or below the mean.[8] In such instances, the assay of routine samples is halted until possible causes are checked and corrected. As a typical example of quality controls, our current plasma and whole-blood ascorbic acid lysate controls have the following values (mean ± SD):

P1: 0.95 ± 0.23 ng/ml (2.15 ± 0.52 pmol/ml)
P2: 2.51 ± 0.55 ng/ml (5.69 ± 1.25 pmol/ml)
P3: 6.10 ± 0.78 ng/ml (13.82 ± 1.77 pmol/ml)
R1: 53 ± 4 ng/ml (120 ± 9 pmol/ml)
R2: 89 ± 9 ng/ml (202 ± 20 pmol/ml)
R3: 176 ± 12 ng/ml (399 ± 27 pmol/ml)

To obtain a value for erythrocyte folates (as concentration per milliliter of erythrocytes) the measured whole-blood value is divided by the PCV obtained from an automated (Coulter) counter or hematocrit, or by an estimate of the PCV derived from analysis of hemoglobin in the ascorbic acid lysate samples (O'Connor *et al.*[3]).

[8] E. Mullins, *Analyst* **119,** 369 (1994).

[6] Determination of Tetrahydrobiopterin Biosynthetic Activities by High-Performance Liquid Chromatography with Fluorescence Detection

By ERNST R. WERNER, HELMUT WACHTER, and GABRIELE WERNER-FELMAYER

Introduction

Tetrahydrobiopterin [6*R*-6(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin] serves as cofactor of phenylalanine 4-monooxygenase, tyrosine 3-monooxygenase, glyceryl-ether monooxygenase, and nitric oxide synthase.[1] Because intracellular tetrahydrobiopterin concentrations affect the amount of metabolites of, e.g., nitric oxide synthase formed by intact cells,[2] regulation of the biosynthesis of tetrahydrobiopterin is of interest. Assays of the three biosynthetic enzymes (Fig. 1) involved in the formation of tetrahydrobiopterin from guanosine 5′-triphospate (GTP) based on high-performance liquid chromatography (HPLC) with fluorescence detection

[1] S. Kaufman, *Annu. Rev. Nutr.* **13,** 261 (1993).
[2] G. Werner-Felmayer, E. R. Werner, D. Fuchs, A. Hausen, G. Reibnegger, and H. Wachter, *J. Exp. Med.* **172,** 1599 (1990).

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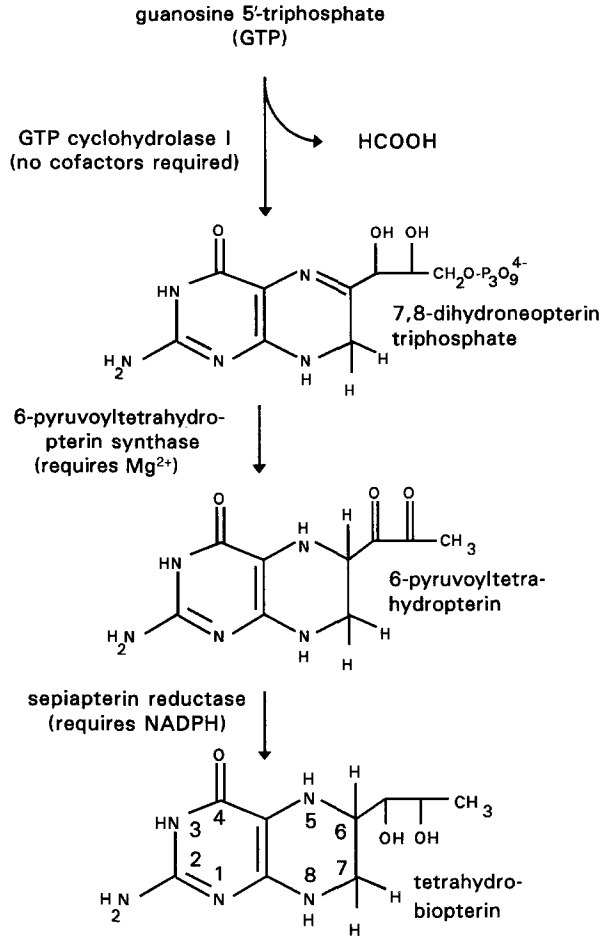

FIG. 1. Biosynthesis of tetrahydrobiopterin from guanosine 5'-triphosphate.

are detailed in this chapter. While the assay of GTP cyclohydrolase I, the first enzyme of the pathway that is regulated by cytokines,[3] as well as the assay for sepiapterin reductase, can be run with materials that are commercially available, 6-pyruvoyltetrahydropterin synthase assays are performed with two purified enzymes, GTP cyclohydrolase I and sepiapterin reductase, to synthesize the labile substrate and to help convert the unstable product to a stable metabolite.

[3] E. R. Werner, G. Werner-Felmayer, D. Fuchs, A. Hausen, G. Reibnegger, G. Wels, J. J. Yim, W. Pfleiderer, and H. Wachter, *J. Biol. Chem.* **265,** 3189 (1990).

Preparation of Extracts from Mammalian Cells and Tissues for Measurement of Cytosolic Activities of Tetrahydrobiopterin Biosynthetic Enzymes

All three assays are performed with extracts from cultured cells prepared in the same manner: 10^7 cells are collected and frozen at −80° in 1 ml of distilled water containing 5 m*M* dithioerythritol (Serva, Heidelberg, Germany) and phenylmethylsulfonyl fluoride (10 μg/ml; Serva). Cells are broken by thawing (normally one cycle is sufficient) and the amount of broken cells estimated by trypan blue staining. Tissue specimens are best homogenized with a pestle in the same solution; 1 g wet weight per 5 ml of solution is used.

Crude extracts are centrifuged for 15 min at 13,000 *g* and 4°, and the supernatant collected. The protein concentrations of the extracts thus prepared should be in the range of 1 to 5 mg/ml. For further processing, extracts are subjected to gel filtration in the respective assay buffer: 500 μl of cell extract is loaded onto an NAP-5 column (Pharmacia, Uppsala, Sweden) and eluted with 1000 μl of assay buffer. Together with providing the correct buffer for the respective enzyme incubations, this step removes low molecular weight substances, in particular pteridines, which interfere with the assay or may alter the enzyme activities, e.g., via feedback inhibition mechanisms.

Assay for GTP Cyclohydrolase I

Principle

The procedure described here is adapted from work by Viveros *et al.*[4] Extracts containing GTP cyclohydrolase I activity are incubated with GTP (K_m ~50 μ*M*) in the presence of EDTA, the 7,8-dihydroneopterin triphosphate formed is then oxidized to neopterin triphosphate with iodine in acidic solution, the excess iodine is destroyed with ascorbic acid, the mixture is rendered slightly alkaline, and the triphosphate group is cleaved off by alkaline phosphatase. Neopterin is then determined by HPLC with fluorescence detection. The presence of EDTA inhibits further conversion of the product 7,8-dihydroneopterin triphosphate because the enzyme 6-pyruvoyltetrahydropterin synthase requires Mg^{2+} to react.

Enzyme Reaction and Conversion of Product to Neopterin

The assay buffer used is 50 m*M* Tris-HCl, pH 7.8, containing 300 m*M* KCl, 2.5 m*M* EDTA, 10% (v/v) glycerol. NAP-5 eluate (250 μl; see Prepara-

[4] O. H. Viveros, C. L. Lee, M. M. Abou-Donia, J. C. Nixon, and C. A. Nichol, *Science* **213,** 349 (1981).

tion of Extracts) in this buffer is incubated with 2 m*M* GTP for 30 to 90 min at 37° in the dark in a final volume of 300 μl. The reaction is started by addition of GTP and stopped with 40 μl of a mixture of 20 μl of 1 *N* HCl and 20 μl of 0.1 *M* I_2 dissolved in 0.25 m*M* KI. After a 45- to 60-min incubation in the dark at room temperature, the precipitate is removed by centrifugation (2 min, 13,000 *g*, 25°); the clear supernatant should have a yellow color due to excess iodine, otherwise the iodine concentration must be increased. On subsequent addition of 20 μl of 0.1 *M* freshly dissolved ascorbic acid the yellow color should disappear. The pH is then adjusted to pH 7.5–9 by addition of 20 μl of 1 *N* NaOH and the mixture is incubated with 10 μmol of alkaline phosphatase per minute (ca. 3000 μmol mg^{-1} min^{-1}) at 37° for 60 min in the dark. Incubation mixtures are then stored at −20° until assayed by HPLC for concentration of neopterin.

High-Performance Liquid Chromatography Determination of Neopterin and Biopterin with Fluorescence Detection

Assay mixtures are analyzed by HPLC with fluorescence detection as follows: 10 μl of reaction mixture is injected onto a column (250 mm long, 4-mm i.d.) containing octadecyl-modified silica gel (ODS, 5-μm particle size, e.g., Merck Lichrospher RP-18; Merck, Darmstadt, Germany) protected with a 4-mm guard column filled with the same material. Pteridines are isocratically eluted at a flow rate of 0.8 ml/min with 15 m*M* potassium phosphate buffer, pH 6.0, and quantified using a fluorescence detector (excitation at 350 nm, emission at 440 nm). Depending on the sensitivity of the fluorescence detector, a detection limit of 10 fmol of neopterin at a signal-to-noise ratio of 5 : 1 can be reached. Column and injection loop are rinsed daily with 3 ml of distilled water followed by 15 ml of methanol or 2-propanol and 3 ml of water, at a flow rate of 0.3 ml/min. Care should be taken in the preparation of standards of neopterin and biopterin, because the oxidized pterins show only limited solubility in water. We stir 10 mg of neopterin in 10 liters of distilled water neutralized with NaOH containing 0.1 m*M* dithioerythritol overnight to ensure quantitative solution.

Notes

While the assay is straightforward for use with tissues with high activities such as rat liver (about 1 pmol mg^{-1} min^{-1}), some points should be considered when trying to measure activities in cultured cells, which even when treated with cytokines may be one or two orders of magnitude lower. We observed that the quality of the GTP (some batches contain traces of dihydroneopterin phosphates) and of the alkaline phosphatase (some batches contain traces of GTP cyclohydrolase I activity) is crucial, thus

reagent blanks should be run before precious samples are used. Repeated thawing of the incubation mixtures should be avoided, because this leads to loss of analyte at low concentrations (<10 n*M* neopterin). Ascorbic acid should be prepared freshly and not exposed to light, otherwise fluorescent compounds are formed, which interfere with neopterin determination. As an alternative to phosphatase cleavage, direct determination of neopterin phosphates with ion-pair HPLC has been used by Blau and Niederwieser.[5] This has the advantage of fewer steps required, but poses problems at very low activities owing to the chemical instability of the neopterin phosphates.

Assay for 6-Pyruvoyltetrahydropterin Synthase

Principle

The assay described here is adapted from work by Shintaku *et al.*[6] Extracts containing 6-pyruvoyltetrahydropterin synthase activity are incubated with 7,8-dihydroneopterin triphosphate (K_m 10 μM) in the presence of Mg^{2+}, sepiapterin reductase, and NADPH. 7,8-Dihydroneopterin triphosphate is prepared immediately before the assay, using purified GTP cyclohydrolase I and GTP. Mg^{2+} is required by 6-pyruvoyltetrahydropterin synthase to operate.[7] Sepiapterin reductase reduces the labile 6-pyruvoyltetrahydropterin product with NADPH to 5,6,7,8-tetrahydrobiopterin. This is subsequently oxidized by iodine in acid to the fluorescent biopterin, and the excess iodine is destroyed by addition of ascorbic acid. Biopterin is quantified by HPLC with fluorescence detection. Owing to the presence of a high excess of fluorescent neopterin phosphates originating from the substrate, the HPLC determination requires special methods to separate these from the small amounts of biopterin.

Sources of GTP Cyclohydrolase I and Sepiapterin Reductase

Several protocols for the purification of the two enzymes required, GTP cyclohydrolase I and sepiapterin reductase, have been published, and both have been cloned and expressed. Both enzymes are also active as fusion proteins, thus for their use in these assays the recombinant fusion proteins need not necessarily be cleaved. GTP cyclohydrolase I may be best purified from *Escherichia coli.*[8] Purification is simplified to a one-column procedure

[5] N. Blau and A. Niederwieser, *Anal. Biochem.* **128,** 446 (1983).
[6] H. Shintaku, A. Niederwieser, W. Leimbacher, and H. C. Curtius, *Eur. J. Pediatr.* **147,** 15 (1988).
[7] S. I. Takikawa, H. C. Curtius, U. Redweik, W. Leimbacher, and S. Ghisla, *Eur. J. Biochem.* **161,** 295 (1986).
[8] J. J. Yim and G. M. Brown, *J. Biol. Chem.* **251,** 5087 (1976).

when *E. coli* strains overexpressing GTP cyclohydrolase I are used.[9-11] Sepiapterin reductase is best purified from erythrocytes[12] and is also available in recombinant form.[13,14]

Generation of 7,8-Dihydroneopterin Triphosphate

In a first step, a solution of 7,8-dihydroneopterin triphosphate is prepared from GTP. The actual amount prepared will depend on the number of assays performed subsequently. The following concentrations are used: 0.1 *M* Tris-HCl (pH 8.5), 0.1 *M* KCl, 2.5 m*M* EDTA, 500 μM GTP, and sufficient GTP cyclohydrolase I (>10 nmol min^{-1} ml^{-1}) to metabolize virtually all GTP in 120 min at 37°. If GTP cyclohydrolase I is abundantly available, concentrations might be increased and incubation times reduced. This solution is used directly as substrate, because the presence of GTP cyclohydrolase I does not interfere with the 6-pyruvoyltetrahydropterin synthase reaction.

Enzyme Reaction and Conversion of Product to Biopterin

The assay buffer used for 6-pyruvoyltetrahydropterin synthase is 100 m*M* Tris-HCl (pH 7.4), containing 20 m*M* $MgCl_2$. Seventy microliters of the NAP-5 eluate (see Preparation of Extracts, above) in this assay buffer is incubated with 2 m*M* NADPH, sepiapterin reductase (2 nmol min^{-1}), and 100 μM freshly prepared 7,8-dihydroneopterin triphosphate in a final volume of 100 μl for 30 to 90 min in the dark. The reaction is started by addition of 7,8-dihydroneopterin triphosphate and stopped with 20 μl of a mixture of 10 μl of 1 *N* HCl and 10 μl of 0.1 *M* I_2 dissolved in 0.25 *M* KI. After a 45- to 60-min incubation in the dark at room temperature and subsequent removal of solid by centrifugation (13,000 *g*, 2 min, 25°) 10 μl of freshly prepared ascorbic acid is added and the mixture stored at −20° until analyzed for biopterin content by HPLC.

[9] G. Katzenmeier, C. Schmid, and A. Bacher, *FEMS Microbiol. Lett.* **54,** 231 (1990).
[10] M. Gütlich, E. Jaeger, K. P. Rucknagel, T. Werner, W. Rodl, I. Ziegler, and A. Bacher, *Biochem. J.* **302,** 215 (1994).
[11] H. Ichinose, T. Ohye, E. Takahashi, N. Seki, T. Hori, M. Segawa, Y. Nomura, K. Endo, H. Tanaka, S. Tsuji, K. Fujita, and T. Nagatsu, *Nature Genet.* **8,** 236 (1994).
[12] T. Sueoka and S. Katoh, *Biochim. Biophys. Acta* **717,** 265 (1982).
[13] B. A. Citron, S. Milstien, J. C. Gutierrez, R. A. Levine, B. L. Yanak, and S. Kaufman, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 6436 (1990).
[14] H. Ichinose, S. Katoh, T. Sueoka, K. Titani, K. Fujita, and T. Nagatsu, *Biochem. Biophys. Res. Commun.* **179,** 183 (1991).

High-Performance Liquid Chromatography Analysis of Biopterin in Presence of Abundant Neopterin Phosphates with Fluorescence Detection

The analysis of the small amounts of biopterin formed (nmol/liter) in the presence of 100 μM neopterin phosphates by fluorescence detection requires the use of solid-phase extraction[15,16] or column-switching techniques.[6,17] We use a convenient combination of solid-phase extraction and on-line elution HPLC, which enables enrichment of the analyte and extraction in one step. Rippin[18] has adapted this procedure for conventional solid-phase extraction setups.

For solid-phase extraction with on-line elution HPLC, 100 μl of the acidic reaction mixture is loaded onto a strong cation-exchange solid-phase extraction column (SCX, 2.1 × 17 mm; Varian, Palo Alto, CA) that has been preequilibrated with two 500-μl washes with 0.1 M H_3PO_4. The solid phase does not bind the negatively charged neopterin phosphates, but quantitatively extracts biopterin. This is then eluted by an automated processor using a high-pressure tight chamber (AASP; Varian) directly onto the HPLC column. Elution of biopterin is facilitated by a pulse of 100 μl of 0.6 M potassium phosphate buffer, pH 6.8, which is delivered with the injection valve of the AASP from a 100-μl loop continuously refilled with the buffer using a peristaltic pump.[15] Biopterin is then separated on a reversed-phase system with fluorescence detection, similar to the system described above (see Assay for GTP Cyclohydrolase I, above) for the determination of neopterin; the detection limit for biopterin is 100 fmol.

Notes

Activities in human cells are comparatively low, ranging from ~0.1 to 10 pmol mg^{-1} min^{-1}. For accurate determinations, the concentration of 100 μM 7,8-dihydroneopterin triphosphate, which is 10 times the K_m, should not be underscored even if GTP cyclohydrolase I for the preparation of the substrate is scarce. It is also essential to run reagent controls, because purified sepiapterin reductase may contain residual activities of 6-pyruvoyltetrahydropterin synthase. 7,8-Dihydroneopterin triphosphate has also been prepared with recycling of GTP cyclohydrolase I.[19] This compound

[15] E. R. Werner, D. Fuchs, A. Hausen, G. Reibnegger, and H. Wachter, *Clin. Chem.* **33,** 2028 (1987).

[16] E. R. Werner, G. Werner-Felmayer, D. Fuchs, A. Hausen, G. Reibnegger, G. Wels, J. J. Yim, W. Pfleiderer, and H. Wachter, *J. Chromatogr.* **570,** 43 (1991).

[17] A. Niederwieser, W. Staudenmann, and E. Wetzel, *J. Chromatogr.* **290,** 237 (1984).

[18] J. J. Rippin, *Clin. Chem.* **38,** 1722 (1992).

[19] J. Ferre, E. W. Naylor, and K. B. Jacobson, *Anal. Biochem.* **176,** 15 (1989).

is exceptionally labile, so that prolonged storage of these solutions at −80° is feasible only when the 7,8-dihydroneopterin triphosphate content is monitored before each assay. Some protocols use HPLC with electrochemical detection of tetrahydrobiopterin[7]; these show a selectivity for the tetrahydro compound but are less robust than fluorescence detection.

Sepiapterin Reductase Assay

Principle

The assay presented here is adapted from Ferre and Naylor.[20] Extracts containing sepiapterin reductase activities are incubated with the artificial substrate sepiapterin, which is converted by sepiapterin reductase to 7,8-dihydrobiopterin with the aid of NADPH. In the presence of 7,8-dihydrofolate reductase activity in the extract, this is further converted to 5,6,7,8-tetrahydrobiopterin. Both compounds are then oxidized to the fluorescent biopterin by iodine in acid, and excess iodine is reduced with ascorbic acid. Biopterin is determined by HPLC with fluorescence detection.

Enzyme Reaction and Conversion of Product to Biopterin

The assay buffer used is 100 m*M* potassium phosphate, pH 6.4. Seventy microliters of NAP-5 eluate (see Preparation of Extracts, above) prepared in this buffer is incubated with 1 m*M* sepiapterin (K_m ~10 μM) in the presence of 2 m*M* NADPH for 10 min at 37°. The reaction is stopped by adding 20 μl of a mixture of 10 μl of 1 *N* HCl and 10 μl of 0.1 *M* I_2 dissolved in 0.25 *M* KI. After incubation for 45 to 60 min at room temperature in the dark, solid is removed by centrifugation (13,000 *g*, 2 min, 25°), and the mixture is stored at −20° until analyzed for biopterin content by HPLC.

Reversed-Phase High-Performance Liquid Chromatography Analysis of Biopterin with Fluorescence Detection

An HPLC setup similar to the one described above for neopterin is used (see Assay for GTP cyclohydrolase I, above). Depending on the sensitivity of the fluorescence detector used, a detection limit of 20 fmol for biopterin may be reached. Usually sepiapterin incubation mixtures must be diluted 10- to 100-fold to ensure that the signals do not exceed the maximal capacity of the fluorescence detector. Alternatively, the sensitivity range of the fluorescence detector may be changed accordingly.

[20] J. Ferre and E. W. Naylor, *Biochem. Biophys. Res. Commun.* **148,** 1475 (1987).

Notes

Compared to GTP cyclohydrolase I and 6-pyruvoyltetrahydropterin synthase, human cells contain higher sepiapterin reductase activities (~500 pmol mg^{-1} min^{-1}), which can be measured easily without approaching the sensitivity limits of HPLC with fluorescence detection. If material is abundant and activities are high, reduction of sepiapterin may alternatively be monitored by decrease in absorbance at 420 nm,[21] e.g., in a microplate photometer.

Owing to the use of the artificial substrate sepiapterin, the activity may not necessarily be identical to the activity toward the labile natural substrate, 6-pyruvoyltetrahydropterin. For comparative purposes, however, the assay using sepiapterin as substrate is useful.

Acknowledgment

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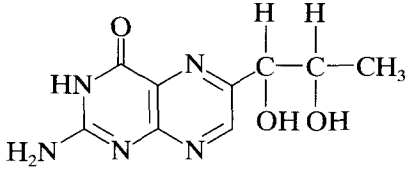
[21] S. Katoh, *Arch. Biochem. Biophys.* **146,** 204 (1971).

[7] Microtiter Plate Assay for Biopterin Using Cryopreserved *Crithidia fasciculata*

By ROBERT J. LEEMING



L-*erythro*-Biopterin
(M_r 237)

The protozoan *Crithidia fasciculata* requires biopterin for normal metabolism. Growth in a biopterin-free culture medium is proportional to added biopterin. The defined medium and the culture conditions used in this assay

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