Pteridine biosynthesis and nitric oxide synthase in *Physarum polycephalum*

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Physarum polycephalum, an acellular slime mould, serves as a model system to study cell-cycle-dependent events since nuclear division is naturally synchronous. This organism was shown to release isoxanthopterin which is structurally related to tetrahydrobiopterin, a cofactor of aromatic amino acid hydroxylases and of nitric oxide synthases (NOSs) (EC 1.14.13.39). Here, we studied *Physarum* pteridine biosynthesis in more detail and found that high amounts of tetrahydrobiopterin are produced and NOS activity is expressed. *Physarum* pteridine biosynthesis is peculiar in as much as 7,8-dihydroneopterin aldolase (EC 4.1.2.25), an enzyme of folic acid biosynthesis usually not found in organisms producing tetrahydrobiopterin, is detected in parallel. NOS purified from *Physarum* depends on NADPH, tetrahydrobiopterin and flavins. Enzyme activity is independent of exogenous Ca^{2+} and is inhibited by arginine analogues. The purified enzyme (with a molecular mass of 130 kDa) contains tightly bound tetrahydrobiopterin and flavins. During the synchronous cell cycle of *Physarum*, pteridine biosynthesis increases during S-phase whereas NOS activity peaks during mitosis, drops at telophase and peaks again during early S-phase. Our results characterize *Physarum* pteridine biosynthesis and NOS and suggest a possible link between NOS activity and mitosis.

INTRODUCTION

Pteridines are a class of pyrazino[2,3-*d*]pyrimidine compounds which play a role as pigments and cofactors such as tetrahydrobiopterin, molybdopterin or folic acid. Molybdopterin is a cofactor of several molybdenum-containing enzymes, e.g. xanthine oxidase (reviewed in [1]). The reduced forms of folic acid are cofactors for a number of vital reactions in cell metabolism, including synthesis of purines [1]. In contrast with tetrahydrobiopterin, folic acid is a so-called 'conjugated' pteridine since *p*-aminobenzoylglutamate is linked to the pterin ring. Both tetrahydrobiopterin and folic acid are formed from GTP and the first step of the two biosynthetic pathways is catalysed by GTP cyclohydrolase I (EC 3.5.4.16) [1,2]. The ability to form folic acid was lost in vertebrates and a number of other eukaryotic organisms during evolution so that folic acid is a vitamin for these species [1]. On the other hand, tetrahydrobiopterin could not be detected in *Escherichia coli* [3] and growth of the protozoan *Crithidia fasciculata* depends on biopterin supply [1].

In mammals, tetrahydrobiopterin was shown to be a cofactor of aromatic amino acid hydroxylases [4], alkyl glycol ether mono-oxygenase [4] and, more recently, of nitric oxide synthases (NOSs) (EC 1.14.13.39) (for review see [4,5]). A further as yet undefined function of tetrahydrobiopterin is its participation in cell proliferation and differentiation of certain cell types [6,7].

Formation of tetrahydrobiopterin, which is constitutively found in hepatic and neuronal tissues [1], can be induced by cytokines (mainly interferon-γ, tumour necrosis factor-α and interleukin-1) or by lipopolysaccharide in a wide variety of peripheral cells not involved in aromatic amino acid-derived neurotransmitter biosynthesis including macrophages, fibroblasts and endothelial cells (reviewed in [8]). In human cells, cytokine-induced biosynthesis of tetrahydrobiopterin is paralleled by formation of neopterin, a marker for immune activation (for review see [9]). Accumulation of neopterin is caused by the comparatively low activity of 6-pyruvoyl tetrahydropterin synthase found in human but not in mouse cells [8]. Using intact fibroblasts [10], vascular-smooth-muscle cells [11], macrophages [12], and endothelial cells [13], it was shown that a functional role of cytokine-induced tetrahydrobiopterin biosynthesis is to provide a cofactor for NOSs.

Due to its spontaneously synchronous cell cycle, the myxomycete *Physarum polycephalum* is an attractive model system in cell biology. Cell cycle (8–10 h) of the *Physarum* macroplasmodium, a multinucleated giant cell, consists of the S-phase (about 3 h), which immediately follows mitosis, and of a G_2-phase, whereas the G_1-period found in other eukaryotic systems is lacking. Several years ago, release of isoxanthopterin, an oxidation product of tetrahydrobiopterin, into supernatants from cultured macroplasmodia was reported and involvement of pteridines in growth regulation was suggested [14]. Studying formation of pteridines during cell cycle showed that intracellular concentrations of an unidentified pteridine X decreased at the S/G_2 border [15].

The present report examines *Physarum* pteridine biosynthesis in more detail and shows that this organism has NOS activity. Purification and characterization of NOS was performed and both pteridine biosynthesis and NOS activity were studied during the cell cycle.

EXPERIMENTAL

Materials

Pteridines were purchased from Schircks Laboratories (Jona, Switzerland). Molecular-mass markers (rainbow markers) for SDS/PAGE and L-[2,3,4,5-^{3}H]arginine monohydrochloride (35–70 Ci/mmol) were from Amersham Corp. (Amersham, Bucks., U.K.). [^{3}H]Arginine was purified by h.p.l.c. over a Nucleosil 10 SA column from Macherey and Nagel (Düren, Germany) in 100 mM sodium acetate buffer, pH 4.5. Molecular-mass markers for gel filtration, arginine analogues and Dowex-50W were from Sigma (Munich, Germany). Flavins, NADPH,

Abbreviations used: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; DTE, 1,4-dithioerythritol; PMSF, phenylmethanesulphonyl fluoride; IPTG, isopropyl-β-D-thiogalactopyranoside; NMA, N^G-monomethyl-L-arginine; NNA, N^G-nitro-L-arginine.

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the calmodulin inhibitor R24571, L-arginine, 1,4-dithioerythritol (DTE), phenylmethanesulphonyl fluoride (PMSF), alkaline phosphatase from calf intestine (3077 units/mg), ampicillin, kanamycin, isopropyl-β-D-thiogalactopyranoside (IPTG), lysozyme from chicken egg white, and DNAase I from bovine pancreas (3000 units/mg) were from Serva (Heidelberg, Germany). GTP cyclohydrolase I was purified from *Escherichia coli* strain M15 which was a kind gift from Dr. C. Schmid and Dr. A. Bacher (Technical University of Munich, Munich, Germany). This strain carries the plasmid pNCO113 and expresses GTP cyclohydrolase I under control of the lac-repressor. The enzyme was purified from IPTG-treated *E. coli* M15 lysates, obtained by treatment with 1 mg of lysozyme and 0.1 mg of DNAase I per 1 g (wet weight) of bacteria for 1.5 h at 37 °C, using a DEAE Superspher column (Fractogel EMD DEAE 650-S, 25–40 μm particle size; 150 mm × 10 mm; Merck, Darmstadt, Germany) and a KCl gradient from 10–300 mM (the h.p.l.c. system used is detailed below for NOS). Sepiapterin reductase, purified from *Drosophila* (strain Oregon R) by a procedure outlined in [16], was kindly provided by Dr. J. J. Yim (University of Seoul, Seoul, Korea). All other chemicals used were of the highest available quality and were obtained from Merck.

Culture techniques

Microplasmodia of *Physarum polycephalum* (strain M_3b, a Wis 1 isolate), used for purification of NOS, were maintained in submersed shake-culture in semi-defined medium [17], supplemented with 0.013 % (w/v) haemoglobin instead of haematin, at 25 °C. Macroplasmodia, used for studying enzyme activities during the cell cycle, were prepared by coalescence of exponentially growing microplasmodia and cultivated in plastic dishes on filter paper supported by glass beads. Explants (about 4 cm^2) of the same macroplasmodium were harvested at indicated time points. Mitosis (telophase) was determined in ethanol-fixed smears under phase contrast. Freshly harvested material was washed once with 50 mM Tris/HCl, pH 8.0, containing 1 mM PMSF and 5 mM DTE and then stored in 1 vol. of the same buffer at −80 °C until required for purification or determination of enzyme activities and pteridine content.

Preparation of cell extracts

Cell extracts were prepared for determination of pteridines, pteridine biosynthetic activities and NOS activity. Frozen macroplasmodia (see above) were rapidly thawed at 25 °C and homogenized using a Teflon homogenizer. After centrifugation at 350000 *g* (TLA 120.2 fixed-angle rotor, Beckman tabletop ultracentrifuge) for 10 min at 4 °C for removal of cell debris and slime, the supernatants were used for pteridine determination. For detection of enzyme activities, eluates freed from low-molecular-mass compounds by Sephadex G25 chromatography (NAP-5, Pharmacia LKB, Uppsala, Sweden) were prepared from these supernatants. Protein was determined in cell extracts and in eluates according to the method of Bradford [18], using BSA as a standard and the protein–dye reagent from Bio-Rad.

H.p.l.c. system

The h.p.l.c. system used for pteridine determination in cell extracts and enzyme incubation mixtures consisted of a liquid chromatograph (LC 5500, Varian, Palo Alto, CA, U.S.A.), an LS 4 fluorescence detector (Perkin–Elmer, Beaconsfield, Bucks., U.K.), and an AASP module (Varian) for direct insertion of solid-phase cation-exchange cartridges (Varian). Fluid connections were modified as described previously [19]. For purification of NOS, we used a 1050 titanium h.p.l.c. system from Hewlett–Packard (Vienna, Austria), equipped with a 10 ml superloop (Pharmacia) for sample application.

Determination of pteridines

Intracellular pteridines were quantified as described elsewhere [16] using reversed-phase C_{18} columns (Lichrosorb RP 18, 7 μm particle size, 250 mm × 4 mm, Merck) and h.p.l.c. with fluorescence detection. Total amounts of pteridines were detected in samples after oxidation with iodine in acidic medium [20]. For determination of 7,8-dihydrobiopterin and biopterin, iodine oxidation was carried out at basic pH values [20]. Chemical identity of biopterin was confirmed by isolation and derivatization to pterin-6-carboxylic acid by oxidation with alkaline potassium permanganate as reported previously [21].

Determination of pteridine biosynthetic enzymes

GTP cyclohydrolase I, assayed by a method modified from [22], 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase, assayed by a method modified from [23], were determined in cell extracts (see above) as described before [16]. The product of GTP cyclohydrolase I is 7,8-dihydroneopterin trisphosphate which is oxidized by iodine in HCl and cleaved by alkaline phosphatase to neopterin. GTP (2 mM) was used as a substrate and 5 mM EDTA was included in the incubation buffer for inhibition of 6-pyruvoyl tetrahydropterin synthase which requires Mg^{2+}. GTP cyclohydrolase I activity is expressed as the amount of neopterin formed/min per mg of protein. 6-Pyruvoyl tetrahydropterin synthase was measured after incubation of 7,8-dihydroneopterin trisphosphate, freshly prepared by GTP cyclohydrolase I purified from *E. coli* (see the Experimental section) in the presence of excess sepiapterin reductase, NADPH and Mg^{2+}. The product of the reaction, 6-pyruvoyl tetrahydropterin, is thus converted into tetrahydrobiopterin which is then detected, after iodine oxidation at acidic pH, as the fluorescent biopterin. 6-Pyruvoyl tetrahydropterin reductase activity is given as pmol of biopterin formed/min per mg of protein. Sepiapterin reductase, determined with sepiapterin as a substrate, was measured by quantifying the amount of 7,8-dihydrobiopterin, detected as the fluorescent biopterin derivative after iodine oxidation at acidic pH, and is given as pmol of biopterin/min per mg of protein. 7,8-Dihydroneopterin aldolase activity was assessed according to [24]. 7,8-Dihydroneopterin or 7,8-dihydromonapterin (100 μM each) were used as substrates. 7,8-Dihydroneopterin aldolase activity (pmol/min per mg of protein) is the amount of 6-hydroxymethyl-7,8-dihydropterin formed which was detected as the fluorescent 6-hydroxymethylpterin after iodine oxidation in acidic medium. All enzyme assays were carried out at 25 °C, the growth temperature of *Physarum*, instead of 37 °C.

NOS assay

Determination of NOS activity was performed by measuring formation of [^{3}H]citrulline from [^{3}H]arginine by a method modified from [25,26] as detailed elsewhere [13]. Briefly, homogenates, eluates (see above) or fractions from various purification steps were incubated with 100 μM L-arginine, 25 μM FAD, 25 μM FMN, 5 μM 6*R*-tetrahydrobiopterin, 2 mM NADPH, and 60000–80000 c.p.m. of purified [2,3,4,5-^{3}H]-arginine (see the Experimental section) in 50 mM Tris/HCl,

pH 8.0, containing 1 mM PMSF and 5 mM DTE. In the case of fractions from the purification steps, 10% (v/v) glycerol and 10 mM 2-mercaptoethanol were included. The final volume was 200 μl. In some experiments, the free Ca^{2+} concentration was adjusted to 3 μM by adding 0.15 mM EGTA, 0.9 mM EDTA, 1.78 mM $MgCl_2$ and 0.27 mM $CaCl_2$ according to [27]. Ca^{2+}-free conditions were achieved by addition of 0.15 mM EGTA, 0.9 mM EDTA and 2.05 mM $MgCl_2$ without addition of $CaCl_2$ [27]. The calmodulin inhibitor R24571 was included at concentrations up to 10 μM. The arginine analogues N^G-monomethyl-L-arginine (NMA) and N^G-nitro-L-arginine (NNA) were tested at concentrations up to 250 μM. The EC_{50} for FAD was determined in the absence of FMN and vice versa. EC_{50} determination for FAD plus FMN was carried out by varying the concentrations of both at a constant molar ratio. After 30 min at 25 °C, the reaction was stopped with 800 μl of 20 mM sodium acetate, pH 5.0, containing 200 μM EDTA and 1 mM L-citrulline. [^{3}H]Citrulline was quantified after separation from [^{3}H]arginine by the cation-exchanger Dowex-50W. NOS is given as the amount of citrulline formed/min per mg of protein.

Determination of nitrite, nitrate and citrulline

Formation of nitrite and nitrate was determined using the NOS standard assay (see above) with some modifications [28]: the incubation time was extended to 3 h at 25 °C, an NADPH-regenerating system (0.25 unit/ml glucose-6-phosphate dehydrogenase and 20 mM glucose 6-phosphate) was included in order to keep the assay linear over the incubation time, and the reaction was stopped by rapid freezing. For analysis, samples were thawed one by one and applied immediately to the h.p.l.c. system. Nitrite and nitrate were detected according to [29]. Samples (50 μl) were applied to a reversed-phase C_{18} column (Lichrospher, 5 μm particle size, 250 mm × 4 mm, Merck) and eluted with 5% NH_4Cl, pH 7.0. Nitrate was reduced to nitrite by a cadmium reactor (cadmium 0.3–0.8 mm, 20–50 mesh ASTM, Merck, washed with 0.1 M HCl and packed into a Pharmacia HR 5/5 glass column). Nitrite was quantified after post-column mixing with the stable Griess–Ilosvay reagent from Merck by measurement of u.v. absorption at 546 nm. Citrulline in these incubation mixtures was determined after derivatization with *o*-phthalaldehyde at pH 9.0, using a C_{18} reversed-phase column and fluorescence detection.

Purification of NOS

NOS from *Physarum* microplasmodia was purified by a method modified from refs. [25,26,30]. Microplasmodia were used in order to obtain sufficient material for purification. Frozen material (about 200 ml of sedimented microplasmodia in 1 vol. of 50 mM Tris/HCl, pH 8.0, containing 1 mM PMSF and 5 mM DTE) was thawed rapidly at 25 °C and disintegrated by means of a high-speed tissue homogenizer for 20 s at 4 °C. After centrifugation for 20 min at 48000 *g* and 4 °C, the supernatant was further cleared by ultracentrifugation for 1 h at 100000 *g* and 4 °C. The supernatant was stirred with 2′-5′-ADP–Sepharose 4B (Pharmacia, 1 g per g of protein) for 1 h on ice. 2′-5′-ADP–Sepharose 4B was collected by centrifugation for 5 min at 750 *g* and 4 °C and washed successively with buffer A [50 mM Tris/HCl, pH 8.0, 1 mM PMSF, 5 mM DTE, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol], containing 0.5 M NaCl and with buffer A alone (80 ml per 1 g of 2′-5′ ADP–Sepharose 4B). NOS was eluted from 2′-5′-ADP–Sepharose 4B with buffer A containing 3 mM NADPH (10 ml per 1 g of 2′-5′-ADP–Sepharose 4B) by stirring for 5 min on ice. The solution was clarified from the gel by centrifugation and immediately applied to an h.p.l.c.–DEAE-column (Lichrospher 1000, 5 μm particle size, 50 mm × 10 mm, Merck), equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 5 mM DTE, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol by means of a 10 ml superloop (Pharmacia). After 15 min of isocratic flow, NOS was eluted by a linear gradient of increasing KCl concentration to 0.3 M in 35 min (flow rate 1 ml/min). Active fractions were pooled, concentrated by ultrafiltration (Microcon 30, Amicon, Beverly, MA, U.S.A.) and applied to a Superose-6 HR 1030 column (30 μm particle size, 300 mm × 10 mm, Pharmacia), equilibrated with 50 mM Tris/HCl, pH 7.2, containing 5 mM DTE, 10 mM 2-mercaptoethanol, 150 mM KCl and 10% (v/v) glycerol. NOS was eluted at a flow rate of 0.3 ml/min. Whereas fractions after DEAE-chromatography were stable when supplemented with 25% (v/v) glycerol and stored at −80 °C for at least 3 weeks, the activity was lost in highly purified fractions after Superose-6 chromatography within a few days at −80 °C. To characterize the enzyme, DEAE-purified fractions were therefore used for the assays.

RESULTS

Determination of intracellular pteridines in *Physarum polycephalum* cell extracts showed two major fluorescent peaks which were identified as biopterin and 6-hydroxymethylpterin (Figure 1a). The identity of both compounds was checked by spiking with synthetic reference compounds. Furthermore, the identity of biopterin was confirmed by isolation and derivatization to pterin-6-carboxylic acid [21]. Along with biopterin and 6-hydroxymethylpterin, which were present at equal amounts in cell extracts, low levels of pterin and isoxanthopterin, two degradation products of tetrahydrobiopterin were observed (Figure 1a). Dictyopterin, a stereoisomer of L-biopterin which is the major pterin produced by the cellular slime mould *Dictyostelium discoideum* [31], could not be detected (< 20 pmol per mg of protein). As determined by iodine oxidation in alkaline medium, 76±9% (mean of 18 experiments ± S.D.) of biopterin was present as the active tetrahydro derivative. Likewise, 73.8±7% (mean of 18 experiments ± S.D.) of 6-hydroxymethylpterin occurred as alkali-labile reduced derivatives.

Cell extracts from *Physarum* contained GTP cyclohydrolase I (EC 3.5.4.16), 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase (EC 1.1.1.153) activities (Figure 1b), which are the three enzymes required for biosynthesis of tetrahydrobiopterin [1,4]. In addition, 7,8-dihydroneopterin aldolase (EC 4.1.2.25), an enzyme involved in folic acid biosynthesis [2,24], was detected which is responsible for formation of 6-hydroxymethyl-7,8-dihydropterin, detected as 6-hydroxymethylpterin after iodine oxidation at acidic pH (Figure 1b). 7,8-Dihydroneopterin and 7,8-dihydromonapterin were equally metabolized by 7,8-dihydroneopterin aldolase in *Physarum* cell extracts (Figure 1b). The conversion of 7,8-dihydroneopterin or 7,8-dihydromonapterin into its respective 2′-epimer reflects epimerase activity of cell extracts (Figure 1b). Furthermore, cell extracts contained phosphatase activity which cleaved freshly prepared 7,8-dihydroneopterin trisphosphate into 7,8-dihydroneopterin, a substrate for 7,8-dihydroneopterin aldolase (Figure 1b).

Having detected tetrahydrobiopterin in *Physarum* cell extracts, we then checked for NOS activity. We found that protein fractions from *Physarum* converted [^{3}H]arginine into [^{3}H]citrulline. Further, nitrite and nitrate could be detected in NOS assays. A typical experiment using macroplasmodia

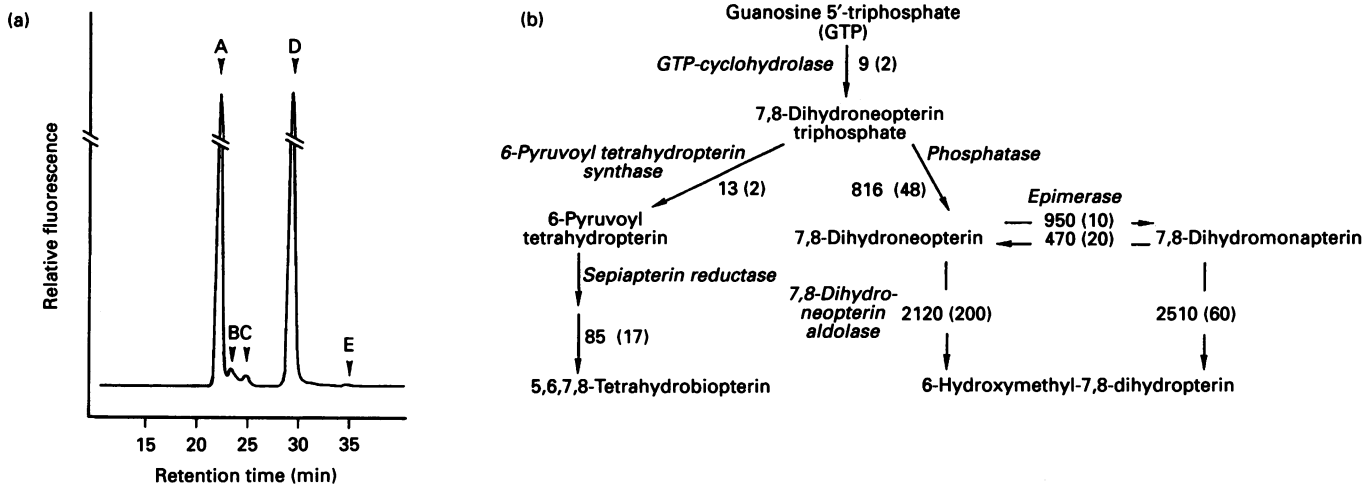

Figure 1 H.p.l.c. profile of pteridines (a) and pteridine biosynthetic activities (b) determined in homogenates of *Physarum polycephalum*

Macroplasmodia of *Physarum*, grown on filter paper, were harvested 1.5 h after second mitosis (M2). Determination of pteridine contents and of enzyme activities were carried out in cell extracts as detailed in the Experimental section. (**a**) Retention times: A, biopterin; B, pterin; C, isoxanthopterin; D, 6-hydroxymethylpterin; E, retention time of dictyopterin standards. (**b**) Scheme of pteridine biosynthetic activities determined in *Physarum*. Values are enzyme activities in pmol/mg per min of three experiments, values in brackets are S.E.M.

Table 1 Purification of NOS from *Physarum polycephalum*

Physarum microplasmodia were cultivated in submersed shake-culture in semi-defined medium, harvested by centrifugation at 500 *g* and stored at −80 °C in 1 vol. of 50 mM Tris/HCl, pH 8.0, containing 1 mM PMSF and 5 mM DTE. For purification (methods modified from [25,26,30]) frozen material (about 200 ml of sedimented microplasmodia) was thawed rapidly at 25 °C and disintegrated by means of a high-speed tissue homogenizer for 20 s at 4 °C. After centrifugation for 20 min at 48000 *g* and 4 °C, the supernatant was further cleared by ultracentrifugation for 1 h at 100000 *g* and 4 °C. NOS was purified by successive chromatography with 2′-5′-ADP–Sepharose 4B, DEAE Lichrospher and Superose 6. Determination of NOS activity was done by measuring formation of [^{3}H]citrulline from [^{3}H]arginine using the standard procedure (for details of purification and assay conditions see the Experimental section). One representative example of five purifications is shown.

Fraction	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/mg per min)	Yield (%)	Purification factor
Homogenate	2496	54.9	0.022	100	1
100000 *g* supernatant	1751	49.9	0.028	91	1.3
2′,5′-ADP–Sepharose 4B	6.2	47.8	7.72	87	351
H.p.l.c.					
DEAE	0.05	35.9	665	36	30227
Superose 6	0.005	7.8	1552	8	70545

harvested 1 h after second mitosis yielded the following NOS activities: when measured as formation of [^{3}H]citrulline using the standard assay, the activity was 109±6 pmol/mg per min (mean±S.D. from triplicate incubations). In parallel incubations (3 h assay, see the Experimental section), NOS activity detected as nitrite plus nitrate was 94±7 pmol/mg per min and 88±12 pmol/mg per min, when determined as citrulline formation. Like pteridine biosynthetic activities, NOS activity was optimal at 25 °C as would be expected from this soil-inhabiting organism. At 37 °C, a 36% reduction and at 4 °C, a 75% reduction of [^{3}H]citrulline formation was observed. Enzyme activity was located in the cytosol rather than in membranes, nuclei or microsomes (results not shown).

For further characterization of *Physarum* NOS, we purified the enzyme by successive chromatography with 2′-5′-ADP–Sepharose, DEAE and Superose-6 columns as outlined in Table 1. The final specific activity was 1552 nmol/min per mg of protein as determined by formation of [^{3}H]citrulline. The final recovery was 8%.

Table 2 summarizes the biochemical characteristics of *Physarum* NOS. The enzyme had a strict requirement for NADPH, FAD and 6*R*-tetrahydrobiopterin. Omission of NADPH from the assay reduced enzyme activity by about 90%. The purified enzyme contained tetrahydrobiopterin (Table 2). Omitting tetrahydrobiopterin from the enzyme incubation led to a 5-fold decrease in enzyme activity (results not shown). Whereas FAD was absolutely required for the reaction and omission of this cofactor reduced activity by 90%, leaving out FMN from the standard assay mixture which contained 25 μM FAD did not influence enzyme activity (results not shown). Accordingly, FMN applied in the absence of FAD stimulated the reaction only poorly (about 10% of $V_{max.}$ in the presence of FAD). However, FMN reduced the requirement for FAD about 10-fold when applied at equimolar concentrations (Table 2), demonstrating a cooperative effect of both flavins. Studying flavin content revealed that FAD and FMN were bound to the enzyme after purification (Table 2). The ratio of enzyme-bound FAD to FMN was about 1:6, thus explaining the requirement for addition of FAD.

Table 2 Biochemical characteristics of NOS from *Physarum polycephalum*

DEAE-purified fractions were used to characterize NOS from *Physarum*. Enzyme activity was determined as formation of [^{3}H]citrulline as described in the Experimental section. IC_{50} and K_i values for arginine analogues were determined in the presence of 100 μM L-arginine. The EC_{50} for FAD was obtained in the absence of FMN and that for FMN in the absence of FAD. EC_{50} determination for FAD plus FMN was carried out by varying the concentrations of both at a constant molar ratio. Values are means of three independent experiments $\pm$ S.D. Determination of cofactors bound was carried out by h.p.l.c. analysis as detailed in [44]. Abbreviation: 6*R*-tetrahydrobiopterin, 6*R*-BH_4.

Parameter	Value
EC_{50} NADPH	5.7 $\pm$ 1.5 μM
EC_{50} FAD	1.7 $\pm$ 0.3 μM
EC_{50} FMN	12.2 $\pm$ 1.0 μM
EC_{50} FAD + FMN	380 $\pm$ 120 nM
EC_{50} 6*R*-BH_4	175 $\pm$ 35 nM
K_m L-arginine	13.5 $\pm$ 2.2 μM
IC_{50} NMA	6.7 $\pm$ 1.8 μM
K_i NMA	0.9 $\pm$ 0.4 μM
IC_{50} NNA	13.9 $\pm$ 4.9 μM
K_i NNA	1.6 $\pm$ 0.7 μM
Cofactor bound	
FAD	0.018 mol/mol of NOS
FMN	0.106 mol/mol of NOS
6*R*-BH_4	0.050 mol/mol of NOS

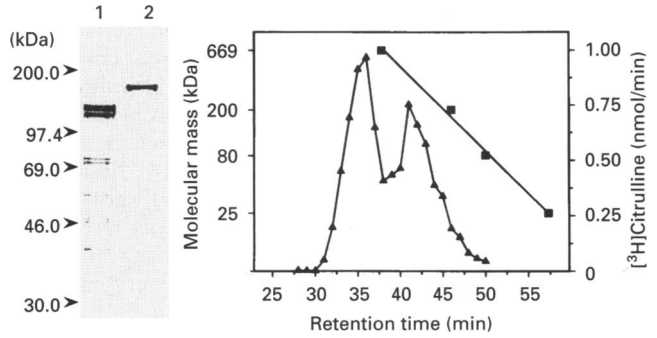

Figure 2 SDS/PAGE profile and elution profile of *Physarum* NOS after Superose-6 gel filtration

SDS/PAGE according to Laemmli was performed in 7.5% (w/v) acrylamide slab gels stained with Coomassie Blue. Samples of NOS purified from *Physarum* (10 μg) after DEAE chromatography (lane 1) and of NOS purified from pig brain (1 μg) ([25], lane 2) were applied. Gel filtration by Superose-6 column and determination of NOS activity (▲, total activity per fraction, 300 μl fraction size) were carried out as detailed in the Experimental section. Molecular-mass markers (■) used were thyroglobulin (669 kDa), β-amylase (200 kDa), transferrin (80 kDa), and chymotrypsinogen (25 kDa).

Purified NOS from *Physarum* was inhibited by both NMA and NNA, NMA being about twice as effective as NNA (Table 2). Applying Ca^{2+}-free conditions or addition of the calmodulin antagonist R23571 (not shown) did not affect *Physarum* enzyme activity, indicating that the enzyme does not require exogenous Ca^{2+} for activity.

Under denaturing conditions, two closely related bands with molecular masses of 130 kDa and 120 kDa were determined for *Physarum* NOS (Figure 2). The native protein showed peaks at 400 and 800 kDa in gel filtration (Figure 2); the corresponding Stoke's radii are 7.0 and 8.7 nm.

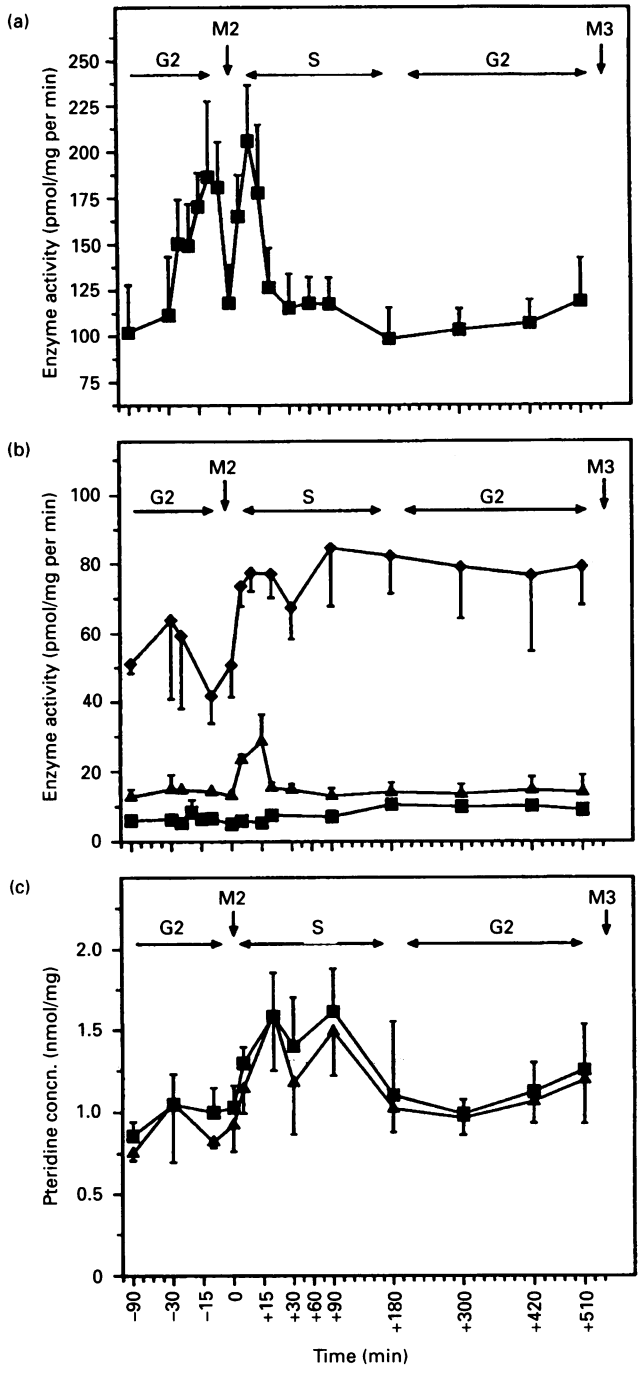

Figure 3 NOS activity (■, a), tetrahydrobiopterin biosynthetic activities [(b): GTP cyclohydrolase I (■), 6-pyruvoyl tetrahydropterin synthase (▲), and sepiapterin reductase (◆)] and intracellular pteridines [(c): biopterin (■) and 6-hydroxymethyl pterin (▲)] during the cell cycle of *Physarum polycephalum* macroplasmodia between second (M2) and third (M3) mitosis

Homogenates of *Physarum* macroplasmodia from different time points were obtained and enzyme assays (triplicate incubations) and pteridine determinations were carried out as detailed in the Experimental section. Values of each time point given are means of six to nine independent experiments for NOS and of four to six independent experiments for pteridine biosynthesis $\pm$ S.E.M.

We then studied pteridine biosynthesis and NOS activity during the cell cycle. As can be seen from Figure 3, NOS activity showed a biphasic up- and down-regulation during prophase of

mitosis and during early S-phase with a sharp drop within 5 min in telophase. Levels of tetrahydrobiopterin biosynthetic enzyme activities did not follow the same pattern. GTP cyclohydrolase I slightly increased during S-phase and remained somewhat higher during G_2-phase (Figure 3b). Sepiaperin reductase increased about 2-fold during S-phase and also remained high during the second G_2-phase. 6-Pyruvoyl tetrahydropterin synthase, in contrast, peaked during early S-phase but remained unchanged during late S-phase and during the G_2-period. Intracellular concentrations of tetrahydrobiopterin and 6-hydroxymethylpterin were maximal during S-phase but dropped at the S/G_2 border. The ratio of these two compounds did not change during the cell cycle (Figure 3).

Statistical evaluation of the data shown in Figure 3 showed the following significant differences: comparison of NOS activities (Figure 3a) at -5 min, 0 min and $+10$ min yielded a P value of 0.072 (Kruskal–Wallis non-parametric analysis of variance). The two peaks observed close to mitosis (-5 min, $+10$ min) were significantly higher than levels during late S-phase and G_2-phase ($P < 0.02$, Student's t-test, -5 min versus $+180$ min, $+10$ min versus $+180$ min). Sepiapterin reductase (Figure 3b) was increased by about 40 % when comparing levels at -90 min and at $+5$ min ($P < 0.05$, Student's t-test). The short increase in 6-pyruvoyl tetrahydropterin synthase activity between 0 min and $+5$ min yielded a $P < 0.01$ (Student's t-test). Comparison of pteridine levels (Figure 3c) at -90 min versus $+20$ min and $+20$ min versus $+300$ min, showed a 2-fold significant increase during S-phase ($P < 0.05$ in both cases, Student's t-test).

DISCUSSION

So far, endogenous NO formation has been described for highly differentiated eukaryotes like mammals (reviewed in [5,32]), chick [33], the horseshoe crab *Limulus polyphemus* [34], and the blood-sucking insect *Rhodnius prolixus* [35]. In mammals, nitric oxide (NO) fulfills essential roles in regulation of blood pressure, neurotransmission and cytotoxicity (for review see [5,32]). Various isoforms of mammalian NOSs have been characterized at the protein as well as at the DNA level [5,32]. Constitutive NOSs depend on addition of exogenous Ca^{2+} for binding of calmodulin, and different isoforms are found in the nervous system (nNOS) and in endothelium (eNOS). Inducible NOSs (iNOSs) which require cytokines like interferon-γ, interleukin-1 or tumour necrosis factor-α or lipopolysaccharide for expression do not depend on exogenous Ca^{2+}. As was shown for macrophages [36], iNOS contains tightly bound calmodulin. All NOS enzymes studied so far depend on NADPH, bind FAD, FMN, haem and tetrahydrobiopterin, and are active as homodimers (reviewed in [37]). For iNOS from macrophages it was shown that haem, tetrahydrobiopterin and L-arginine support subunit dimerization [38].

In this study, we purified and characterized NOS from the low eukaryote *Physarum polycephalum*. Like nNOS from cerebellum [39], the enzyme produced citrulline and NO, detected as nitrite plus nitrate, at a stoichiometry of 1:1, indicating that the citrulline-generating enzyme activity observed is indeed a NOS. For iNOS from mouse macrophages [26,30] and for eNOS from bovine endothelial cells [40], a molecular mass of about 130 kDa was determined for the monomer. The enzyme purified from *Physarum* showed bands at 130 and at 120 kDa. Such multiple banding was also reported for iNOS from cytokine-treated macrophages [26]. Whether these closely related bands are caused by differential cofactor-binding or enzyme modification remains to be studied. Active mammalian NOS enzymes are dimer with a molecular mass of about 260 kDa in the case of macrophages [26,30]. For nNOS from pig brain, a Stoke's radius of 8.1 nm was reported for the dimer [41]. In contrast, *Physarum* native NOS had two active peaks which corresponded to molecular masses of 400 and 800 kDa or to Stoke's radii of 7.0 and 8.7 nm. It remains to be seen whether these two active peaks are monomer and dimer or two conformers of the protein.

Dependence of *Physarum* NOS on L-arginine and NADPH was within the range reported for mammalian NOS enzymes [26,30,40,42,43]. Flavins and tetrahydrobiopterin remained bound to the enzyme after purification. Whereas mammalian NOSs contain about 1 mol of FAD and 1 mol of FMN per mol of NOS subunit [26,30,42,44], FMN and in particular FAD are only weakly bound to NOS from *Physarum* and FAD especially seems to be lost during purification. The mechanism underlying these different efficiencies in cofactor binding remains to be elucidated. In contrast, the amount of enzyme-bound tetrahydrobiopterin was only slightly smaller for *Physarum* compared with nNOS from pig brain [44].

As was shown for cytokine-treated macrophages [45] or for purified eNOS [40], and unlike nNOS [41], NMA was more effective in inhibiting *Physarum* NOS than NNA. Thus with regard to the rank order of inhibition by arginine analogues, the behaviour in SDS gel electrophoresis and its independence of exogenous Ca^{2+}, NOS from *Physarum* resembles iNOS. Further characterization of the enzyme will show whether calmodulin is an integral part of the protein, as was demonstrated for iNOS from macrophages [36].

In the vegetative state studied here, *Physarum* is an undifferentiated giant cell containing multiple, synchronously dividing nuclei. Previous reports indicated that pteridines are formed by *Physarum* [14] and cell-cycle-linked changes in pteridine contents were observed [15]. We identified these pteridines to be tetrahydrobiopterin and 6-hydroxymethyl-7,8-dihydropterin. Interestingly, the latter compound is an intermediate of folic acid biosynthesis formed by 7,8-dihydroneopterin aldolase [2,24], an enzyme activity usually not found in organisms producing tetrahydrobiopterin. It was recently reported for the eukaryote *Pneumocystis carinii*, that a single gene is encoding for a polypeptide responsible for three enzyme activities in folic acid biosynthesis, i.e. 7,8-dihydroneopterin aldolase, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase, and dihydropteroate synthase [2]. Studying molecular regulation of these pathways in *Physarum* will elucidate a possible interdependence of tetrahydrobiopterin and folic acid biosynthesis which both utilize the same intermediate, i.e. 7,8-dihydroneopterin trisphosphate.

In the course of the cell cycle both pteridines showed their maximum levels during S-phase but decreased at the S/G_2 border. The ratio of tetrahydrobiopterin and 6-hydroxymethyl-7,8-dihydropterin remained 1:1 throughout the cell cycle. This ratio may be regulated by phosphatase activity and the K_m values of 6-pyruvoyl tetrahydropterin synthase and 7,8-dihydroneopterin aldolase respectively.

Although we cannot exclude the possibility that tetrahydrobiopterin also exerts other functions in *Physarum*, one of its roles may be to support formation of NO. It is not clear at present which function NO might have in *Physarum*. However, the finding of elevated NOS activity during mitosis suggests that formation of NO may be linked to this process. It is remarkable that the constitutive enzyme found in *Physarum* seems to be similar to iNOS, an isoform which is regulated by cytokines and requires *de novo* protein biosynthesis. It is still unclear how NOS activity is regulated in *Physarum*. A possible mechanism for regulating NOS activity could be enzyme modification by phosphorylation, which was shown to modulate NOS activity

[46–48]. The target for NO in *Physarum* has not yet been identified. Further work will show whether NO acts by stimulation of guanylate cyclase, by interaction with other iron-containing enzymes (for review see [5,32]), by enzyme modification through binding of NAD as reported for glyceraldehyde-3-phosphate dehydrogenase (reviewed in [49]) or actin [50], or by regulation of gene expression at the post-transcriptional level as observed for cellular iron metabolism [51,52]. Characterization of NOS and tetrahydrobiopterin formation in *Physarum polycephalum* may thus permit us to study novel aspects of NO physiology and to clarify previous observations on the involvement of tetrahydrobiopterin in cell-proliferation and differentiation processes [6,7].

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