Effects of sepiapterin and 6-acetyldihydrohomopterin on the guanosine triphosphate cyclohydrolase I of mouse, rat and the fruit-fly *Drosophila*

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The regulation of GTP cyclohydrolase I would lead to the regulation of tetrahydrobiopterin, an important cofactor for synthesis of neurotransmitters. In an attempt to extend a previous finding [Bellahsene, Dhondt, & Farriaux (1984) *Biochem. J.* **217**, 59–65] that GTP cyclohydrolase I of rat liver is inhibited by subnanomolar concentrations of reduced biopterin and sepiapterin, we found that this could not be verified with the enzyme from mouse liver, fruit-fly (*Drosophila*) heads or, indeed, from rat liver. It was shown, however, that 12 μM -sepiapterin inhibited mouse liver GTP cyclohydrolase I. Another compound, namely 6-acetyldihydrohomopterin, was also employed in the present study to explore its effect on enzymes that lead to its synthesis in *Drosophila* and for effects on mammalian systems; at 2–5 μM this compound was shown to stimulate one form of mouse liver GTP cyclohydrolase I and then to inhibit at higher concentrations (40 μM). Neither sepiapterin nor 6-acetyldihydrohomopterin caused any effect on the *Drosophila* head enzyme. On the other hand, the sigmoid GTP concentration curve for the *Drosophila* enzyme may indicate a regulatory characteristic of this enzyme. Another report, on the lower level of GTP cyclohydrolase I in mutant mouse liver [McDonald, Cotton, Jennings, Ledley, Woo & Bode (1988) *J. Neurochem.* **50**, 655–657], was confirmed and extended. Instead of having 10 % activity, we find that the *hph-1* mouse mutant has less than 2 % activity in the liver. These studies demonstrate that micromolar levels of reduced pterins may have regulatory effects on GTP cyclohydrolase I and that a mouse mutant is available that has low enough activity to be considered as a model for human atypical phenylketonuria.

INTRODUCTION

Tetrahydrobiopterin is the cofactor for oxidation of aromatic amino acids in the pathway to neurotransmitters, e.g. adrenaline (epinephrine) and 5-hydroxytryptamine ('serotonin'). As shown in Scheme 1, the synthesis of tetrahydrobiopterin involves four enzymes, GTP cyclohydrolase I, pyruvoyltetrahydropterin synthase, pyruvoyltetrahydropterin reductase and sepiapterin reductase [1–3]. A report by Bellahsene *et al.* [4] raised the possibility of a very sensitive feedback-control mechanism in this pathway; they obtained a form of GTP cyclohydrolase I from rat liver that was inhibited over 90 % by 0.2 nM-sepiapterin and by other reduced pterins, such as dihydro- and tetrahydro-biopterin. In *Drosophila*, 6-acetyldihydrohomopterin (Ahp) is produced from the same precursor as sepiapterin [5,6], so a comparison of the metabolic effects of these two compounds was one of the goals of the present study.

The choice of mouse liver and *Drosophila* was based on the availability of mutants: the *hph-1* mutant of mouse was reported to have ~ 10 % of normal activity of GTP cyclohydrolase I [7] and the Punch mutant of *Drosophila* has very low activity [8]. Humans with atypical phenylketonuria produce insufficient tetrahydrobiopterin and, in some cases, this is due to a deficiency of GTP cyclohydrolase I [9,10]. Furthermore, this enzyme varied in mice with genetically based neurological and immunological deficiencies [11] and with hormone treatment [12].

The goals of the present study were to determine whether Ahp and sepiapterin were effective inhibitors of GTP cyclohydrolase I from mouse liver, rat liver and *Drosophila* heads and to re-examine the low activity of the mouse mutant *hph-1*.

MATERIALS AND METHODS

Biological materials

Mouse livers were obtained from C₃Hf, C57Bl/6 $\times$ CBA F₁ hybrids, and *hph-1/hph-1* mutant males (5–6 months old). The C57Bl/6 $\times$ CBA F₁ hybrids and the *hph-1/hph-1* mutant were generously given by Dr. V. Bode and Dr. J. D. McDonald at Kansas State University, Manhattan, KS, U.S.A. Rat livers were from 6-month-old Wistar rats. All animals had free access to food and water.

The wild-type *Drosophila* Oregon-R was grown at 25 °C on a cornmeal/yeast/sucrose-containing medium [13], harvested every second day and stored at –80 °C. Only the heads were utilized for the preparation of the enzyme extract; the isolation of the heads was as described previously [5].

Chemicals

[8-¹⁴C]GTP (sp. radioactivity 57 mCi/mmol) was purchased from Amersham International. [8-¹⁴C]GMP was prepared by heating [8-¹⁴C]GTP in 1.25 M-HCl at 100 °C for 10 min, drying under N₂ at 50 °C, dissolving in water,

Abbreviation used: Ahp, 6-acetyldihydrohomopterin.

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Scheme 1. Pathways for biosynthesis of tetrahydrobiopterin (H₄B) and drosopterin (Dp)

Other abbreviations: GTP, guanosine triphosphate; H₂NTP, dihydroneopterin triphosphate; Sp, sepiapterin; PH₄P, pyruvoyltetrahydropterin; LH₄P, lactoyltetrahydropterin; H₂Ahp, 6-acetyldihydrohomopterin; 1, GTP cyclohydrolase I.

drying again under N₂, and dissolving the contents in the original volume with water. [8-¹⁴C]Guanine was produced by boiling [8-¹⁴C]GTP in 1.25 N-HCl for 60 min, and the same procedure as above applied to remove the HCl. D-erythro-Dihydroneopterin triphosphate was produced by incubating GTP with partially purified GTP cyclohydrolase I from *Escherichia coli* [14]. Neopterin and sepiapterin were purchased from Dr. B. Shirkes (Jano, Switzerland). Ahp was purified from *Drosophila* heads [5].

Sephadex-6B and Sephadex G-25 (coarse grade) were obtained from Pharmacia; monobasic potassium phosphate, dibasic potassium phosphate and formic acid were from Fisher Scientific; (NH₄)₂SO₄, allopurinol and Tris base were from Sigma; activated charcoal and ascorbic acid were from MCB Reagents, Norwood, OH, U.S.A.; EDTA was from Mallinckrodt; and aqueous counting scintillant was from Amersham International.

Enzyme assay procedures

GTP cyclohydrolase I was assayed by incubating the enzyme with GTP and measuring the products, dihydroneopterin triphosphate and formate, by two methods: (a) fluorescence of the neopterin in the dihydroneopterin

triphosphate and related hydrolysis products after iodine oxidation [15], and (b) release of C₈ compounds from [8-¹⁴C]GTP [14]. The standard conditions for assaying the mouse and rat enzyme were those used by Bellahsene *et al.* [4]: the enzyme was added to a solution containing 250 μM-GTP, 50 mM-potassium phosphate, pH 8.0, and 0.67 mM-EDTA and incubated for 60 min at 37 °C in the dark. The standard conditions for assaying the *Drosophila* enzyme were as follows: the enzyme was added to a solution containing 200 μM-GTP, 50 mM-potassium phosphate, pH 7.5, and 10 mM-EDTA and incubated for 30 min at 42 °C in the dark. For both enzymes the volume of the assay mixture was 600 μl. To determine fluorescence of neopterin from the dihydroneopterin triphosphate the incubation was terminated by adding 1000 μl of 0.1 M-HCl and 100 μl of 1% iodine/2% KI. After 15 min, the excess iodine was removed by adding 100 μl of 2% ascorbic acid, followed by 500 μl of 0.4 M-Tris base to adjust the pH to 8. Fluorescence was determined (excitation at 356 nm, emission at 454 nm) using a Perkin-Elmer 650S spectrofluorimeter. A standard curve for neopterin in the same solution was used for quantification of the fluorescence.

The release of the C₈ compounds in the form of

formate and other products from $[8-^{14}\text{C}]\text{GTP}$ (20000 c.p.m.) was measured as described by Ferre & Jacobson [16].

Before the enzyme assay, all enzyme solutions were desalted by chromatography over Sephadex G-25 equilibrated with 50 mM-potassium phosphate at pH 8.0 (mouse) or pH 7.5 (*Drosophila*) using the centrifugation procedure of Neal & Florini [17].

A unit of activity is defined as the release of 1 nmol of product (C_8 or dihydroneopterin triphosphate)/h per ml.

Gel chromatography of GTP cyclohydrolases

Enzyme preparation involved molecular-sieve chromatography as described by Bellahsene *et al.* [4]. Livers were homogenized in a Kontes glass tissue grinder in 4 vol. of 0.05 M-potassium phosphate buffer, pH 8.0, and the homogenate was centrifuged at 12000 *g* for 10 min. 'Heat-treated' homogenates were obtained by rapidly heating homogenates in a 100 °C water bath until the temperature reached 70 °C. They were immediately cooled in an ice/water bath to 5 °C and centrifuged at 12000 *g* for 10 min. Rapid heating and cooling was accomplished by using a flask large enough so the extract was no more than 1 cm deep. The supernatant of the heated or unheated extract was adjusted to 45% saturation of $(\text{NH}_4)_2\text{SO}_4$ and maintained at 0 °C with stirring for 20 min and without stirring for an additional 20 min. The resulting precipitate was collected by centrifuging at 20000 *g* for 30 min, and the pellet resuspended in a small volume of 0.05 M-potassium phosphate, pH 8.0, and dialysed overnight at 4 °C against 100–200 vol. of the same buffer. The dialysed sample was applied to a Sepharose-6B column that had been equilibrated with 0.05 M-potassium phosphate buffer, pH 8.0 at 4 °C. The column was developed in the same buffer at a flow rate of 0.2 ml/min, and 2 ml fractions were collected. The eluate was analysed by measuring its A_{280} , by measuring GTP cyclohydrolase I activity by both assays, fluorescence from neopterin triphosphate and C_8 release. The main-activity fractions from either peak I or peak II 'unheated' and peak I 'heated' were pooled and used for Ahp and sepiapterin inhibition studies.

Drosophila heads (5 g) were homogenized in a glass tissue grinder in 5 vol. of 0.05 M-potassium phosphate buffer, pH 7.5, and fractionated as described in [8]. The homogenate was centrifuged for 7 min at 7000 rev./min. To the supernatant was added 300 mg of acid-washed Darco G-60 charcoal. The charcoal-treated extract was gently mixed and then centrifuged at 10000 *g* for 2 min. The resulting supernatant was filtered through glass wool to remove the last traces of charcoal. Next, the filtered supernatant was centrifuged at 100000 *g* for 45 min and then refiltered through Whatman no. 1 paper. This supernatant was adjusted to 35% satd. $(\text{NH}_4)_2\text{SO}_4$. The solution was stirred for 20 min and left for an additional 20 min at 4 °C unstirred. The resulting precipitate was collected by centrifugation at 20000 *g* for 30 min. The pellet was resuspended in a small volume of 0.05 M-potassium phosphate, pH 7.5, and dialysed overnight against 100–200 vol. of the same buffer at 4 °C. The same column procedures were followed for the *Drosophila* GTP cyclohydrolase as with the mouse liver enzyme. Active fractions were pooled and used as the enzyme source for the inhibition study.

Protein assay

Proteins were quantified by the method of Bradford [18], with bovine serum albumin as standard.

Inhibition studies

The inhibitor (sepiapterin or Ahp), enzyme and standard reaction components were preincubated for 2 min at 22 °C before adding GTP. The enzyme was obtained from gel chromatography of both heated and unheated mouse liver homogenate, heated rat liver homogenate and unheated *Drosophila* homogenate. Incubation was for 30 min at 42 °C for *Drosophila* head GTP cyclohydrolase and 60 min at 37 °C for the liver enzyme. The fluorescence assay of neopterin triphosphate production was performed in duplicate for each inhibitor concentration, and the fluorescence obtained in the absence of GTP was considered as the blank. To determine concentration, the millimolar absorption coefficients were taken to be 10.4 for sepiapterin at 410 nm and 12.8 for Ahp at 383 nm, pH 7.0.

RESULTS

Rat liver GTP cyclohydrolase I

The fractionation of rat liver GTP cyclohydrolase was performed as described by Bellahsene *et al.* [4] using the heated homogenate; the results are shown in Fig. 1. Two peaks were obtained by chromatography on Sepharose 6B, one at the exclusion volume (fraction 14), and another larger peak (fraction 26). However, no peak was found in the region of the chromatogram that corresponds to the 40–70 kDa proteins (fractions 40–60), which Bellahsene *et al.* [4] reported and designated as 'fraction A3'.

Fig. 1. Chromatography of rat liver GTP cyclohydrolase I on Sepharose 6B

The homogenate was heated and fractionated as described in [4]. The column dimensions were 1.3 cm × 91 cm. The rates of appearance of the fluorescence of the neopterin from dihydroneopterin triphosphate and the $[^{14}\text{C}]$ formate and other compounds from $[8-^{14}\text{C}]\text{GTP}$ were measured as described in the Materials and methods section

Fig. 2. Chromatography of mouse liver GTP cyclohydrolase I on Sepharose 6B

(a) The homogenate was not heated, as described in the Methods and materials section. The 15 ml sample represented 14.5 g of livers from 6-month-old C3H male mice. (b) The homogenate was heated. The 7 ml sample represented 49 g of liver from the same type and age of mice. The column was 2.8 cm $\times$ 91 cm in both cases. Assays were performed as described in Fig. 1.

The ability of sepiapterin to inhibit the rat liver GTP cyclohydrolase I was tested using four pooled fractions of Fig. 1: pool I consisted of fractions 14–19, II of 20–22, III of 25–30, and IV of 31–34. In marked contrast with the findings of Bellahsene *et al.* [4], sepiapterin at 0.2, 2.0 or 250 nM caused no measurable inhibition when assayed by either the neopterin or C_8 release assay. At 6.25 μ M-sepiapterin, pool I was inhibited by 25%, but the other pools were uninhibited.

Effects of sepiapterin and Ahp on mouse liver GTP cyclohydrolase I

The mouse liver was fractionated without heating (Fig. 2a) or after heating the homogenate (Fig. 2b). The unheated preparation separated into two partially resolved forms on Sepharose 6B chromatography, whereas the heated form consisted of a single peak. In Fig. 2(a) the fractions were combined into two pools, as shown in the Figure, after the neopterin and C_8 release assays had been performed. On the basis of the fluorescence assay, the two peaks were comparable in size. The greater extent of C_8 release as compared with neopterin tri-

Fig. 3. Effects of sepiapterin on GTP cyclohydrolases from mouse liver and *Drosophila*

The enzyme fractions were obtained from chromatograms on Sepharose 6B. (a) mouse liver, heated, main peak; (b) mouse liver, unheated, peak I; (c) mouse liver, unheated, peak II; (d) *Drosophila*, unheated, main peak; The rate of formation of dihydroneopterin triphosphate was measured using the fluorescence assay for neopterin.

phosphate formation was investigated. [8- 14 C]GMP and [8- 14 C]guanine were as reactive as [8- 14 C]GTP for C_8 release and all three substrates were inhibited by allopurinol. It seems likely that xanthine oxidase leads to allantoin formation, a compound that is not adsorbed by charcoal and thus appears to be a release of the C_8 from the guanine ring. As shown in Fig. 2(b), these activities are apparently absent after heating.

The effects of sepiapterin and Ahp were determined by using the three pools of mouse liver: pools I and II of Fig. 2(a) and a pool of the heated preparations of Fig. 2(b). In the case of the heated mouse liver preparation (Fig. 3a), sepiapterin at 11.4 μ M caused 50% inhibition of GTP cyclohydrolase I. In the unheated pools, the enzyme in pool I was more responsive to sepiapterin [IC_{50} (concn. causing half-maximal inhibition) = 12 μ M] than that in pool II. The effects of Ahp on these mouse liver pooled fractions is shown in Fig. 4. The heated mouse liver fraction (Fig. 4a) was consistently stimulated by 2–5 μ M-Ahp and then inhibited at higher concentrations; 40 μ M-Ahp caused 50% inhibition. Pools I and II (Fig. 4b and 4c) of the unheated preparation showed only a gradual decline with increasing concentrations of Ahp, the latter reaching 50% inhibition at 40 μ M-Ahp. Repetition of this experiment gave very similar results.

Effects of sepiapterin and Ahp on *Drosophila* GTP cyclohydrolase I

When an unheated sample from *Drosophila* heads was chromatographed on Sepharose 6B (Fig. 5), the GTP cyclohydrolase I emerged as a single peak, as measured by the neopterin assay; the C_8 release assay showed a main peak that corresponded to the neopterin assay, but the stoichiometry showed 2–3 times greater activity. We did not explore the effects of heating on this organism's enzyme.

The peak fractions (19–26) were pooled and examined

Fig. 4. Effects of Ahp on GTP cyclohydrolase I from mouse liver and *Drosophila*

See Fig. 3 for sources of enzyme and assay.

Fig. 5. Chromatography of *Drosophila* head GTP cyclohydrolase I on Sepharose 6B

See Fig. 1 for assays used. Abbreviation: N_p, neopterin.

for response to sepiapterin and Ahp. As shown in Figs. 3 and 4, neither compound caused appreciable inhibition or stimulation.

Responses to GTP concentration

The response to substrate concentration was determined for the heated mouse liver GTP cyclohydrolase obtained after chromatography on Sepharose 6B and for the *Drosophila* preparation after the (NH₄)₂SO₄ fractionation. As shown in Fig. 6, neither demonstrated an

Fig. 6. Relationship between rate and GTP concentration

(a) The *Drosophila* enzyme was the 0–35% -satd (NH₄)₂SO₄ fraction of the 100000 g supernatant that was desalted on Sephadex G-25. The incubation for the assay was for 15 min. (b) The mouse liver enzyme was from pooled fractions of a Sepharose 6B chromatogram. The incubation for the assay was for 60 min. The liver extract was heated to 60 °C before fractionation and chromatography. See Fig. 3 for the assay used.

ordinary hyperbolic response. The *Drosophila* enzyme had a sigmoidal response, and a Hill coefficient (*h*) of 1.7 was calculated from these data. The mouse liver enzyme showed a different kind of non-hyperbolic response. Both of these types of curves were obtained repeatedly.

GTP cyclohydrolase I in the *hph-1* mouse mutant

Enzyme activity was determined in livers from the *hph-1* mutant and mice with the C57BL/6 × CBA genetic background on which the mutant was induced. Both were examined after Sepharose 6B chromatography of the unheated homogenate. As shown in Fig. 7, the normal enzyme emerged in two peaks, as seen previously for the C3H genotype, and with even greater activity. The enzyme from *hph-1* exhibited less than 2% of the total control activity; these assay results could be even lower, since the values were close to background.

Fig. 7. Chromatography of mutant mouse liver GTP cyclohydrolase I on Sepharose 6B

The homogenates were unheated in both cases and the livers were from 4–6-month-old male mice of the (a) C57 BL/6 × CBA F1 genotype (a 3 ml sample that represents 6.8 g of liver) and (b) *hph-1* mutant (a 3 ml sample that represents 5.4 g of liver). The column was 1.3 cm × 91 cm in both cases. See Fig. 1 for assays used.

DISCUSSION

The regulatory properties of GTP cyclohydrolase I were considered after purification from *E. coli*, but no inhibition by a variety of pterins was found [14]. When 0.2 nM-sepiapterin was reported to inhibit rat liver GTP cyclohydrolase I [4], the regulatory significance was apparent. With the enzyme from mouse liver and *Drosophila* we found no inhibition by sepiapterin at less than 1 μ M, so the rat liver GTP cyclohydrolase was prepared also, as described by Bellahense *et al.* [4]. After chromatography on Sepharose 6B we found activity in two peaks, but none in the region of the A3 peak described by Bellahense *et al.* [4], the peak they reported to be inhibited by 0.2 nM-sepiapterin. Whether the appearance of A3 is related to the age or nutritional state of the rat or some other factor remains to be seen. Subsets of the two rat liver enzyme peaks showed no inhibition by sepiapterin up to 250 nM. When GTP cyclohydrolase was purified more extensively from rat liver, it exhibited no inhibition by several pterins at concentrations less than 1 μ M [19]. GTP cyclohydrolase from *Drosophila* was not inhibited by sepiapterin or Ahp at any concentration tested. The enzyme from mouse liver was mildly inhibited in some cases; the heated enzyme and the larger of the two unheated peaks (peak I) showed 50% inhibition by 12

μ M-sepiapterin. The smaller of the mouse liver unheated peaks was less susceptible to inhibition by sepiapterin.

Inhibition by Ahp was slight for all forms of the mouse liver enzyme tested, and was completely absent in the case of the *Drosophila* enzyme. Ahp was isolated from *Drosophila* heads [5] and shown to stimulate DNA synthesis and biopterin production in mammalian cells (I. Ziegler & K. B. Jacobson, unpublished work). Ahp and sepiapterin are similar in reaction with nickel ions [20]. Since the chemical synthesis of Ahp has been accomplished [21], further information on its effects can be expected.

The *Drosophila* enzyme showed no response to sepiapterin, but gave a sigmoid response to GTP. Such a response is often seen for allosteric enzymes with negative co-operativity. A Hill coefficient of 2.1 ± 0.6 was determined on the basis of three separate titrations with GTP concentrations from 10 to 100 μ M. Between 100 and 400 μ M-GTP the Hill coefficient was found to be 1.1 ± 0.1 . Thus, over the concentration range for GTP that may be reasonable to expect *in vivo*, the *Drosophila* GTP cyclohydrolase exhibits negative co-operativity, but at higher concentrations this property is lost. Weisberg & O'Donnell [8] had previously shown a Hill coefficient of 2.1 for the *Drosophila* enzyme if measured in phosphate buffer, but 1.2 if measured in Tris buffer; clearly the regulatory property of this enzyme could be profitably explored further.

A suggestion of regulatory possibility comes also from the response of the mouse liver enzymes to GTP concentration. The mouse liver (heated) gave a non-hyperbolic response curve that contained multiple inflections. As pointed out by Wong [22], this may indicate that both positive and negative co-operativity may be involved. A study on CTP synthase showed clear indications of this mixed type of co-operativity [23].

Finally, although not directly relevant to enzyme regulation, we found that the *hph-1* mouse mutant has much less GTP cyclohydrolase I activity in its liver than had been reported previously; McDonald *et al.* [24] found about 10% of normal activity, whereas our chromatographic fractions had less than 2%. The actual value was not well defined, since the assay values are quite close to background. Having no more than 2% of normal GTP cyclohydrolase I activity, the *hph-1* mutant may be quite interesting as a model for atypical phenylketonuria, certainly more so than if it had 10% of normal activity for this enzyme. Whether this mutation causes less enzyme to be produced or causes a defect at the catalytic site is yet to be determined.

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