

Detection of pterins and enzymatic activities of GTP-cyclohydrolase I and sepiapterin reductase in *Neurospora crassa* and *Phycomyces blakesleeanus*

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The best known pterin in *Neurospora crassa* is molybdopterin, which is a constituent of the flavohemoenzyme nitrate reductase and participates in blue-light promoted conidiation (Klemm and Ninnemann 1979 Photochem. Photobiol. 29:629-632, Ninnemann 1991 J. Photochem. Photobiol. 9B:189-199). There is evidence that additional pterin derivative(s) occur in *N. crassa* mycelium (Siefermann-Harms, Fritz and Ninnemann 1985 Photochem. Photobiol. 42:771-778) as well as in crude extracts of the zygomycete *Phycomyces blakesleeanus* (Kiewisch and Fukshansky 1991 Photochem. Photobiol. 53:407- 409). For this reason, we have purified and characterized unconjugated pterins from mycelium of *N. crassa* and from mycelium and sporangiophores of *P. blakesleeanus*.

In addition we have determined the activities of two related enzymes: GTP- cyclohydrolase I and sepiapterin reductase. GTP-cyclohydrolase I converts GTP to dihydroneopterin triphosphate, which is the precursor for unconjugated pterins and folates, but not for molybdopterin. Sepiapterin reductase catalyzes the last step in biosynthesis of biopterin from GTP and has previously been reported only in vertebrates and arthropods (for review see Brown 1985 In: Folates and Pterins. Vol. 2. Blakely and Benkovic eds., John Wiley and Sons, New York, pp. 115-154; Nichol, Smith and Duch 1985 Ann. Rev. Biochem. 54:729-764; Duch and Smith 1991 J. Nutr. Biochem. 2:411-423).

N. crassa wild type (strain 987 from the Fungal Genetics Stock Center, University of Kansas) was grown on nitrate reductase-inductive Vogel's medium (with 10g/l glucose) modified so that 50 mM NaNO₃ was the only nitrogen source. *P. blakesleeanus* (strain F813, wild type, +-strain, a generous gift from Prof. Dr. Oberwinkler, Univ. Tübingen, Germany) was grown on malt medium (2% malt extract) in 300 ml flasks for the production of mycelium or with additional 1.2% agar in Petri dishes for the production of sporangiophores (state V).

The mycelia were harvested by filtration through nylon meshes. About 0.5 g of frozen sample was triturated in the dark under N₂ to a fine powder and solubilized with 1 ml of 0.1 N HCl. Pterins were analyzed by cation-exchange chromatography and HPLC determination as described initially by Fukushima and Nixon 1980 Anal. Biochem. 102:176- 188 and as modified by Ziegler 1985 J. Cell. Biochem. 28:197-206. The following modifications were used: monapterin was added as an internal standard prior to the oxidation step and pterins were eluted from the cation-exchange resin with 1 M NH₄-acetate dissolved in a 1:4 mixture (v/v) of methanol and water. The following instrumentation assembly was used for the HPLC analysis:

Pharmacia pump 2248, Pheodyne loop injector (20 μ l); Hypersil ODS C-18 column (5 μ m particle size, 4.6 x 250 mm) and pre-column (4.6 x 20 mm), Shimadzu fluorescence monitor type RF-551 and Spectraphysics integrator ChromJet. Identification of pterins was done by co-chromatography with the respective standard pterins (Schircks Laboratories, Jona, Switzerland) in different solvent systems as described by Ziegler 1985 J. Cell. Biochem. 28:197-206. Pterins were quantified by fluorescence yield comparison with the corresponding standards in concentrations within the linear response range of the fluorescence monitor.

Protein extracts for enzyme tests were made by sonification of the harvested mycelia or sporangiophores in 2.5 mM EDTA, 50 mM Tris-HCl (pH 8.0). After centrifugation at 12,000 g for 5 min the supernatant was desalted on Sephadex G-25 columns (NAP-5, Pharmacia, Freiburg, Germany) equilibrated with the sonification buffer. Protein concentrations were estimated by the Coomassie blue dye assay (Bio-Rad, München, Germany), with bovine serum albumin as standard. Enzyme tests for GTP-cyclohydrolase I and sepiapterin reductase were performed as described by Kerler et al. 1990 J. Cell. Physiol. 142:268-271.

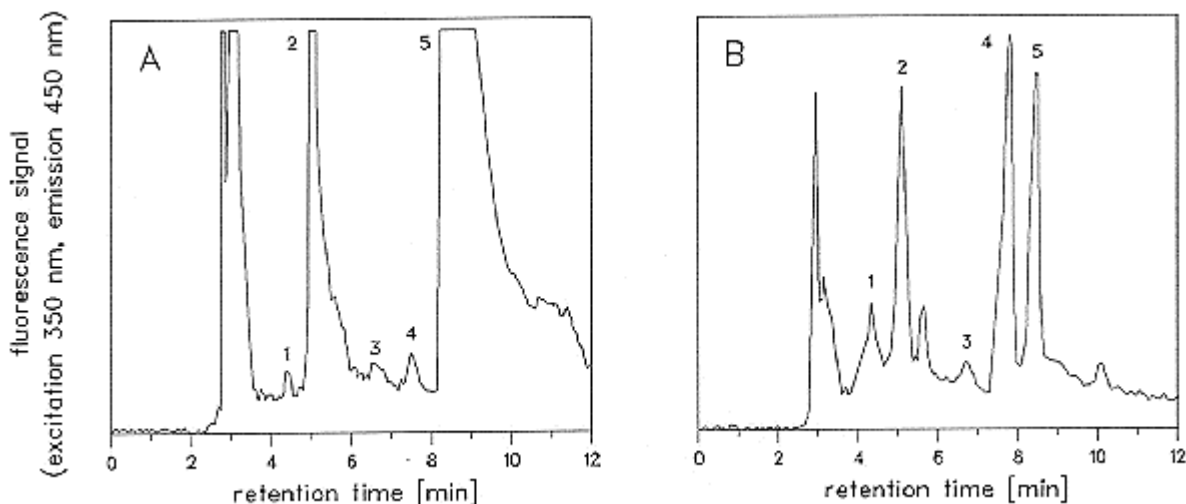


Figure 1. Elution profile for pterins isolated from *Neurospora crassa* (A) and *Phycomyces blakesleeanus* (B). 1) neopterin; 2) monapterin (added as internal standard); 3) xanthopterin; 4) biopterin; 5) 6- hydroxymethylpterin. For peak quantification we diluted the sample until the peak area fitted in the linear range of a standard curve.

Figure 1 shows elution profiles of pterins from *N. crassa* and *P. blakesleeanus*. The dominant unconjugated pterin in *N. crassa* was found to be 6-hydroxymethylpterin, accompanied by minor amounts of neopterin and biopterin (Figure 1A and Table 1). In *P. blakesleeanus*, the main pterin was biopterin. It was present in the same concentration in mycelium and in sporangiophores; in addition neopterin, xanthopterin and 6- hydroxymethylpterin were present (Figure 1B and Table 1). GTP-cyclohydrolase I activities were 7.6 times higher in mycelia of *N. crassa* than in mycelia of *P. blakesleeanus* and was not found in sporangiophores of *P. blakesleeanus* (Table 1). Sepiapterin reductase showed a different activity pattern: it was high in *P. blakesleeanus* and low in *N. crassa* (Table 1).

GTP-cyclohydrolase I produces dihydroneopterin triphosphate from GTP, the precursor for the biosynthesis of pterins. The product is dephosphorylated to dihydroneopterin and yields, after removal of a C2-residue from the C3-side chain 6-hydroxymethyldihydropterin, which is the precursor for folate biosynthesis. The reduced pterins were detected after oxidation to fluorescent compounds. Analysis of *N. crassa* revealed large amounts of 6-hydroxymethylpterin, accompanied by a high GTP-cyclohydrolase I activity. In *P. blakesleeanus* the total amounts of pterins were decreased and the level of GTP-cyclohydrolase I was also relative to those in *N. crassa*. In sporangiophores of *P. blakesleeanus* no GTP-cyclohydrolase I was detectable; the amounts of neopterin and 6-hydroxymethylpterin were decreased compared with mycelia of *P. blakesleeanus*.

Species	<i>Neurospora crassa</i> mycelia	<i>Phycomyces blakesleeanus</i> mycelia	<i>Phycomyces blakesleeanus</i> sporangiophores
<u>pterins [pmol mg⁻¹]</u>			
neopterin	20±5	257±14	26±6
biopterin	15±4	540±27	510±22
6-hydroxymethylpterin	9480±470	1120±71	420±20
<u>enzyme activities [pmol min⁻¹ mg⁻¹]</u>			
GTP-cyclohydrolase I	4.95±0.32	0.65±0.10	b.d.
sepiapterin reductase	0.56±0.12	18.9±1.20	22.4±1.41

Table 1. Estimation of pterin concentrations (per mg wet weight of mycelia or sporangiophores) and activities of GTP-cyclohydrolase I and sepiapterin reductase (per mg of soluble protein in crude extracts after desalting with Sephadex G-25). b.d.= below detection limit. Values are means of three experiments.

In animals dihydroneopterin triphosphate is converted to 6-pyruvoyl tetrahydropterin, which is sequentially reduced by the action of sepiapterin reductase to yield tetrahydrobiopterin. Interestingly, in fungi, sepiapterin reductase was detectable and its activity seems to correlate with the amount of biopterin (Table 1): mycelia of *N. crassa* showed low concentrations of biopterin and low activities of sepiapterin reductase, for mycelia and sporangiophores of *P. blakesleeanus* higher activities of sepiapterin reductase corresponded with higher amounts of biopterin.

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