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Inhibition of GTP cyclohydrolase I by pterins

Rong-sen Shen, Aftab Alam and Yixian Zhang

Division of Biochemistry, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX (U.S.A.)

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Pterins inhibit rat liver GTP cyclohydrolase I activity noncompetitively. Reduced pterins, such as 7,8-dihydro-D-neopterin, (6*R*,*S*)-5,6,7,8-tetrahydro-D-neopterin, 7,8-dihydro-L-biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin, L-sepiapterin, and DL-6-methyl-5,6,7,8-tetrahydropterin are approximately 12-times more potent as inhibitors than are oxidized pterins, such as D-neopterin, L-biopterin, and isoxanthopterin. They are also 12-times more potent than folates, such as folic acid, dihydrofolic acid, (±)-L-tetrahydrofolic acid, and aminopterin. The K_i values for 7,8-dihydro-D-neopterin, 7,8-dihydro-L-biopterin, and (6*R*)-5,6,7,8-tetrahydro-L-biopterin are 12.7 μM, 14.4 μM, and 15.7 μM, respectively. These results suggest that mammalian GTP cyclohydrolase I may be regulated by its metabolic end products.

Introduction

GTP cyclohydrolase I (EC 3.5.4.16) catalyzes the formation of D-*erythro*-7,8-dihydroneopterin (dihydroneopterin) triphosphate and formate from GTP. Dihydroneopterin triphosphate is a key intermediate in the biosynthesis of various substances: folic acid in microorganisms [1,2]; several pteridines in insects and amphibians [3,4]; and L-*erythro*-5,6,7,8-tetrahydrobiopterin (tetrahydrobiopterin) in mammals [5–8]. Tetrahydrobiopterin is the obligatory cofactor for tyrosine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4), which are rate-limiting enzymes during biogenic amine synthesis, and for phenylalanine hydroxylase (EC 1.14.16.1), which converts L-phenylalanine to L-tyrosine [9].

For several reasons, it appears that tetrahydrobiopterin or its metabolites, or both, may play some role in the central-nervous and immune systems [10]. First, because deficiency of GTP cyclohydrolase I has caused a variant form of hyperphenylalaninemia, in which neurologic deterioration progresses rapidly despite a strict dietary regimen [11,12]. Further, recent studies have found that tetrahydrobiopterin stimulates concanavalin A-induced lymphocyte activation [13], and that neopterin is released from macrophages upon stimulation with certain factors that are derived from activated T-cells, such as interferon-γ [14].

Biopterin and other pteridines are widely distributed in microorganisms, insects, plants, and animals [15]. GTP cyclohydrolase I activity and biopterin have been found in organs and biological fluids of several mammalian species [16]. The regulation of GTP cyclohydrolase I activity and the function of biopterin in these organs are largely unknown. GTP cyclohydrolase I activity in primary cultures of adrenomedullary chromaffin cells is increased by treatment with compounds that increase levels of cyclic AMP, and by treat-

Correspondence: R.-s. Shen, Division of Biochemistry, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

ment with agents that deplete cellular catecholamines [17]. Hypophysectomy decreases GTP cyclohydrolase I activity and tetrahydrobiopterin levels in spleen and bone marrow, but not in brain of rats, whereas thyroidectomy decreases tetrahydrobiopterin levels and GTP cyclohydrolase I activity in spleen only [18]. Moreover, adrenalectomy or castration has no effect on biopterin metabolism in these tissues [18], whereas administration of adrenocorticotropic hormone to intact and hypophysectomized rats elevates GTP cyclohydrolase I activity and tetrahydrobiopterin levels in adrenal cortex [19], but not in spleen and bone marrow [18]. It appears that GTP cyclohydrolase I activity and tetrahydrobiopterin levels in spleen and bone marrow, but not in brain, may be regulated by factors secreted from the pituitary.

In vitro studies have shown that GTP cyclohydrolase I activities in rat liver [20] and in *Escherichia coli* [21] may be inhibited by their metabolic end products, such as tetrahydrobiopterin, 7,8-dihydro-L-biopterin (dihydrobiopterin), L-sepiapterin, dihydroneopterin, tetrahydrofolic acid, and dihydrofolic acid. Others, however, have found no evidence of end-product inhibition of bacterial GTP cyclohydrolase I [22,33]. These contradictory reports on the inhibition of GTP cyclohydrolase I activity by pterins are probably a result of the use of a [^{14}C]formate-releasing method for assaying GTP cyclohydrolase I activity. This method is not specific for assaying GTP cyclohydrolase I activity, unless the enzyme preparation is homogeneous and thus free of contamination of other forms of GTP cyclohydrolase or formate-releasing enzyme(s). We, therefore, used the fluorometric method of Blau and Niederwieser [24] in our systematic investigation of the inhibition of rat liver GTP cyclohydrolase I activity by pterins.

Materials and Methods

L-Biopterin, dihydro- and tetrahydrobiopterins, D-neopterin, dihydroneopterin, (6*R*,*S*)-5,6,7,8-tetrahydro-D-neopterin (tetrahydroneopterin), L-sepiapterin, 6-methylpterin, 6-hydroxymethylpterin, 6-formylpterin, and dihydroxanthopterin were purchased from Dr. B. Schircks Labs. (Buechstr. 17a, CH-8645 Jona, Switzerland). (±)-L-tetrahydrofolic acid (tetrahydrofolic acid), folic acid, aminopterin (4-aminofolic acid), DL-6-methyl-5,6,7,8-tetrahydropterin (6-methyltetrahydropterin), 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine (6,7-dimethyltetrahydropterin), pterin, pterin-6-carboxylic acid, xanthopterin, isoxanthopterin, GTP, and alkaline phosphatase (type VII-S from bovine intestinal mucosa) were purchased from Sigma (St. Louis, MO). Pterin solutions were prepared fresh in 0.01 M sodium phosphate buffer (pH 7.7) or 0.01 M HCl, and serial dilutions were made in sodium phosphate buffer.

[8-^{14}C]GTP was obtained from Amershan (Arlington Heights, IL). The specific radioactivity was reduced to 0.05 mCi/mmol by adding unlabeled GTP. 2,5-Diphenyloxazole (PPO) and *p*-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) were purchased from New England Nuclear Corp. (Boston, MA). Scintillation fluid was prepared by dissolving 16 g PPO and 2 g POPOP in a mixture of 20 ml absolute alcohol and 4 l toluene (Fisher).

Rat liver was obtained from Pel-Freez Biologicals (Rogers, AR) and GTP cyclohydrolase I was purified by acid precipitation (pH 5.0), heat treatment (65 °C), ammonium sulfate precipitation (30–40%), ultracentrifugation (2 h at 190000 × *g* and then 4.5 h at 190000 × *g*), and DEAE-Sephacel chromatography. The enzyme preparation used in this study had a specific activity of 11 units/mg of protein, which represents purification from the crude extract of approximately 1300-fold. One unit is the amount of enzyme that catalyzes the formation of 1 nmol of dihydroneopterin triphosphate per h at 37 °C.

GTP cyclohydrolase I activity was determined radiochemically, by extraction of [^{14}C]formate [25], and fluorometrically, by conversion of dihydroneopterin triphospate to neopterin triphosphate or neopterin [24]. The reaction mixture and conditions for the formation of [^{14}C]formate and dihydroneopterin triphosphate were essentially the same (3–75 μg enzyme protein; 1 mM GTP; 0.01 M sodium phosphate buffer, pH 7.7; 1 mM 2-mercaptoethanol; 2.5 mM Na_2-EDTA; 5% glycerol; 37 °C for 30 min).

For the [^{14}C]formate extraction method, 0.05 ml 4 M HCl and 1 ml ethyl acetate were added to the reaction mixture (total 1.25 ml) and [^{14}C]for-

mate was extracted into ethyl acetate by using a Glas-Col shaker. One-half milliliter of the ethyl acetate phase was then mixed with 4 ml of scintillation fluid and the radioactivity was counted (Beckman LS 6800 Liquid Scintillation System). The efficiency of [^{14}C]formate extraction in ethyl acetate was approximately 60%.

For the fluorometric method, 0.05 ml of a mixture of 1.1% I_2 and 2.2% KI in 1.1 M HCl was added to the reaction mixture (total 0.55 ml) to oxidize dihydroneopterin triphosphate to neopterin triphosphate. At the end of 8 min at 25°C, excess iodine was removed by adding 0.05 ml of 1% ascorbic acid solution to the mixture. After centrifugation (Eppendorf 5413, 5 min), 20 μl of supernatant was injected into the HPLC column to measure neopterin triphosphate. Alternatively, the pH of the supernatant was adjusted to 8.0 by the addition of 1 M NaOH (approximately 0.06 ml), and neopterin triphosphate was dephosphorylated to neopterin by alkaline phosphatase (5 units/0.01 ml) at 37°C for 30 min. After centrifugation, 20 μl of supernatant was injected onto the HPLC column to measure neopterin.

The HPLC system consisted of an SCL-6A system controller, an LC-6A pump, an RF-530 fluorescence detector, and a C-R3A recording data processor (Shimadzu Scientific Instruments, Inc., Columbia, MD). The following conditions were set for chromatography: column, Spherisorb 5 μ C18 (250 × 4.6 mm) (Phenomenex, Rancho Palos Verdes, CA); mobile phase 3.5 mM K_2HPO_4 (pH 7.4); flow rate, 1 ml/min; temperature, ambient, and excitation/emission wavelengths, 365/446 nm. The fluorometric determination of neopterin triphosphate and neopterin yielded essentially the same results. In this study, GTP cyclohydrolase I preparations, assayed by the [^{14}C]formate extraction method, yielded activities that were comparable to those obtained by the fluorometric method.

Enzyme inhibition studies were conducted by preincubating the enzyme with inhibitors for 5 min at 37°C, and then adding the substrate (1 mM GTP) to determine the residual enzyme activity. The dissociation constants of the enzyme-inhibitor complexes (K_i values) were determined by plotting the reciprocal of the apparent maximum velocities ($1/V_{max,app}$) against the inhibitor concentrations. To obtain data for these plots, four concentrations of the test compounds were incubated with the enzyme, and the activities were determined at five GTP concentrations (0.1–1 mM). The percent inhibition was obtained by measuring the enzyme activities at 1 mM GTP after incubating 3–75 μg of enzyme, with and without inhibitors, for 5 min at 37°C. The dose-response curves for the inhibitors were constructed by plotting percent inhibition against the logarithm of at least five concentrations of the test compounds. The I_{50} value is defined as the concentration of inhibitor that inhibits 50% of the enzyme activity.

Results

The inhibition of each pterin against rat liver GTP cyclohydrolase I was initially tested at a fixed substrate concentration of 1 mM GTP. No substrate inhibition of the enzyme was detected at this concentration, which is approximately 8-times the K_m value of the enzyme. Fig. 1 shows two groups of dose-response curves which were separated by a concentration factor of approximately 10. The reduced pterins, sepiaterin, dihydro- and tetrahydroneopterins, dihydro- and tetrahydrobiopterin, and 6-methyltetrahydropterin, had a mean I_{50} value of 12.7 ± 1.8 μM. The oxidized pterins, biopterin, neopterin, and isoxanthopterin, as well as the folates, oxidized and reduced, had a mean I_{50} value of at least 151 ± 39 μM. The reduced pterins are, therefore, at least 12-times more potent than the oxidized pterins and the folates. Other pterins, such as pterin, 6-methylpterin, 6-hydroxymethylpterin, 6-formylpterin, pterin-6-carboxylic acid, xanthopterin, dihydroxanthopterin, and aminopterin, on the other hand, had less than 10% inhibitory effect at 100 μM (data not shown).

Three reduced pterins, dihydroneopterin, and dihydro- and tetrahydrobiopterin, were then chosen and tested for their inhibition of the enzyme at various GTP concentrations. All three compounds displayed similar patterns of inhibition. Fig. 2 shows Lineweaver-Burk plots of the inhibition of GTP cyclohydrolase I activity with four concentrations of dihydrobiopterin (1, 2, 4 and 8 μM), against various concentrations of GTP (0.1–1 mM). The kinetic pattern indicates noncompetitive inhibition, and the replot of dihydrobiopterin

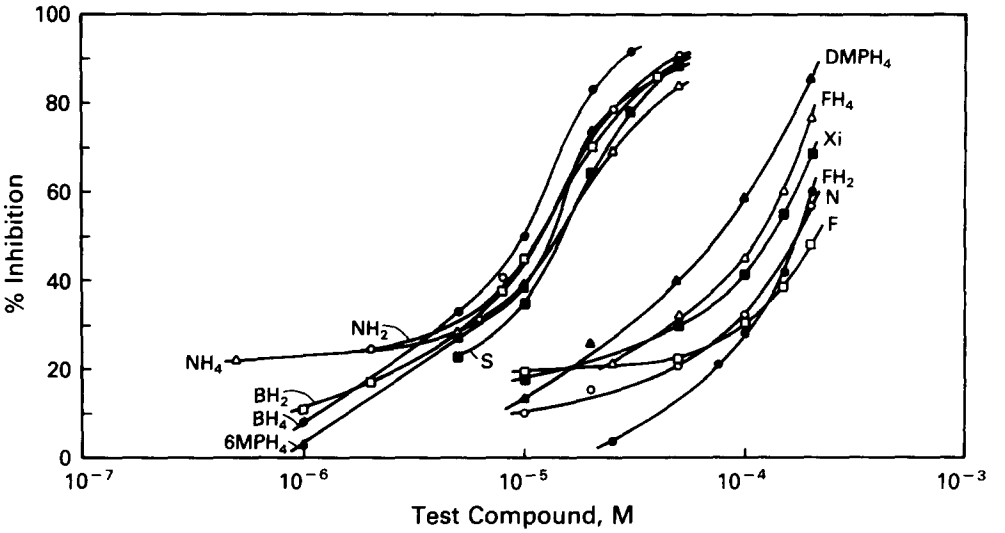


Fig. 1. Dose-response curves of the inhibitory effects of pterins on rat liver GTP cyclohydrolase I. Partially purified rat liver GTP cyclohydrolase I preparations (3–75 μg protein) were preincubated for 5 min with the indicated compound at 37 °C. Residual enzyme activity was assayed by the fluorometric method. Compounds tested: NH_2, 7,8-dihydro-D-neopterin; NH_4, (6*R*,*S*)-5,6,7,8-tetrahydro-D-neopterin; BH_2, 7,8-dihydro-L-biopterin; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; $6MPH_4$, DL-6-methyl-5,6,7,8-tetrahydropterin; S, L-sepiapterin; $DMPH_4$, 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine; FH_4, (±)-L-tetrahydrofolic acid; N, D-neopterin; Xi, isoxanthopterin; FH_2, dihydrofolic acid; F, folic acid.

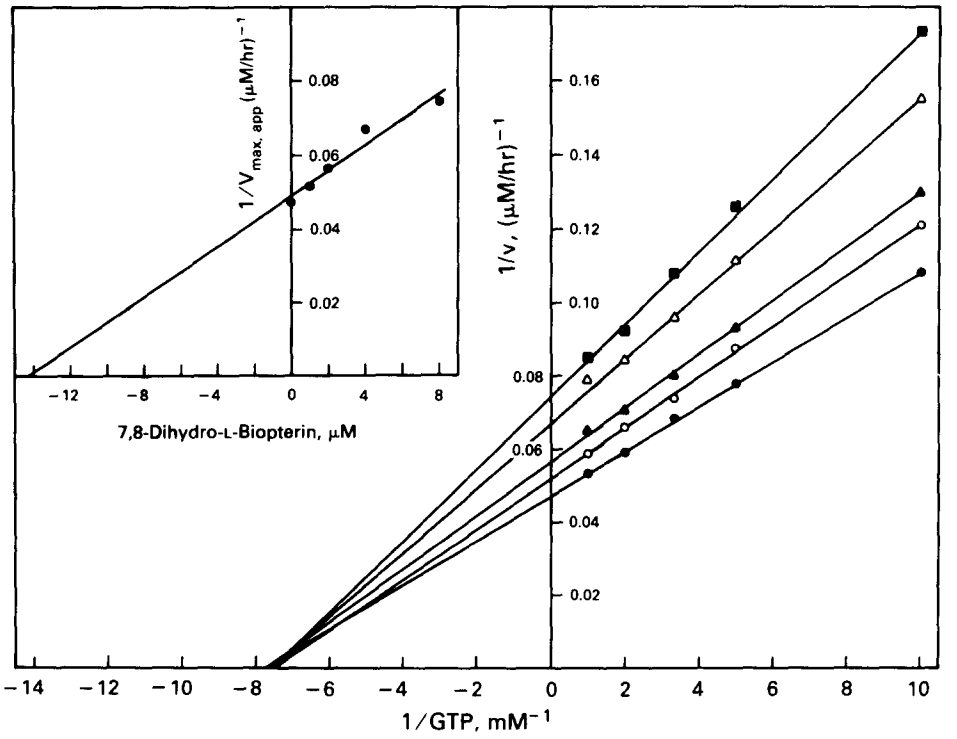
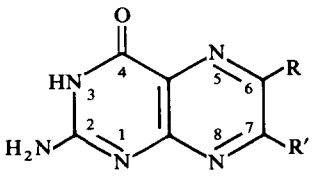


Fig. 2. Double-reciprocal plot of the inhibition of rat liver GTP cyclohydrolase I by 7,8-dihydro-L-biopterin. Partially purified rat liver GTP cyclohydrolase I (3 μg protein) was incubated with varying concentrations of 7,8-dihydro-L-biopterin for 5 min at 37 °C: no inhibitor (●), 1 μM (○), 2 μM (▲), 4 μM (△), and 8 μM (■). Residual enzyme activity was determined at varying GTP concentrations (0.1–1 mM) by the HPLC-fluorometric method. Inset: Replot of the reciprocal of apparent maximal velocity ($1/V_{max,app}$) versus 7,8-dihydro-L-biopterin concentrations.

TABLE I

I_{50} VALUES OF PTERINS AS INHIBITORS OF RAT LIVER GTP CYCLOHYDROLASE I

Partially purified rat liver GTP cyclohydrolase I preparations (3–75 μg protein) were incubated for 5 min with at least five concentrations of the test compound at 37 °C. Residual enzyme activity was assayed by the fluorometric and the [^{14}C]formate extraction methods. The I_{50} value was obtained from the dose-response curve and expressed in μM. Because of a paucity of enzyme sample, values obtained at each concentration point were the single or the average of duplicate determinations.



R	R′	Pterin	HPLC method	[^{14}C]Formate method
CHOH-CHOH-CH_3	H	L-biopterin	> 200	> 200
CHOH-CHOH-CH_3	H;H	7,8-dihydro-L-biopterin	12	22
CHOH-CHOH-CH_3;H	H;H	(6*R*)-5,6,7,8-tetrahydro-L-biopterin	10	40
CHOH-CHOH-CH_2OH	H	D-neopterin	130	> 200
CHOH-CHOH-CH_2OH	H;H	7,8-dihydro-D-neopterin	12	27
CHOH-CHOH-CH_2OH;H	H;H	(6*R*,*S*)-5,6,7,8-tetrahydro-D-neopterin	14	13
CH_2-NH-C_6H_4-CO-Glu	H	folic acid	210	> 200
CH_2-NH-C_6H_4-CO-Glu	H;H	dihydrofolic acid	170	180
CH_2-NH-C_6H_4-CO-Glu;H	H;H	(±)-L-tetrahydrofolic acid	114	130
CO-CHOH-CH_3	H;H	L-sepiapterin	15	35
CH_3;H	H;H	DL-6-methyl-5,6,7,8-tetrahydropterin	13	29
CH_3;H	CH_3;H	2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine	76	112
H	OH	isoxanthopterin	130	> 200

concentrations against the reciprocal of $V_{max,app}$ yielded a K_i of 14.4 μM (Fig. 2 inset). The K_i values obtained for tetrahydrobiopterin and dihydroneopterin were 15.7 μM and 12.7 μM, respectively.

The I_{50} values for pterins were also obtained by using the [^{14}C]formate extraction method. The results are summarized in Table I. The I_{50} values obtained with the [^{14}C]formate method were generally equal to or twice as high as those obtained by the fluorometric method. Moreover, the [^{14}C]formate extraction method also revealed that the reduced pterins were more potent inhibitors than the oxidized pterins and the folates.

Discussion

Tetrahydrobiopterin is known to be significant in the nervous system of mammals, because it participates in catechol neurotransmitter synthesis [9], and possibly also in the immune system, where it may well stimulate the activation and proliferation of lymphocytes [10]. The rate-limiting enzyme in its biosynthesis, GTP cyclohydrolase I, has been the subject of considerable interest in recent years, because of its importance to tetrahydrobiopterin. The mammalian enzyme, however, has not been purified to homogeneity, and its kinetic properties, protein chemistry, and mechanism of action in regulation are still largely unknown.

We have found in this study that pterins inhibit rat liver GTP cyclohydrolase I noncompetitively, and that the reduced pterins are more potent inhibitors than the oxidized pterins. This is in agreement, fundamentally, with the findings of Bellahsene et al. [20], although they found that the I_{50} values for dihydro- and tetrahydrobiopterins, 6-methyl- and 6,7-dimethyltetrahydropterin, and dihydro- and tetrahydrofolic acids ranged from 10^{-10} M to 10^{-9} M, which are approximately

10^5-fold lower than our values. These tremendous differences in the inhibitory potency of pterins between the two studies are difficult to explain, since the GTP cyclohydrolase I preparations used in both studies were heat-stable, Mg^{2+}-inhibitory, GTP- and dihydroneopterin triphosphate-specific. The enzyme preparation used in our study had a specific activity of 11.4 units/mg of protein, a K_m of 124 μM, and a molecular weight of 509 000 ± 15 500 (unpublished data), in comparison to the specific activity of 2.7 units/mg of protein, K_m of 50 μM, and $M_r \geq 160\,000$ of the enzyme preparation used by Bellahsene et al. [20]. Because of the great difference in molecular weight, it is likely that different forms of GTP cyclohydrolase I may be involved in these studies.

GTP cyclohydrolase is known to exist in multiple forms in microorganisms (for review, see Ref. 26) and in rat liver [20]. All of them release formate from GTP as a common product. The [^{14}C]formate-releasing method, therefore, may not be specific for the determination of the activities of GTP cyclohydrolase I, which produces dihydroneopterin triphosphate along with formate. This is particularly true if the enzyme preparation is crude or only slightly purified. We used both the [^{14}C]formate extraction and the fluorometric methods in assaying GTP cyclohydrolase I activity. The stoichiometric ratio between the two products, dihydroneopterin triphosphate and formate, produced by our enzyme preparation was 1 : 1 (data not shown). This result indicates that the enzyme preparation used in this study was free of contamination by other nonspecific formate-forming activity. The high purity of the enzyme preparation explains the general agreement in the I_{50} values obtained by the two methods.

GTP cyclohydrolase I activities in human liver [27] and *E. coli* [21,28] are also inhibited by reduced pterins. Noncompetitive inhibition was reported for 6-hydroxymethylpterin [21] and uncompetitive inhibition was found for dihydrobiopterin [28] against *E. coli* enzymes. Other studies, however, found that reduced pterins did not inhibit GTP cyclohydrolase I in *E. coli* [22] and *Lactobacillus plantarum* [23]. The results that we obtained, and those of others [20,27], support the notion that mammalian GTP cyclohydrolase I activity is inhibited by its end products. The controversy over the inhibition of bacterial GTP cyclohydrolase I by pterins remains to be resolved. Since homogeneous enzyme was used in the study by Yim and Brown [22], it is conceivable that the regulation mechanisms of GTP cyclohydrolase I in mammals are different from those in *E. coli*.

The inhibition properties of pterins depend on the reduced state of the compounds. The 3-carbon alkyl substitution at the C-6 atom of the pterin ring appears to have no effect on the inhibitory potency of these compounds. If the C-6-substituent is bulky, such as the aminobenzoyl-L-glutamyl group, the inhibitory potency of the compound decreases by 12-fold. Since L-dihydrobiopterin and D-dihydrineopterin are equally effective inhibitors, the stereoisomerism of the C-6-substituents may not play an important role in the binding of pterins to the enzyme.

The detection of hyperphenylalaninemia due to GTP cyclohydrolase I deficiency depends on the determination of the activity of this enzyme in biopsy samples. Since pterins in major organs and biological fluids are present predominantly in the reduced forms, they must be removed by gel filtration or other means before the crude homogenates can be used in assying GTP cyclohydrolase I activity.

The inhibitory action of reduced pterins makes them ideal ligands for the purification of GTP cyclohydrolase I by affinity chromatography. Dihydrofolate-Sepharose was used successfully in the purification of bacterial GTP cyclohydrolase I [28,29], whereas dihydrobiopterin-Sepharose did not work well for the mammalian enzymes [20,27]. The ineffectiveness of pterin-Sepharose in the purification of mammalian GTP cyclohydrolase I is probably not a result of its lack of or low affinity for the enzyme molecules. It may well be due to the loss of the enzyme binding site during coupling of pterins to gels. Efforts are being directed in our laboratory toward improving the coupling technique so that the functional groups on pterin molecules remain available for binding GTP cyclohydrolase I.

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