Guanosine triphosphate cyclohydrolase activity in rat tissues

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The GTP cyclohydrolase activity of rat tissues has been studied by means of the measurement of formic acid release and neopterin synthesis from GTP. After gel filtration of a 45%-satd.-(NH₄)₂SO₄ fraction of liver homogenates, three enzyme fractions were separated and named A₁, A₂ and A₃ according to the order of their elution. Fractions A₁ and A₃ displayed an 8-formyl-GTP deformylase activity; no proof of cyclized product has yet been established. This activity was heat-labile and required Mg²⁺ for maximal activity. Fraction A₂ displayed a 'neopterin-synthetase' activity, with dihydroneopterin triphosphate and formic acid formed in stoichiometric amounts. Fraction A₁* isolated from heat-treated homogenates also produced dihydroneopterin triphosphate. Neopterin synthetase activity in fractions A₁* and A₂ was heat-resistant and inhibited by Mg²⁺. In liver the A₂ fraction represented 70–75% of the neopterin synthetase capacity and was inhibited by reduced pterines (sepiapterin, dihydrobiopterin and tetrahydrobiopterin) and to a lesser extent by reduced forms of folic acid. In kidney and brain, fraction A₁ and A₃ GTP 8-formylhydrolase activities were found in significant amounts, in contrast with the neopterin synthetase activity, which was low and appeared to be confined to the A₁* fraction.

BH₄ is an essential cofactor for the pterin-dependent aromatic amino acid hydroxylases (Kaufman, 1964). BH₄ is synthesized from GTP with dihydroneopterin triphosphate as an intermediate. Burg & Brown (1968) described an enzyme, GTP cyclohydrolase I (EC 3.5.4.16), from *Escherichia coli* that catalyses the removal of the carbon at ring position 8 of GTP to form formate and dihydroneopterin triphosphate. The occurrence of a GTP cyclohydrolase acting as the first step of pterin synthesis was then demonstrated in a number of bacteria (Cone & Guroff, 1971; Jackson & Shiota, 1971; Yim & Brown, 1976; Iwai *et al.*, 1980) and in higher animals such as Syrian golden hamster (Fukushima *et al.*, 1975a,b), chicken (Fukushima *et al.*, 1977), rat (Gal *et al.*, 1978; Lee *et al.*, 1979; Viveros *et al.*, 1981) and human (Blau & Niederwieser, 1983). Recent observations designated this enzyme as the rate-limiting step for BH₄ synthesis in

rats (Viveros *et al.*, 1981) and in human (Blau & Niederwieser, 1983). Niederwieser *et al.* (1982b) reported the observation of a child with hyperphenylalaninaemia and atypical neurological signs who excreted low amounts of neopterin and biopterin indicating a defect between GTP and dihydroneopterin triphosphate.

The present paper describes properties and distribution of GTP cyclohydrolase activities in rat tissues.

Experimental

Biological material

Liver, brain and kidney were obtained from male Wistar-strain rats (200–300 g) that had free access to food and water and *E. coli* from C.N.R.S. (Gif sur Yvette, France).

Chemicals

[8-¹⁴C]GTP (sp. radioactivity 46 Ci/mol) was purchased from New England Nuclear. Alkaline phosphatase, GTP, GDP, GMP, guanosine, tetrahydrofolic acid, dihydrofolic acid and (±)-N₅-methyltetrahydrofolic acid were obtained from

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Abbreviations used: BH₄, tetrahydrobiopterin [2-amino-6-(1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridin-4(3H)-one]; h.p.l.c., high-performance liquid chromatography.

Sigma Chemical Co. and biopterin, 6-methyltetrahydropteridine and 6,7-dimethyltetrahydropteridine from Calbiochem. Neopterin, sepiapterin, dihydrobiopterin and BH₄ were prepared by Dr. B. Schircks (Wettswill, Switzerland), and D-erythro-dihydroneopterin triphosphate was produced by incubation of GTP with partially purified GTP cyclohydrolase I from *E. coli* (Yim & Brown, 1976). D-erythro- and D-threo-neopterin were gifts from Dr. K. Fukushima (Wellcome Research Labs., Research Triangle Park, NC, U.S.A.).

Enzyme assay procedures

Enzyme activity was assayed by the fluorescence method described by Fukushima *et al.* (1977) and by the liberation of radioactive formic acid from [8-¹⁴C]GTP by the method of Burg & Brown (1968). Unless otherwise specified, the standard reaction mixture contained enzyme, 150 nmol of GTP, 30 μ mol of potassium phosphate buffer, pH 8.0, 6 mg of bovine serum albumin and 0.4 μ mol of EDTA (adjusted to pH 8.0 with KOH) or 30 μ mol of MgCl₂ in a total volume of 0.6 ml. Incubation was for 60 min at 37°C.

For the fluorescence method, the incubation was terminated by the addition of 1 ml of 0.1 M-HCl. Oxidation of pterins was performed by addition of 100 μ l of iodine solution (1 g of iodine and 2 g of KI in 100 ml of water) for 15 min. Then 100 μ l of 0.1 M-ascorbic acid and 0.5 ml of 0.4 M-Tris base were added. The fluorescence was measured with a Jobin-Yvon JY3 spectrofluorimeter (excitation, 356 nm; emission, 454 nm). Standards of neopterin were used for quantification.

For the radioactive method, the incubation was followed by the addition of 1 ml of 1 M-formic acid and 1.5 ml of ethyl acetate. The mixture was shaken and centrifuged, and 0.75 ml of the organic layer was analysed for radioactive formate in an LKB Rack-beta scintillation counter with 5 ml of Biofluor (NEN). Formic acid release was calculated on the basis of a 40% level of extraction of formate by ethyl acetate (39.8 $\pm$ 0.9% from 10 determinations with H¹⁴COOH).

Identification of reaction products

Using similar conditions to those previously described, except the use of 0.05 M-Tris/HCl, pH 8.0, as buffer, a reaction mixture of 1.5 ml–4.5 ml was incubated at 37°C for 60 min. Then 2 ml of 1 M-HCl was added and oxidation was performed by addition of iodine. After addition of ascorbic acid to reduce the excess iodine, the mixture was adjusted to pH 8.0 with 2 M-Tris base. Alkaline phosphatase (2 mg/ml) was added and the mixture was incubated at 37°C for 60 min in the dark. Then 1 ml of 2 M-trichloroacetic acid and 50 μ l of 6-methylpteridine (5 mg/100 ml of 0.1 M-HCl) as internal standard

were added. After mixing and centrifugation, pterins in the supernatant were isolated by ion-exchange chromatographies [Dowex 50W (X8, H⁺ form; 1X2, OH⁻ form)]. The final acid eluate was analysed by an h.p.l.c. procedure as described previously (Dhondt *et al.*, 1981) on Partisil-10 SCX (Whatman).

Gel chromatography of GTP cyclohydrolases

Unless otherwise specified, all operations were carried out at 4°C. Rat tissues were homogenized in 4 vol. of 0.05 M-potassium phosphate buffer, pH 8.0, and the homogenate was centrifuged at 12 000 g for 10 min.

'Heat-treated' homogenates were obtained by heating homogenates in a water bath at 100°C until the temperature reached 60°C. Then, homogenates were cooled in an ice bath, centrifuged at 12 000 g for 10 min and subjected to the same procedures described below.

To the supernatant fluids was added a cold saturated solution of (NH₄)₂SO₄ to give a 45%-saturated solution. After the mixture had been stirred for 20 min, the resulting precipitate was recovered by centrifugation (20 000 g for 30 min) and suspended in a small volume of 0.05 M-potassium phosphate buffer, pH 8.0, and then dialysed overnight against the same buffer. The dialysed samples were applied to a Sepharose 6B (Pharmacia) column (1.6 cm $\times$ 40 cm or 2.6 cm $\times$ 80 cm) that had been equilibrated with 0.05 M-potassium phosphate buffer, pH 8.0. The column was developed in the same buffer at a flow rate of 30 ml/h and 2 ml fractions were collected.

Miscellaneous

Dihydrobiopterin affinity chromatography. The coupling of dihydrobiopterin to CH-Sepharose 4B (Pharmacia, Uppsala) was performed by a method derived from that of Cotton (1974).

Inhibitor studies. When GTP cyclohydrolase activity was assayed in the presence of the tetrahydro-form of pteridines, they were protected from spontaneous oxidation by addition of 4 mM-dithiothreitol.

Protein concentrations were estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

M_r was estimated by gel filtration (Sephadex G-100) with Blue Dextran, aldolase (*M_r* approx. 160 000) and bovine serum albumin (*M_r* approx. 67 000) as markers.

Results

Liver GTP cyclohydrolase activity

Formic acid formation. On the basis of the formic acid release activity, three protein fractions were

Fig. 1. Sepharose 6B chromatography (2.6 cm $\times$ 80 cm) of rat liver GTP cyclohydrolase activity

Each fraction was assayed using the formic acid release method in the presence of 50 mM-MgCl₂ (● and ▲) or 0.66 mM-EDTA (○ and △) and the fluorescence method (●—●) in the presence of 0.66 mM-EDTA. Native (● and ○) and 'heat-treated' homogenates (▲ and △) served as enzyme source.

separated on a Sepharose 6B column (2.6 cm $\times$ 80 cm) (Fig. 1) on the basis of their requirement for Mg²⁺ and their heat-stability. They have been termed A₁, A₂ and A₃ in the order of their elution.

A₁ was heat-labile and a slight increase (1.4–1.8-fold) in activity occurred when Mg²⁺ (50 mM) was used instead of EDTA.

A₂ was strongly inhibited by Mg²⁺; half-maximal activity was observed at 2.5 mM-Mg²⁺. Therefore maximal velocity was obtained in the EDTA-containing reaction mixture. This fraction was heat-stable; thus, studies of A₂ were easily performed on A₂ fractions isolated from 'heat-treated' homogenates.

A₃ required Mg²⁺ for maximal activity (EDTA at 0.7 mM completely inhibited the activity) and was heat-labile.

No change in the profile of elution and activities was noted when the homogenization and following steps were performed in the presence of dithiothreitol (5 mM) or KCl (2.5 mM).

Dihydroneopterin triphosphate formation. Fluorescent material was produced only by A₁ and A₂ fractions. The fluorescent material was identified as *erythro*-dihydroneopterin triphosphate on the basis of a stronger fluorescence after oxidation and the h.p.l.c. separation of oxidized and dephosphorylated products. Standards of *D-erythro*-neopterin and *D-threo*-neopterin eluted respectively at 7 min and 7.3 min and enzymic product co-eluted with the first peak.

Dihydroneopterin triphosphate synthesis was

strongly inhibited by Mg²⁺, whatever the enzyme fraction tested, and the lack of a pterin production was confirmed by h.p.l.c. analysis. The two dihydroneopterin triphosphate synthesis activities were heat-stable. The formation of dihydroneopterin triphosphate and of formic acid from GTP was stoichiometric for the A₂ fraction (formic acid release/fluorescent product = 1.02 ± 0.14 ; seven experiments), whereas the reaction stoichiometry was greater than 1.5 with the A₁ fraction separated from 'heat-treated' homogenates (termed A₁* fraction). Moreover, the formic acid release activity of fraction A₁* remained stimulated by Mg²⁺ (Table 1). Of the total dihydroneopterin triphosphate synthesis in liver 70–75% was linked to the A₂ fraction.

Properties of A₂. A large-scale preparation of A₂ enzyme has been obtained from 'heat-treated' homogenates (Table 1). A₂ was most active with GTP as substrate. GDP was approximately one-sixth as efficient a substrate as GTP. GMP and guanosine did not exert a substrate or an inhibitory effect. The *K_m* for GTP was $47 \pm 10 \mu\text{M}$ from a double-reciprocal plot (six experiments), whatever the assay method.

The following compounds displayed neither stimulatory nor inhibitory effects: dithiothreitol (5.0 mM), β -mercaptoethanol (2.5 mM), *p*-hydroxy-mercuribenzoate (2.5 mM), NH₄Cl (2.5 mM), KCl (2.5 mM) and phenylalanine (1.2 mM). Formate (ammonium or sodium salt) (2.5 mM) and dihydro-neopterin triphosphate (0.5 mM), obtained from *E. coli*, had no effect.

Table 1. *Purification of dihydroneopterin triphosphate synthetase from rat liver*

Dihydroneopterin triphosphate synthesis was measured by fluorescence assay in the presence of 0.66 mM-EDTA. The Sepharose 6B column dimensions were 2.6 cm × 80 cm. Abbreviation: nd, not determined.

	Total protein (mg)	Formic acid release (nmol/60 min)		Dihydroneopterin triphosphate synthesis	
		+EDTA (0.66 mM)	+Mg ²⁺ (50 mM)	Total activity (nmol/60 min)	Specific activity (nmol/60 min per mg of protein)
Homogenate	6040	7610	9290	60	0.010
Heat supernatant fluid	1630	382	420	245	0.15
Dialysed (NH ₄) ₂ SO ₄ - satsd. fraction	505	366	310	240	0.48
Sepharose 6B					
fraction 48-63 (A ₁)	107	77	122	52	0.49
fraction 75-94 (A ₂)	50	148	13	136	2.72
fraction 99-110 (A ₃)	69	17	72	nd	—

Fig. 2. Influence of reduced pteridines on the release of [¹⁴C]formic acid by enzyme A₂.

The standard incubation mixture plus various concentrations of pteridines was used (●, BH₄; □, dihydrobiopterin; ■, sepiapterin; ▼, tetrahydrofolate; ▽, dihydrofolate; ◇, 6-methyltetrahydropteridine; ◆, 6,7-dimethyltetrahydropteridine). Enzyme A₂ was obtained from gel chromatography of 'heat-treated' liver homogenates. Assays were performed in triplicate for each point and the average values were used and expressed as percentages of activity without effector.

Tetrahydrobiopterin, dihydrobiopterin and sepiapterin inhibited [¹⁴C]formic acid release (Fig. 2) and neopterin production, whereas totally oxidized biopterin was inefficient. The affinity of dihydrobiopterin for A₂ was verified by the capacity of a dihydrobiopterin-Sepharose matrix to retain enzyme activity. A Sepharose 6B A₂ fraction (10 ml) was applied to an affinity column (1 cm × 1.5 cm) and washed with phosphate buffer. Elution was performed with the same buffer containing 0.2 M-NaCl. A blank control was achieved with a CH-Sepharose-4B column (1 cm × 1.5 cm). The enzyme activity appeared only when the salt elution was applied, with a 50% recovery but with only a 2-fold increase of the specific activity.

6-Methyl- and 6,7-dimethyl-tetrahydropteridine were also inhibitory but to a lesser extent. Tetrahydrofolate and dihydrofolate were found to be inhibitors, in contrast with the N₅-methyltetrahydrofolate, which had no effect (Fig. 2).

GTP cyclohydrolase activity in kidney and brain

Fig. 3 compares the profiles obtained from 4 g tissues samples; activities were measured by the radioactive method.

In kidney and brain the A₂ fraction was not identified, whereas A₁ and A₃ were present. In kidney, the effects of Mg²⁺ and of heat treatment were similar to those described for liver fractions. In contrast, the brain A₁ fraction appeared to be more heat-resistant and more sensitive to Mg²⁺. In the three tissues, the amount of A₃ was in the same range of activity when expressed per g wet wt. of tissue.

Dihydroneopterin triphosphate formation was very low in kidney and in brain, and appeared to be confined to the A₁ fraction (data not shown). As in the liver, this synthesis was heat-stable.

Discussion

GTP cyclohydrolase activity has been located in many species. However, conflicting results have been obtained in tissues from higher species. A GTP cyclohydrolase that catalyses the synthesis of dihydroneopterin triphosphate from GTP in a one-step reaction was found in chicken liver (Fukushima *et al.*, 1977), rat adrenal gland (Viveros *et al.*, 1981) and rat and human liver (Blau & Niederwieser, 1983), whereas Gal *et al.* (1978) isolated from rat brain three enzymes: two GTP cyclohydrolases ('AI and AII') forming a formamidopyrimidine derivative from GTP and a dihydroneopterin triphosphate synthetase catalysing dihydroneopterin triphosphate synthesis from the formamidopyrimidine ('enzyme B').

Fig. 3. *Sepharose 6B chromatographic profiles of GTP cyclohydrolase activity of liver, kidney and brain tissues* Dialysed (NH₄)₂SO₄ extracts of homogenates (● and ○) or 'heat-treated' homogenates (▲ and △) from 4 g of the respective tissues were applied to a Sepharose 6B column (1.6 cm × 40 cm). In each fraction formic acid release activity was measured in the presence of 50 mM-MgCl₂ (● and ▲) or 0.66 mM-EDTA (○ and △).

Table 2. *Characteristics of GTP cyclohydrolases isolated from chicken and rat tissues*
Abbreviations used: H₂NP₃, dihydroneopterin triphosphate; FP₂PyDP₃, formamidopyrimidine derivative; nd, not determined.

	Chicken liver (Fukushima <i>et al.</i> , 1977)	Rat brain (Gal <i>et al.</i> , 1978)		Rat liver (the present study)
		A I	A II	
Product	H ₂ NP ₃	FP ₂ PyDP ₃	FP ₂ PyDP ₃	H ₂ NP ₃
Apparent <i>K_m</i> (μM)	14	1.2	58	50
Heat-stability	Stable	Stable	Labile	Stable
Molecular weight	125 000	135 000	34 000	≥160 000
Mg ²⁺ effect	Inhibition	No effect	Dependency	Inhibition
Substrate activity (GDP/GTP)	1/17	nd	nd	1/6
Stoichiometry (H ₂ NP ₃ /formic acid)	1	1	1/280	1

The present findings demonstrated the occurrence in liver of three enzyme fractions catalysing the release of formic acid from GTP. A₁ and A₃ rather displayed a 8-formyl-GTP deformylase activity, as no evidence of cyclized product(s) has yet been established, whereas A₂ presented a true 'neopterin-synthetase' activity with dihydroneopterin triphosphate and formic acid formed in stoichiometric amounts. However, it was also noted that 30% of the neopterin synthetase activity co-eluted with A₁. On the basis of the characteristics of this activity (heat-stability and Mg²⁺ inhibition) it could

be speculated that two enzymes are present in the A₁ fraction.

In kidney and brain fractions A₁ and A₃ were present, whereas A₂ was not identified. In these tissues the 'neopterin-synthetase' activity was linked to the A₁ heat-resistant fraction (A₁*). This finding of efficient 'neopterin-synthetase' activity in liver in contrast with the low activity in other tissues agreed with the data of Fukushima *et al.* (1975*b*) in Syrian golden hamster. The present results suggest that the A₂ enzyme is specific for the liver.

As a result, our attention was focused on the

Fig. 4. Proposed mechanism for the enzymic conversion of GTP into D-erythro-dihydroneopterin triphosphate

characterization of the A_2 enzyme. This fraction presented some similarities with the enzyme isolated by Fukushima *et al.* (1977) from chicken liver: inhibition by Mg^{2+} and heat-stability (Table 2). However, in contrast with the results of Fukushima *et al.* (1977), the A_2 enzyme was found to be strongly inhibited by reduced pteridines and to bind to a dihydrobiopterin–Sephadex matrix. Such behaviour was reported for the GTP cyclohydrolase I from *E. coli* (Then, 1979).

The end product and intermediates of the biosynthetic pathway of BH_4 (Häusermann *et al.*, 1981) are thus potent inhibitors. Consequently a negative-feedback control of the synthesis of BH_4 is conceivable. Indirect evidence of such a regulation has been obtained in man from observations of inborn errors of the metabolism of BH_4 . In the so-called 'dihydrobiopterin-synthetase' deficiency, neopterin accumulates and no, or a reduced amount of, biopterin is synthesized (Niederwieser *et al.*, 1980). The administration of BH_4 or sepiapterin induced a decrease of the excretion of neopterin (Niederwieser *et al.*, 1982a). In dihydropteridine reductase-deficient patients, who are unable to reduce quinonoid-dihydrobiopterin into BH_4 , di-

hydrobiopterin accumulates and the neopterin excretion remained in the normal range (Niederwieser *et al.*, 1980).

The precise mechanism of the inhibition by reduced pteridines is unclear, but evidence has been presented that only hydrogenated forms of pteridines are inhibitory and that 6- and 7-substitutions of the pteridine ring are also of importance.

The intervention of the formamidopyrimidine intermediate (Fig. 4) in the synthesis of dihydroneopterin triphosphate has been extensively discussed but not definitely resolved (Shiota *et al.*, 1967; Yim & Brown, 1976; Gal *et al.*, 1978; Blau & Niederwieser, 1983). Nevertheless, it is clear that with the present A_2 enzyme a direct and stoichiometric production of dihydroneopterin triphosphate was observed.

However, the metabolic role of the A_1 and A_3 8-formyl-GTP deformylase has not been elucidated and the product(s) of the reaction is not yet known. In micro-organisms, GTP cyclohydrolase is thought to participate in riboflavin synthesis (Foor & Brown, 1975). More recently, it has been proposed that queuine, a substance found in animal serum and amniotic fluid and a substrate for queuine–guanosine tRNA transglycosidase, is produced from GTP via GTP cyclohydrolase catalysis (Jacobson *et al.*, 1981). However, the significance of this pathway is not well established and the synthesis of queuine not confirmed in mammals.

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