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6-Pyruvoyl tetrahydropterin synthase assay in extracts of cultured human cells using high-performance liquid chromatography with fluorescence detection of biopterin

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ABSTRACT

An assay for 6-pyruvoyl tetrahydropterin synthase, the second enzyme in the conversion of guanosine triphosphate into tetrahydrobiopterin, has been developed. Cell extracts were incubated with enzymatically prepared dihydroneopterin triphosphate (80 μM) in the presence of Mg^{2+} (12 mM), excess sepiapterin reductase (EC 1.1.1.153) (2 nmol/min) and NADPH (2 mM). 6-Pyruvoyl tetrahydropterin, the product of the reaction, was thus converted into tetrahydrobiopterin. After oxidation of the reduced biopterin derivatives in acidic iodine solution, biopterin was enriched and separated from the abundant neopterin phosphates by solid-phase extraction on a strong cation exchanger. Biopterin was then directly eluted on a reversed-phase liquid chromatographic column and detected fluorimetrically using excitation at 353 nm and emission at 438 nm. The biopterin concentrations formed by the coupled enzyme reaction increased linearly with incubation times up to 90 min. The assay allows the quantification of 6-pyruvoyl tetrahydropterin synthase in cultured human cells.

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INTRODUCTION

6-Pyruvoyl tetrahydropterin synthase converts 7,8-dihydroneopterin triphosphate into 6-pyruvoyl tetrahydropterin. This reaction is the second step in the biosynthesis of 5,6,7,8-tetrahydrobiopterin from guanosine 5-triphosphate (GTP).

Assays to determine 6-pyruvoyl tetrahydropterin synthase require 7,8-dihydroneopterin triphosphate as a substrate, which is enzymically prepared by GTP-cyclohydrolase I (EC 3.5.4.16). In most assays described so far, 6-pyruvoyl tetrahydropterin, the product of the reaction, is converted by sepiapterin reductase (EC 1.1.1.153) into tetrahydrobiopterin, which is then determined by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection [1–3]. However, owing to the low stability of tetrahydrobiopterin, which is easily oxidized to 7,8-dihydrobiopterin, this method cannot be used with the long incubation times that are required to obtain a high sensitivity. Alternatively, 6-pyruvoyl tetrahydropterin is converted by 6-pyruvoyl tetrahydropterin reductase into 6-lactoyl tetrahydropterin. After auto-oxidation, 6-lactoyl tetrahydropterin is then quantified as sepiapterin by HPLC with UV detection [4]. Sepiapterin is much more stable than tetrahydrobiopterin, but the sensitivity is limited by the use of the UV detection. An assay with fluorimetric detection has been described for erythrocytes [5]. However, this assay failed to detect 6-pyruvoyl tetrahydropterin synthase activity in cultured fibroblasts.

This paper describes an assay for 6-pyruvoyl tetrahydropterin synthase based on the fluorimetric detection of total biopterin by fluorescence. Biopterin is determined by solid-phase extraction with on-line elution to a reversed-phase HPLC system. This provides a several-fold improvement in sensitivity, allowing the detection of 6-pyruvoyl tetrahydropterin synthase activity in cultured human cells [6].

EXPERIMENTAL

GTP, NADPH, bovine serum albumin, culture media and supplements for tissue culture were obtained from Serva (Heidelberg, Germany). Fetal calf serum was from Biochrom (Berlin, Germany). Pteridines for calibration were obtained from Schircks Labs. (Jona, Switzerland). Sephadex G-25 gel was from Pharmacia (Uppsala, Sweden). The protein assay dye reagent was obtained from Biorad (Richmond, CA, USA). All other chemicals were from Merck (Darmstadt, Germany).

Sepiapterin reductase was purified from *Drosophila* (strain Oregon-R). Heads (30 g) were homogenized using a pestle homogenizer in 0.1 *M* 1,4-piperazine-diethanesulphonic acid (PIPES), pH 7.0, containing 2 m*M* phenylmethylsulphonylfluoride (4 ml buffer per gram of heads). After centrifugation (10 000 *g* for 60 *min*) and filtration (cheesecloth), protein was precipitated with ammonium sul-

phate (40–75%) and redissolved in 20 m*M* PIPES (pH 7.5) containing 10 m*M* 2-mercaptoethanol and 10% (v/v) glycerol. Dialysed material was then subjected to gel permeation by the use of fast protein liquid chromatography (FPLC) (LKB, Uppsala, Sweden) on a column of Ultrogel AcA44. The column was eluted at a flow-rate of 0.7 ml/min to achieve the baseline separation of sepiapterin reductase from 6-pyruvoyl tetrahydropterin synthase and 6-pyruvoyl tetrahydropterin reductase. Sepiapterin reductase activity in fractions was determined according to Katoh [7]. The preparation was free of 6-pyruvoyl tetrahydropterin synthase activity (see also Fig. 1C). Active fractions were pooled, concentrated by ultrafiltration (Amicon PM 10), and stored in aliquots for up to twelve months at −20°C.

The human transitional cell bladder carcinoma line T 24 [8] was kindly provided by Dr. Feichtinger (Institute of Pathology, University of Innsbruck, Innsbruck, Austria). Normal human fibroblasts, isolated from dermis, were a gift from Professor M. Schweiger (Institute of Biochemistry, University of Innsbruck, Innsbruck, Austria) and were used at passage 20 to 30. T 24 cells were grown in Eagles minimum essential medium containing Earle's salts. Fibroblasts were grown in Dulbecco's modified Eagles medium. Media were supplemented with 2 m*M* glutamine, 100 U/ml penicillin, 0.1 ng/ml streptomycin and 10% (v/v) heat-inactivated fetal calf serum.

For the preparation of extracts, cells were detached by trypsin (0.5 g/l) and EDTA (0.2 g/l) in phosphate-buffered saline (pH 7.4) containing 130 m*M* NaCl, 2 m*M* KCl, 6 m*M* Na_2HPO_4 and 1 m*M* KH_2PO_4, and washed once with complete culture medium and once with phophate-buffered saline. Cells ($2 \cdot 10^7$) were suspended in 1 ml of distilled water and disrupted by freezing and thawing. After centrifugation for 10 min at 10 000 *g* the supernatant was freed from low-molecular-mass compounds by Sephadex G-25 gel permeation: 0.2 ml was applied to columns with 2-ml bed volume and eluted with 0.1 *M* Tris–HCl buffer (pH 7.4) containing 20 m*M* $MgCl_2$. The first 0.2 ml was discarded, and the next 0.55 ml was collected and used to determine 6-pyruvoyl tetrahydropterin synthase activity. Only freshly prepared cell extracts were used for enzyme assays.

Protein was determined in cell extracts according to Bradford [9], with pure bovine serum albumine used as a standard. The optical density was measured at 620 nm with an Anthos 8001 microplate reader (Anthos-Labtec, Salzburg, Austria).

For enzymic preparation of 7,8-dihydroneopterin triphosphate, 0.4 m*M* GTP was incubated in 0.1 *M* KCl, 0.1 *M* Tris–HCl (pH 8.5), 10 m*M* EDTA, with sufficient GTP-cyclohydrolase I (3.3 nmol/min/ml) to convert all GTP into 7,8-dihydroneopterin triphosphate in 2 h at 37°C, immediately before the 6-pyruvoyl tetrahydropterin synthase assay. For details of the determination of GTP-cyclohydrolase I activity see ref. 10. Quantitative conversion of GTP into 7,8-dihydroneopterin triphosphate has been confirmed by simultaneous determination of both compounds with ion-pair HPLC [11]. GTP-cyclohydrolase I was purified

from *Escherichia coli* (strain B) as previously described [10] and stored in aliquots at −20°C for up to twelve months.

For determination of 6-pyruvoyl tetrahydropterin synthase activity, 5 µl of 1 *M* Tris–HCl (pH 7.4) were mixed with 5 µl of 40 m*M* NADPH, 5 µl of sepiapterin reductase (400 nmol/min/ml) and 65 µl of cell extracts (10–200 µg of protein). The reaction was started by the addition of 20 µl of 0.4 m*M* 7,8-dihydroneopterin triphosphate. Incubation was carried out at 37°C for 30–90 min in the dark. The reaction was terminated by the addition of 50 µl of a mixture of 0.2 *M* HCl and 0.02 *M* KI–I_2 (1:1, v/v), which oxidizes all reduced biopterin derivatives to the fluorescent biopterin on incubation at room temperature for 1 h in the dark. Excess iodine was destroyed by the addition of 50 µl of 0.02 *M* ascorbic acid. An aliquot of this mixture was then applied to solid-phase cartridges (SCX, Analytichem, Harbor City, CA, USA), which had been preequilibrated with 0.5 ml of distilled water followed by 1 ml of 0.1 *M* H_3PO_4. Samples were forced through the cartridges by air pressure, and finally the cartridges were washed with 0.5 ml of 0.1 *M* H_3PO_4 and subjected to HPLC analysis.

High-performance liquid chromatography

The HPLC instrument consisted of an LC 5500 liquid chromatograph (Varian, Palo Alto, CA, USA), an LS 4 fluorescence detector (Perkin Elmer, Beaconsfield, UK), and an advanced automated sample processor (AASP, Varian) for direct elution of solid-phase cartridges to the HPLC column. Fluid connections of the AASP were modified as detailed elsewhere [12]. Integration and instrument control was done by a DS 654 data system (Varian). A LiChrosorb RP-18 column (250 mm × 4 mm I.D., 7 µm particle size, Merck) was protected by a 4 mm × 4 mm I.D. guard column of the same material. The column was eluted at a flow-rate of 0.8 ml/min with 0.015 *M* potassium phosphate buffer (pH 6.0) (13.3 m*M* KH_2PO_4 plus 1.7 m*M* K_2HPO_4). Elution of biopterin from the solid-phase cartridges was facilitated by a pulse of 0.1 ml of 0.6 *M* potassium phosphate buffer (pH 6.8) (0.32 *M* KH_2PO_4 plus 0.28 *M* K_2HPO_4, see ref. 12 for details). Biopterin was measured fluorimetrically at an excitation wavelength of 353 nm (slit width 15 nm) and an emission wavelength of 438 nm (slit width 20 nm). The detection limit of biopterin was 0.2 pmol in 100 µl of sample, corresponding to 2 n*M*, at a signal-to-noise ratio of 3:1.

RESULTS AND DISCUSSION

6-Pyruvoyl tetrahydropterin synthase converts 7,8-dihydroneopterin triphosphate into 6-pyruvoyl tetrahydropterin in the presence of Mg^{2+}. The assay of the activity of this enzyme as presented here is based on the quantification of biopterin using fluoresence detection. Tetrahydropbiopterin is formed from 6-pyruvoyl tetrahydropterin, an initial product of the reaction, in the presence of excess sepiapterin reductase and NADPH in the assay mixture. Tetrahydrobiopterin is

then converted into biopterin by oxidation with iodine in HCl. The acidic reaction mixture is directly applied to strong cation-exchange bonded silica columns (SCX, benzenesulphonic acid bound via a *p*-propylene chain). This SCX column quantitatively retains the positively charged pterins (neopterin and biopterin) but not the abundant, negatively charged neopterin phosphates, which originate from the substrate of the enzyme reaction. The cartridges allow the collection of pterins from a comparatively large volume of incubation mixture (up to 3 ml), which are then directly eluted to the HPLC column by means of the AASP instrument. The pterins are eluted from the cartridge with the help of a pulse of 100 μl of 0.6 *M* potassium phosphate buffer (pH 6.8) (0.32 *M* KH_2PO_4 plus 0.28 *M* K_2HPO_4), which neutralizes the charge of the pterins bound to the SCX column and weakens the ionic interactions due to the high salt concentration.

Since biopterin can be enriched and prepurified from a comparatively large sample volume, a several-fold increase in sensitivity for the biopterin determination and thus for the 6-pyruvoyl tetrahydropterin synthase assay is obtained by use of this solid-phase extraction step.

Fig. 1 shows chromatograms from the 6-pyruvoyl tetrahydropterin synthase assay obtained with material derived from T 24 cells, normal dermal human fibroblasts and a reagent control. Neopterin concentrations are 98.5 n*M* (T 24), 76.4 n*M* (fibroblasts) and 9.9 n*M* (reagent control). The corresponding biopterin concentrations were 144.0, 33.7 and 2 n*M*, respectively. The increased amount of

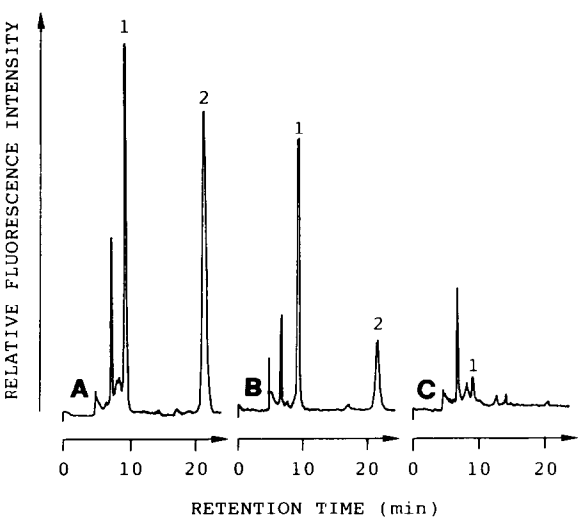

Fig. 1. HPLC profiles of incubation mixtures for 6-pyruvoyl tetrahydropterin synthase assays with extracts of T 24 cells (A), human dermal fibroblasts (B) and a reagent control (C). Amounts of 56 μg (A) and 60 μg (B) of cellular protein or phosphate-buffered saline (C) were used in the assay. Incubation time was 70 min at 37°C. A 100-μl aliquot of the incubation mixture was used. Fluorescence detection was at an excitation wavelength of 353 nm and an emission wavelength of 438 nm. Peaks: 1 = neopterin; 2 = biopterin.

neopterin in the incubation mixtures using cell extracts, compared with the reagent control, originates from the phosphatase activity of the cells, which cleaves the substrate (7,8-dihydroneopterin triphosphate) to produce neopterin [6]. No biopterin was formed when NADPH or 7,8-dihydroneopterin triphosphate was omitted. As can be seen from Fig. 1, the biopterin formed is unambiguously detected with extracts of T 24 cells and fibroblasts. This demonstrates the unique sensitivity of the biopterin determination using the AASP technique compared with conventional techniques, which failed to detect 6-pyruvoyl tetrahydropterin synthase activity in cultured human fibroblasts [5]. 6-Pyruvoyl tetrahydropterin synthase activities were determined to be 7.7 ± 0.8 pmol/mg/min for T 24 cells and 1.5 ± 0.3 pmol/mg/min for dermal fibroblasts (mean ± S.D. of five experiments) using this method [6]. Thus, the reproducibility of the assay ranges from ± 10 to ± 20%, depending on the type of cell used.

The dependence of the amount of biopterin formed on the amount of cellular protein and on incubation time is shown in Fig. 2. Data were obtained with material derived from T 24 cells. As little as 10 μg of T 24 cell extract is sufficient to demonstrate the activity in a 30-min assay. The assay is linear for up to at least 150 μg of cellular protein and an incubation time of 90 min.

Fig. 3 shows a Lineweaver–Burk plot that demonstrates the dependence of 6-pyruvoyl tetrahydropterin synthase activity on the concentration of the substrate 7,8-dihydroneopterin triphosphate. The reaction behaves according to Michaelis–Menten kinetics. The Michaelis constant (K_M) is found to be 10 μM, which is similar to observations made with the liver enzyme [1]. This value re-

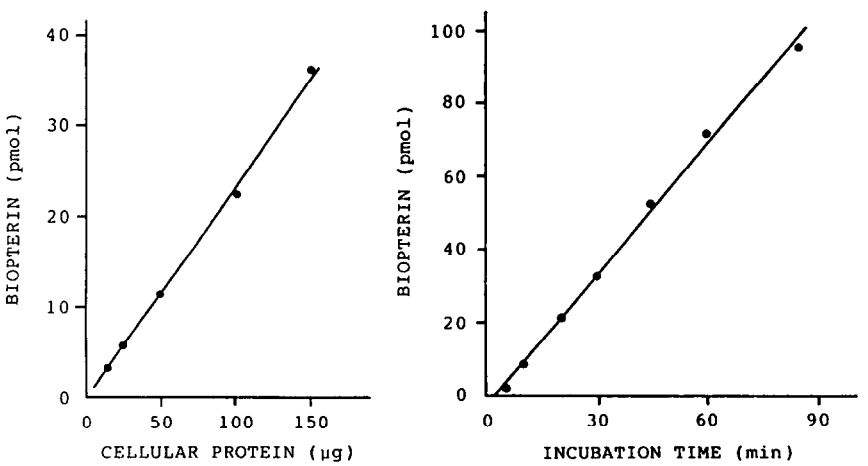

Fig. 2. Dependence of 6-pyruvoyl tetrahydropterin synthase activity on the amount of cellular protein (left) and on incubation time (right). Eluates from T 24 cell extracts were used. Biopterin was determined as described in Experimental. For testing the dependence on the amount of protein, 10–150 μg of cellular protein were applied in a 30-min assay. For testing the dependence on incubation time, 150 μg of protein were used.

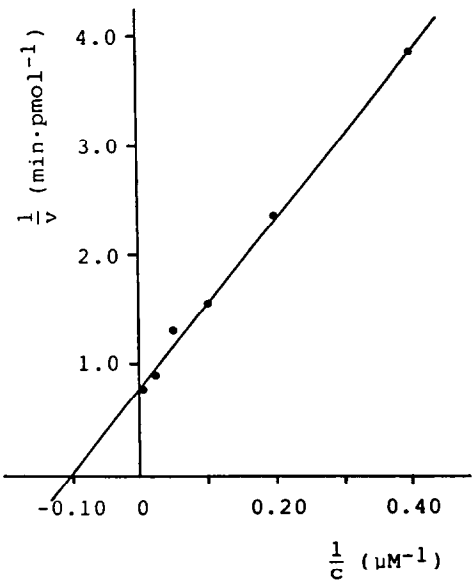

Fig. 3. Lineweaver–Burk plot of 6-pyruvoyl tetrahydropterin activities in T 24 cell extracts. Various concentrations of 7,8-dihydroneopterin triphosphate (2.5–80 μM) were tested. Protein concentration, 150 μg; incubation time, 30 min. Biopterin in incubation mixtures was quantified by reversed-phase HPLC with fluorimetric detection.

mains unchanged following treatment of cells with interferon-gamma, which strongly stimulates pteridine synthesis by inducing the activity of GTP-cyclohydrolase I [6]. In earlier work, 7,8-dihydroneopterin triphosphate has been used at a concentration of 16 μM [13] or 32 μM [1]. Since the K_M of the enzyme is 10 μM, we think that the 80 μM 7,8-dihydroneopterin triphosphate applied in our assay represents the minimal possible substrate concentration to be used. Even when using this concentration, the activity is already estimated to be too low by *ca.* 11% according to the Michaelis–Menten law. Although an improved method for the preparation of 7,8-dihydroneopterin triphosphate has been described [14], the availability of this substrate remains limited owing to the use of GTP-cyclohydrolase I for generation of the compound and also owing to its chemical instability. We therefore did not increase the amount of 7,8-dihydroneopterin triphosphate.

Stimulation of pteridine synthesis is associated with activation of cellular immunity, *e.g.* by viral infections, certain malignancies, autoimmune diseases or treatment with immunomodulators. In humans this leads to accumulation of neopterin in body fluids (reviewed in refs. 15 and 16). A sensitive assay for 6-pyruvoyl tetrahydropterin synthase is a prerequisite to obtain an insight into the regulation of enhanced pteridine synthesis during immune activation. Using this assay, we could also demonstrate the presence of 6-pyruvoyl tetrahydropterin

synthase in human macrophages [6], which has not been possible previously [17]. Furthermore, the assay with improved sensitivity presented here could be a valuable tool to study inborn errors of pteridine metabolism in cultured fibroblasts of patients with atypical phenylketonuria.

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REFERENCES

1 S. Takikawa, H.-Ch. Curtius, U. Redweik, W. Leimbacher and S. Ghisla, *Eur. J. Biochem.*, 161 (1986) 295.
2 G. K. Smith and C. A. Nichol, *J. Biol. Chem.*, 261 (1986) 2725.
3 T. Hasler and H.-Ch.Curtius, *Eur. J. Biochem.*, 180 (1989) 205.
4 Y. S. Park, J.-H. Kim, K. B. Jacobson and J. J. Yim, *Biochim. Biophys. Acta*, 1038 (1990) 186.
5 H. Shintaku, A. Niederwieser, W. Leimbacher and H.-Ch. Curtius, *Eur. J. Pediatr.*, 147 (1988) 15.
6 E. R. Werner, G. Werner-Felmayer, D. Fuchs, A. Hausen, G. Reibnegger, J. J. Yim, W. Pfleiderer and H. Wachter, *J. Biol. Chem.*, 265 (1990) 3189.
7 S. Katoh, *Arch. Biochem. Biophys.*, 146 (1971) 202.
8 J. Bubeník, M. Baresová, V. Viklický, J. Jakoubková, H. Sainerová and J. Donner, *Int. J. Cancer*, 11 (1973) 765.
9 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
10 J. J. Yim and G. M. Brown, *J. Biol. Chem.*, 251 (1976) 5087.
11 N. Blau and A. Niederwieser, *Anal. Biochem.*, 128 (1983) 446.
12 E. R. Werner, D. Fuchs, A. Hausen, G. Reibnegger and H. Wachter, *Clin. Chem.*, 33 (1987) 2028.
13 S. Takikawa, H.-Ch. Curtius, U. Redweik and S. Ghisla, *Biochem. Biophys. Res. Commun.*, 134 (1986) 646.
14 J. Ferré, E. W. Naylor and K. B. Jacobson, *Anal. Biochem.* 176 (1989) 15.
15 H. Wachter, D. Fuchs, A. Hausen, G. Reibnegger and E. R. Werner, *Adv. Clin. Chem.*, 27 (1989) 81.
16 A. Hausen, D. Fuchs, G. Reibnegger, E. R. Werner and H. Wachter, *Pteridines*, 1 (1989) 3.
17 G. Schoedon, J. Troppmair, A. Fontana, C. Huber, H.-Ch. Curtius and A. Niederwieser, *Eur. J. Biochem.*, 166 (1987) 303.