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Purification and Characterization of GTP Cyclohydrolase I from *Streptomyces tubercidicus*, a Producer of Tubercidin

Jin-Cheol Yoo, Ji-Man Han, Ok-Hyun Ko and Hee-Jae Bang

Department of Pharmacy, Chosun University, Kwangju 501-759, Korea

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GTP cyclohydrolase I catalyzing the first reaction in the biosynthesis of pterin moiety of folic acid in bacteria, was purified from *Streptomyces tubercidicus* by at least 203-fold with a yield of 32% to apparent homogeneity, using ammonium sulfate fractionation, DEAE-cellulose, Sepharose CL-6B, and hydroxylapatite column chromatography. The molecular weight of the native enzyme was estimated to be 230,000 daltons by gel permeation chromatography. The purified enzyme gave a single band on sodium dodesyl sulfate-polyacrylamide gel electrophoresis and its molecular weight was apparently 58,000 daltons. These results indicate that the enzyme consists of four subunits with the same molecular weight. The K_m and V_{max} values for GTP of the purified enzyme were determined to be 80 μM and 90 nmol/min (mg protein), respectively. The optimum pH and temperature for the enzyme reaction were pH 7.5~8.5 and 40~42°C, respectively. Coenzyme or metal ion was not required for the enzyme activity. The enzyme activity was inhibited by most divalent cations, while it was slightly activated by potassium ion. In case of nucleotides, CTP, GMP, GDP, and UTP inhibited enzyme activity, among which GDP exhibited the strongest inhibitory effect.

Key words : *Streptomyces tubercidicus*, GTP cyclohydrolase I

INTRODUCTION

Guanosine triphosphate (GTP) is used as a substrate for various biosynthetic pathways, such as the biosynthesis of RNA, riboflavin, folic acid, tetrahydrobiopterin and pyrrolopyrimidine nucleoside antibiotics (De Saizieu *et al.*, 1995; Isono, 1988). GTP cyclohydrolase I (EC 3.5.4.16) is one of the enzymes that uses GTP as a substrate, and catalyzes the conversion of GTP to dihydroneopterin triphosphate and formic acid (Yim and Brown, 1976; Tabraue *et al.*, 1997). Dihydroneopterin triphosphate, the enzymatic product, serve as a key intermediate in the biosynthesis of pteridine moeity of folic acid in prokaryotes, several pteridine compounds (pigments) in insects and amphibians, and tetrahydrobiopterin in higher animals (Nichol *et al.*, 1985; Hatakeyama *et al.*, 1989, 1991). Tetrahydrobiopterin is an essential cofactor for a variety of one-carbon transfer reactions. Tetrahydrobiopterin functions as a cofactor for various amino acid hydoxylase, the O-alkylglycerolipid cleavage enzyme and nitric oxide synthases (Shen *et al.*, 1988; Tabraue *et al.*, 1997). GTP cyclohydrolase I of several different organisms has been described, and the enzyme has been purified to apparent homogeneity from *Escherichia coli, Drosophila melanogaster, Bacillus subtilis*, human, mouse and rat (Yim and Brown, 1976; Bellahsene *et al.*, 1984; Weisberg and O'Donnell, 1986; Cha *et al.*, 1991; Ichinose *et al.*, 1995; De Saizieu *et al.*, 1995). GTP cyclohydrolase I as the first committing step in the biosynthesis of folic acid and the tetrahydrobiopterin is a logical target for regulation of the biosynthesis of these compounds (Jacobson and Manos, 1989). The pteridine moieties of folic acid and riboflavin are synthesized by a series of reactions initiated by removal, as formate, of carbon 8 of GTP. A family of nucleoside antibiotics (pyrrolopyrimidine nucleoside antibiotics) including tubercidin, toyocamycin, sangivamycin, cadeguomycin and kanakawamycin, are characterized by the presence of pyrrolopyrimidine moiety, *i.e.*, either a 7-deaza-adenine ring or a 7-deaza-guanine ring (Isono, 1988). The first reaction to the common biosynthetic pathway of these antibiotics is the removal of carbon 8 of GTP, catalyzed by GTP-formylhydrolase (Smulson and Suhadolnik, 1967), which has similar action mode to that of GTP cyclohydrolase I.

The goal of the current work was to examine the biochemical characteristics of GTP cyclohydrolase I purified from *Streptomyces tubercidicus*, a producer of tubercidin which is a pyrrolopyrimidine nucleoside antibiotic, and to compare the properties of the enzyme

Correspondence to: Hee-Jae Bang, Department of Pharmacy, Chosun University, Kwangju 501-759, Korea

with GTP cyclohydrolase I isolated from other organisms.

MATERIALS AND METHODS

Materials

Guanosine triphosphate (GTP), GDP, GMP, ATP, CTP, TTP, UTP, GTP-agarose, bovine serum albumin, neopterin, polyacrylamide and standard proteins (apoferritin, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, cytochrome c) were purchased from Sigma Chemical Co. (St. Louis, MO. USA). Trizma base, ammonium sulfate, potassium chloride, potassium phosphate and molecular weight markers for SDS-PAGE were purchased from USB (USA). DEAE-cellulose, Sepharose CL-6B and Hydroxylapatite were obtained from Pharmacia LKB Biotechnology INC.

Microrganisms and cultivation

Streptomyces tubercidicus ATCC 25502 was obtained from American Type Culture Collection (ATCC). *Streptomyces tubercidicus* ATCC 25502 was streaked on Bennett's agar medium and incubated at 30°C for 7~10 days. Spores scrapped from the agar plate was used to inoculate tryptic soy broth medium (Difco Co.), and incubated for 3~4 days with rotary shaking (200 rpm) at 30°C.

Enzyme assay

The enzyme assay was based on the method developed by Jacobson and Manos (1989) with some modifications. Unless specified otherwise, the standard reaction mixture contained enzyme, 250 μM GTP, 100 mM Tris/HCl (pH 8.0) and 100 mM KCl in a total volume of 0.6 ml. The reaction was carried out at 40°C for 30 min in the dark. To quantitate the formation of dihydroneopterin triphosphate from GTP, the enzyme reaction was terminated by addition of 0.1 N HCl (1 ml) and 100 μl of iodine solution (1% I_2 and 2% KI). The insoluble materials were removed by centrifugation at 12,000×g for 5 min. After 15 min, excess iodine was reduced by adding 100 μl of 2% ascorbic acid, followed by 500 μl of 0.4 M-Tris base to adjust the pH 8.0. Fluorescence of resulting neopterin triphosphate was determined (excitation at 356 nm, emission at 454 nm) using spectrofluorimeter (Hitachi, 250-0081). A standard curve for neopterin was used for quantification of fluorescence. One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of dihydroneopterin triphosphate for 1 hour.

Protein determination

Protein concentrations was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Purification of the enzyme

72 hour cultured mycelium were harvested by centrifugation (12,000×g, 20 min) and washed with standard buffer (50 mM Tris/HCl, pH 8.0). All procedures were carried out at 0~4°C, unless stated otherwise.

Crude extract: 40 g (wet weight) of mycelium were mixed with 50 ml standard buffer and were disrupted with sonication. The supernatant solution was separated from the mycelial debris by centrifugation at 15,000×g for 30 min. The resulting supernatant solution (77 ml) was referred to as the crude extract.

1st ammonium sulfate fractionation: Ammonim sulfate powder was added with stirring to the crude extract to give a 40% saturated solution and maintained at 0°C with stirring for 20 min. The resulting precipitate was removed by centrifugation (15,000×g, 40 min). Additional ammonium sulfate powder was added to give a 60% saturated solution, and the resulting precipitate was recovered by centrifugation (15,000×g, 40 min). This precipitate contained the enzyme and was redissolved in small volume of standard buffer and dialyzed against the same buffer.

DEAE cellulose ion exchange chromatography: The solution obtained from the 1st ammonium sulfate fractionation step was applied to a column (2.5×32 cm) of DEAE-cellulose equilibrated in standard buffer. After being washed with 150 ml of same buffer, the column was developed with a linear gradient of 0.0~0.5 M KCl in a total volume of 800 ml at a flow rate of 40 ml/h.

The fractions containing enzyme activity (eluents at 0.2 M~0.3 M KCl) were combined (33 ml).

Gel permeation chromatography: The enzyme solution obtained from DEAE-cellulose chromatography was precipitated by addition of ammonium sulfate powder (up to 70% of saturation). The resulting pellet was resuspended in 2 ml of standard buffer containing 0.3 M KCl and then applied to a column of Sepharose CL-6B (1.5×80 cm) equilibrated with standard buffer containing 0.3 M KCl. The proteins were eluted with same buffer, at a flow rate of 6 ml/h. The fractions containing enzyme activity were combined (11.5 ml).

2nd ammonium sulfate fractionation: Ammonim sulfate powder was added with stirring to the active fractions of gel permeation chromatography to give a 43% saturated solution and maintained at 0°C with stirring for 20 min. The resulting precipitate was removed by centrifugation (15,000×g, 40 min). Additional ammonium sulfate powder was added to give a 55% saturated solution, and the resulting precipitate was recovered by centrifugation (15,000×g, 40 min). This

precipitate contained the enzyme and was redissolved in 1 ml of standard buffer and dialyzed against the same buffer.

Hydroxylapatite column chromatography: The active fraction obtained from 2nd ammonium sulfate fractionation step was applied to a column (1.5×25 cm) of hydroxylapatite equilibrated with 5 mM potassium phosphate buffer (pH 7.0). After being washed with 50 ml of same buffer, the column was developed with a linear gradient of 5~200 mM potassium phosphate (pH 7.0) in a total volume of 250 ml, at a flow rate of 20 ml/h.

Molecular weight determination

The molecular weight of the enzyme in its native form was determined by gel filtration on a column of Sepharose CL-6B (1.5×80 cm) equilibrated with 50 mM Tris/HCl (pH. 8.0) buffer containing 0.3 M KCl, at 4°C and a flow rate of 6 ml/h. Molecular weight standards used for the molecular weight determination of native enzyme were:blue dextran (2,000,000), apoferritin (443,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000). To determine the molecular weight of GTP cyclohydrolase I subunit, sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). Phosphorylase b (97,400), albumin (66,200), ovalbumin (42,700), and carbonic anhydrase (31,000) were used for molecular weight markers of SDS-PAGE, and 11% (w/v) separating gel and 5% (w/v) stacking gel were used for protein separation.

Kinetics

K_m and V_{max} values were determined for GTP cyclohydrolase I from Lineweaver-Burk double reciprocal plot of enzyme reactions with the concentration of GTP varied from 10 μM to 500 μM.

Effect of nucleotides and metal ions

To investigate the effects of nucleotides and metal ions on the GTP cyclohydrolase I, various nucleotides and metal ions added to the standard reaction mixture to give a final concentration of 1~2 mM and 0.1~1.0 mM, respectively. Resulting enzyme activities were compared with that of standard enzyme reaction.

RESULTS AND DISCUSSION

Purification of GTP cyclohydrolase I

The enzyme was purified from cells of *Streptomyces tubercidicus* ATCC 25502 as described in methods, and the results of a typical purification are shown in Table I. A single symmetrical peak of the enzyme activity was observed in each elution profile from DEAE-cellulose, sepharose CL-6B, and hydroxylapatite columns. Coincidental elution of the enzyme activity with protein at the final step of purification is shown in Fig. 1. About 203-fold purification in the specific activity of the enzyme from *Streptomyces tubercidicus* was achieved with a yield of 32%. The enzyme preparation obtained at the final step of purification was homogeneous by the criteria of SDS-polyacrylamide gel electrophoresis (Fig. 2).

With phosphate buffer systems, GTP-agarose has been used effectively for the purification of prokaryotic GTP cyclohydrolase I, but it has not been effective for eukaryotic enzymes (Yim and Brown, 1976; Cha *et al.*, 1991). GTP cyclohydrolase I isolated from mouse liver did not bind to GTP-agarose in the presence of phosphate buffer, but showed tight binding to the gel in the PIPES buffer system. Interestingly, GTP cyclohydrolase I isolated from *Streptomyces tubercidicus* did not bind to GTP-agarose both in the

Table I. Scheme for purification of GTP cyclohydrolase I from *Streptomyces tubercidicus*

Fraction step	*Total protein* (mg)	*Total activity* (units)	*Specific activity* (unit/mg)	*Purification fold*	*Recovery* (%)
Crude extract	1087.2	2964	2.7	1	100
1st A. sulfate fractionation	733	3087	4.21	1.6	104
DEAE-Celluloser	27	1567	58	21.48	53
Sepharose CL-6B	8.4	1335	159	59	45
2nd A. sulfate fractionation	3.6	1126	313	116	38
Hydroxylapatite	1.7	948	548	203	32

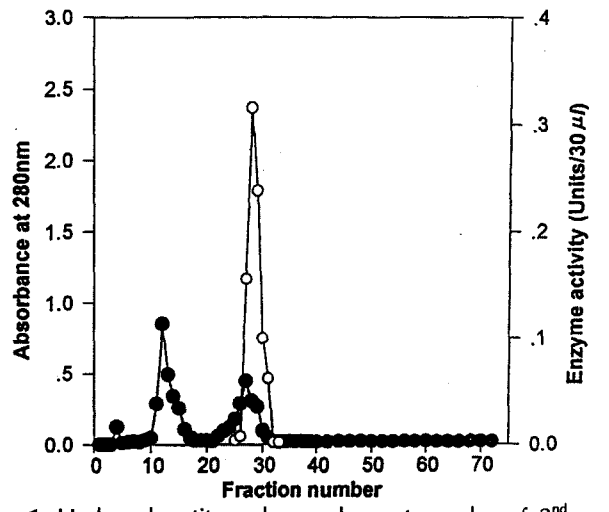

Fig. 1. Hydroxylapatite column chromatography of 2nd ammonium sulfate fraction. The column was developed with a linear gradient of 5~200 mM potassium phosphate (pH 7.0) in a total volume of 250 ml, at a flow rate of 20 ml/h, and 4ml fractions were collected (●: Protein con., ○: Enzyme activity).

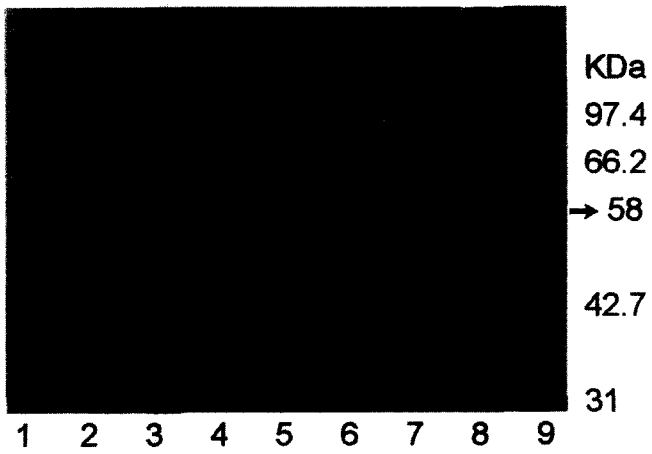

Fig. 2. Purification fractions and molecular weight determination of the purified GTP cyclohydrolase I by SDS-PAGE. Lane 1 and 9, SDS-PAGE standards; Lane 2, Crude extracts; Lane 3, 1st Ammonium sultate; Lane 4, DEAE-cellulose; Lanes 5 and 6, Sepharose CL-6B; Lane 7, 2nd A. sulfate; Lane 8, Hydroxylapatite fraction.

presence of phosphate buffer and PIPES buffer system. These results suggest that GTP cyclohydrolase I isolated from *Streptomyces tubercidicus* have somewhat distinct affinity and/or binding site for GTP compare to enzymes from other sources.

Molecular weight

The molecular weight of the purified enzyme was estimated at 320,000 by comparing its rate of filtration through gel permeation chromatography column of sepharose CL-6B with rate of standard proteins (Fig. 3). In order to establish the number and kinds of subunits present in enzyme, the purified enzyme was

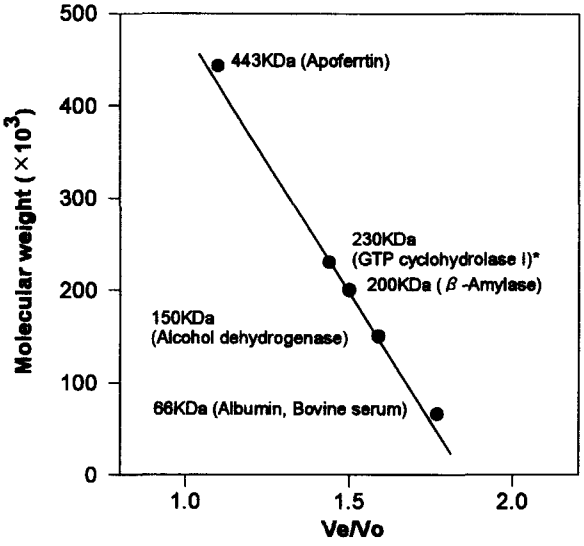

Fig. 3. Determination of molecular weight of native enzyme by gel permeation chromatography on sepharose CL-6B. *GTP cyclohydrolase I purified from *Streptomyces tubercidicus*.

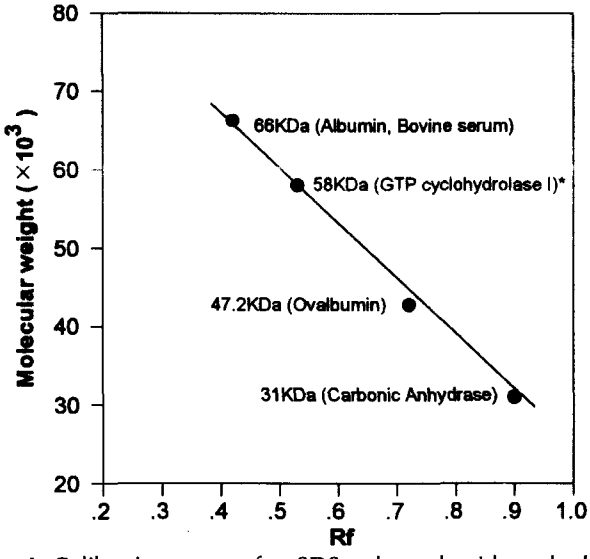

Fig. 4. Calibration curve for SDS-polyacrylamide gel electrophoresis of purified GTP cyclohydrolase I. The purified enzyme was applied to a 11% (w/v) separating and 5% (w/v) stacking gel slab, and protein was stained with coomassie blue.

subjected to electrophoresis on SDS-polyacrylamide gel. The molecular weight of GTP cyclohydrolase I was estimated at 58,000 by comparing its migration position with the mobilities of the standards (Fig. 2, 4). These results suggest that GTP cyclohydrolase I from *Streptomyces tubercdicus* consists of four subunits with the same molecular weight. Thus, this enzyme showed similar high molecular weight and subunit composition to that from other prokaryotes and eukaryotes (Yim and Brown, 1976; Cha *et al.*, 1991; De Saizieu *et al.*, 1995).

Other biochemical characteristics

K_m value for GTP of GTP cyclohydrolase I from *Streptomyces tubercdicus* was 80 μM (Fig. 5), which is relatively high value compared to that of other

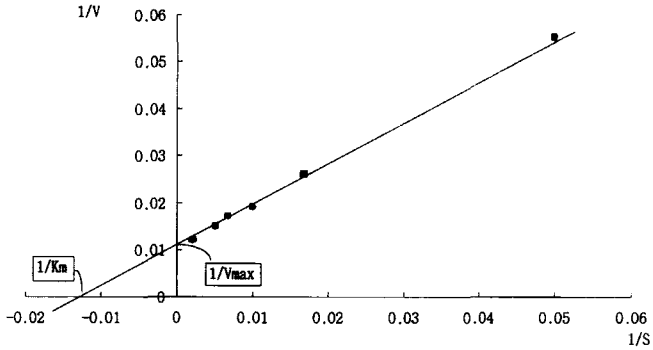

Fig. 5. Determination of K_m and V_{max} values for GTP of GTP cyclohydrolase I purified from *Streptomyces tubercidicus*. Lineweaver-Burk double reciprocal plot of enzyme reactions with the concentration of GTP.

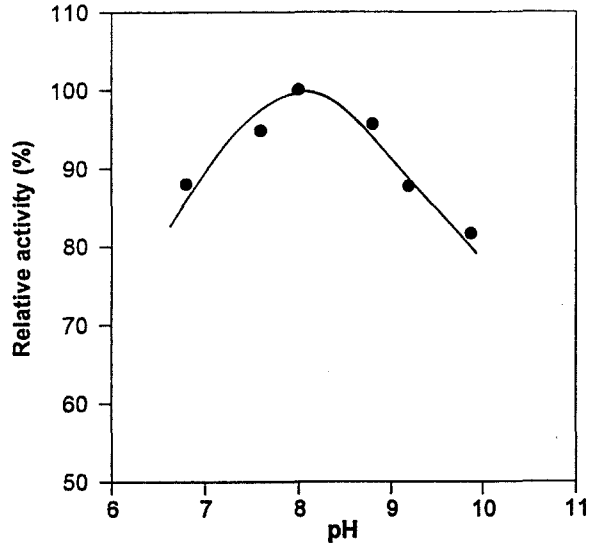

Fig. 6. Effect of pH on GTP cyclohydrolase activity. Enzyme activities were assayed under the standard assay condition with varying pH.

bacteria. The K_m values of *Escherichia coli* and *Bacillus subtilis* enzyme were 0.02 μM and 4 μM, repectively. V_{max} value was 90 nmol/min (mg protein), which is similiar with *Bacillus subtilis* and rat liver enzymes (Cha *et al.*, 1991; De Saizieu *et al.*, 1995). The purified GTP cyclohydrolase I from *Streptomyces tubercidicus* was specific for GTP as a substrate, whereas it does not use ATP, CTP, UTP, and TTP as substrate. The optimum pH of the enzyme reaction was about 7.5~8.5 and the optimum temperature of the enzyme reaction was 40~42°C (Fig. 6).

Effect of nucleotides and metal ions on the enzyme activity

GMP, GDP and UTP inhibited the enzyme activity, among which GDP exhibited the strongest inhibitory activity (Table II). GDP at a concentration of 2 mM inhibited the enzyme activity by 62%. These inhibitory effects of nucleotides on the GTP cyclohydrolase I were similar with *Escherichia coli, Bacillus subtilis* and *Drosophila melanogaster* enzyme. But ATP, which was a strong inhibitor of *Escherichia coli* and *Drosophila melanogaster* enzyme (Yim and Brown, 1976; Cha *et al.*, 1991), showed negligible inhibition effect to GTP cyclohydrolase I isolated from *Sterptomyces tubercidicus*. As described for the enzymes from other sources (Cha *et al.*, 1991), the purified GTP cyclohydrolase I of *Streptomyces tubercidicus* was inhibited by most of divalent cations (Table III). Co^{+2}, Cu^{+2}, Mn^{+2}, Mg^{+2} and Ni^{+2} inhibited the enzyme activity to near completion, at a concentrations of 1 mM. Fe^{+2}, Ca^{+2} and Na^{+} at 1 mM caused relatively weak inhibition (65~15%). The enzyme activity was increased slightly by KCl at 10~100 mM, similar to the enzymes from *Escherichia coli, Bacillus subtilis, Drosophila melanogaster*, rat liver, mouse tissues and human liver.

Table II. Effect of nucleotides on the activity of GTP cyclohydrolase I from *Streptomyces tubercidicus*

Nucleotides	Remaining activity (%)	
	1 mM	2 mM
None	100	100
ATP	102	80.1
CTP	83.6	67.5
GMP	62.7	54.2
GDP	55.4	38.3
TTP	85.8	83.3
UTP	52.3	50.8

Table III. Effect of metal ions on the activity of GTP cyclohydrolase I from *Streptomyces tubercidicus*

Metal ions	Remaining activity (%)	
	0.1 mM	1 mM
None	100	100
K^{+}	90.4	100
Ca^{+2}	83.7	34.3
Cu^{+2}	59.1	0
Co^{+2}	81.9	3
Mn^{+2}	81.5	3
Mg^{+2}	86.1	3
Ni^{+2}	75.8	1
Na^{+}	92	84
Fe^{+2}	83.7	84.5

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