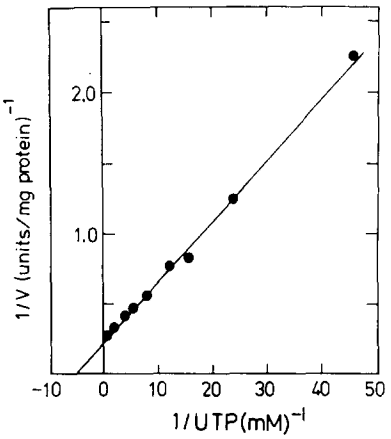

Fig. 2. Double-reciprocal plot of UTP saturation with all other substrates at near saturating concentrations. The standard assay was used except that UTP concentration was varied.

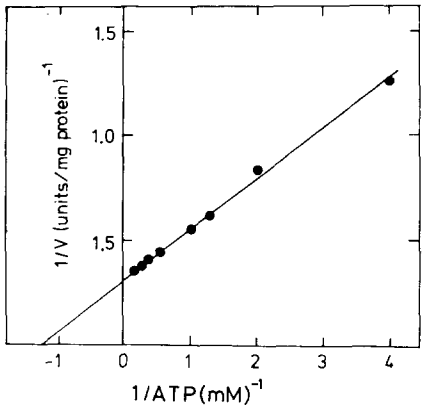

Fig. 3. Double-reciprocal plot of ATP saturation with all other substrates at near saturating concentrations. The standard assay was used except that ATP concentration was varied.

mine reaction were undertaken at various concentrations of the substrates. The saturation curve for UTP was hyperbolic when all other substrates were at saturating concentrations. Double-reciprocal plots gave linear-relationship yielding $K_m = 0.18$ mM (Fig. 2). Similarly, the saturation curve for ATP at saturating concentrations of other substrates was hyperbolic and the K_m was 0.8 mM (Fig. 3).

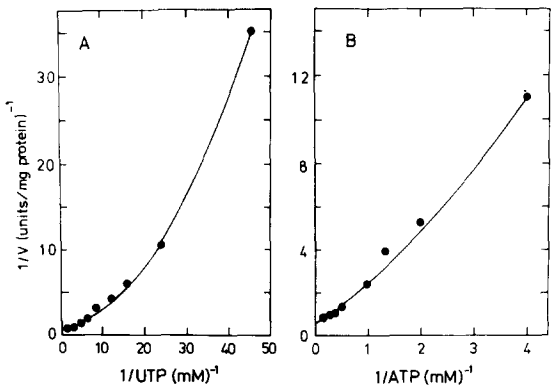

Fig. 4. Double-reciprocal plots of UTP saturation at subsaturating concentration of ATP (A) and of ATP saturation at subsaturating concentration of UTP (B). The UTP saturation was determined under standard conditions except 1.0 mM ATP was used instead of 12 mM ATP, and UTP concentration was varied. The ATP saturation was obtained at various ATP concentrations under standard conditions except that 0.1 mM UTP was used instead of 1.0 mM.

When ATP concentration was 1 mM which was about half-saturating concentration, the double-reciprocal plot for UTP saturation showed sinusoidal character (Fig. 4A) and the Hill plot had a slope with $n = 1.3$. Similarly, the kinetics of ATP saturation at 0.1 mM UTP, which was about half-saturating concentration, departed from Michaelis-Menten kinetics (Fig. 4B) and the n value obtained from the Hill plot was 1.4.

The activity was observed in the absence of GTP, but it was increased about 5-fold by GTP with a maximum activation at 1 mM GTP (Fig. 5) and a double-reciprocal plot gave a K_m value of 0.05 mM. In the

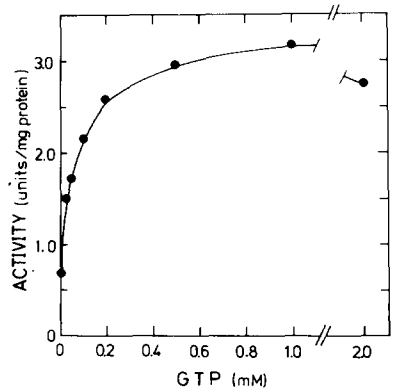

Fig. 5. Effect of GTP on CTP synthetase activity. The activity with glutamine was determined under standard conditions except that the concentration of GTP was varied.

TABLE III

EFFECT OF GTP ON GLUTAMINE AND AMMONIA REACTIONS

The reactions were carried out under the standard conditions using 10 mM glutamine or 10 mM $(NH_4)_2SO_4$ as amino donor in the presence and absence of 1 mM GTP. The kinetic parameters were obtained from double-reciprocal plots.

	Glutamine		Ammonia	
	+GTP	−GTP	+GTP	−GTP
Activity (units/mg protein)	3.23	0.64	3.86	4.76
K_m (mM)	0.13	0.16	2.00	1.70

ammonia reaction no activation was observed at 1 mM GTP; the activity with ammonia in the absence of GTP was 20% higher than in the presence of GTP (Table III). The affinities for both ammonia and glutamine, which were obtained from linear double-reciprocal plots, were not affected by GTP. The K_m value for ammonia was one order higher than that for glutamine.

Discussion

CTP synthetase activity in Ehrlich ascites tumor cells (16–22 nmol/h per mg protein) was higher than that observed in rat and calf liver [6,10] and similar to that in Morris hepatomas of slow and medium growth rates [10]. The high activity in these murine tumor cells suggests that the enzyme might be linked with proliferation [10,11] and involved in the control of intracellular levels of CTP and deoxynucleotides [12,13].

During purification, it has been noted that the enzyme became labile. Glycerol and 2-mercaptoethanol stabilized the enzyme to some extent in parallel with their concentrations up to 30% and 100 mM. The addition of glutamine, dimethylsulfoxide or a proteinase inhibitor, phenylmethylsulfonyl fluoride, did not stabilize the enzyme activity. Because of the instability of the enzyme even in the presence of glycerol and 2-mercaptoethanol, we did not succeed in further purification by conventional methods such as ion-exchange chromatography, hydroxyapatite or phenyl-Sepharose chromatography.

The molecular weight (118 000) in the absence of the substrates estimated by gel filtration was similar to those reported in liver (138 000) [14] and bacteria (105 000) [15]. The determination of the structure of this enzyme awaits further purification.

The liver enzyme, but not the bacterial enzyme, requires a sulfhydryl reagent for its optimal activity and stabilization [3,6]. The enzyme from Ehrlich ascites tumor cells also required a sulfhydryl reagent and *p*-chloromercuribenzoate inhibited the activity which was reversibly restored by a sulfhydryl reagent (Table II). The data indicate that some -SH groups in the enzyme might be involved in the enzyme activity as in other glutamine-requiring enzymes [4]. Magnesium was essential for both the glutamine and ammonia reactions. The activity of the bacterial enzyme also depends absolutely upon the presence of magnesium [2]. The specificity of this enzyme for the amino donor was high; amino acids other than glutamine were ineffective and only ammonia could replace glutamine as is the case with other glutamine amidotransferases [4]. The bacterial enzyme with glutamine exhibits broad pH optima between 7.5 and 9.3 and shows a sharp pH-activity profile with ammonia with a maximum at pH 10.3 [16]. In the enzyme from Ehrlich ascites tumor cells, the pH-activity profile with ammonia was similar to that with glutamine and the activity with ammonia at pH 7.4 was similar to that with glutamine at the same pH. However, the higher K_m value for ammonia indicates that ammonia might not be a physiological amino donor.

The kinetics of UTP, ATP and glutamine saturation followed Michaelis-Menten kinetics when other substrates were saturating (Fig. 2, 3 and Table III). The K_m values for ATP and glutamine were similar to those reported for the liver enzyme [6]. The K_m for UTP was about 2-fold higher than that in the liver enzyme [6], and rather similar to that in the bacterial enzyme [3]. The departure from classical Michaelis-Menten kinetics at subsaturating concentration of UTP or ATP suggests that there might be some cooperative interactions between the enzyme and the substrates, but to a lesser extent than those of the bacterial enzyme in which the cooperativity is prominent [3,4]. In the liver enzyme, however, no cooperative effect has been reported even at subsaturating concentration of the substrates [6].

With glutamine as substrate, GTP is almost abso-

lutely required for the bacterial enzyme with marked effects on both the V (increased) and the K_m value for glutamine (decreased) and it has essentially no effect on the ammonia reaction [4,17]. The precise function of GTP in the enzyme from Ehrlich ascites tumor cells is unkknown at present, but a similar effect of GTP on the glutamine reaction has been reported for the liver enzyme [6].

The present studies showed that (a) the effects of sulfhydryl reagents and GTP on the CTP synthetase from Ehrlich ascites tumor cells were similar to those on the liver enzyme, (b) cooperative interactions which were not observed in the hepatic enzyme were detected in the enzyme from Ehrlich ascites tumor cells, but to a lesser extent than in the bacterial enzyme. These characteristics may suggest that the enzyme from Ehrlich ascites tumor cells had different properties and regulatory mechanisms from those of the liver and bacterial enzymes. The precise regulatory mechanism is at present unknown and further purification and analyses are required for an elucidation of this problem.

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