

Role of Nitric Oxide Synthase in the Light-Induced Development of Sporangiohores in *Phycomyces blakesleeanus*

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Blue light controls the development of sporangiohores in the zygomycete *Phycomyces blakesleeanus* Burgeff. Light represses the production of microsporangiohores and enhances the development of macrosporangiohores. Inhibition of the biosynthesis of tetrahydrobiopterin, a cofactor of NO synthase, inhibits this photomorphogenesis. Light induces production of citrulline from arginine in the mycelium and in sporangiohores. The citrulline-forming activity is dependent on NADPH, independent of calcium, and inhibited by NO synthase inhibitors. It is reduced in tetrahydrobiopterin-depleted mycelium. Light induces emission of NO from the developing fungus in the same order of magnitude as citrulline formation from arginine. The NO donor sodium nitroprusside can replace the light effect on sporangiohore development, and inhibitors of NO synthase repress it. We suggest that a fungal NO synthase is involved in sporangiohore development and propose its participation in light signaling.

Nitric oxide synthases (NOS) catalyze the formation of L-citrulline and NO from L-Arg and oxygen. Tetrahydrobiopterin (BH₄) is a cofactor for certain monooxygenases and an essential factor for the function of NOS as enzymatic studies of BH₄-free expressed enzymes (Gorren et al., 1996; Hurshman et al., 1999) and the crystal structure of mammalian NOS have shown (Crane et al., 1998; Raman et al., 1998; Fischmann et al., 1999). Recent experiments with antipterins have demonstrated that BH₄ of NOS is not participating in a dielectronic redox cycle as in BH₄-dependent monooxygenases (Bömmel et al., 1998; Riethmüller et al., 1999). Most likely a BH₃ radical is formed after a one-electron transfer from BH₄ to the heme ferrous-dioxygen complex, and the BH₃ subsequently is reduced by the NOS flavins (Crane et al., 1998; Hurshman et al., 1999).

BH₄ is present in the ascomycete *Neurospora crassa* Shear et Dodge at low concentrations (up to 10 pmol g⁻¹ mycelia) and at much higher concentrations in sporangiohores and mycelia of the zygomycete *Phycomyces blakesleeanus* Burgeff (up to 2 nmol g⁻¹; Maier and Ninnemann, 1995a). Because fungi lack BH₄-dependent monooxygenases, the function of BH₄ in these organisms is unknown. The biosynthesis of BH₄ starts from GTP, which is converted by GTP-cyclohydrolase I, 6-pyruvoyl-5,6,7,8-tetrahydropterin synthase, and sepiapterin reductase to BH₄ (for review, see Duch and Smith, 1991; Thöny et al., 2000).

This pathway is known in animals and was also shown for bacteria (Son and Rosazza, 2000), cyanobacteria (Lee et al., 1999), *Physarum polycephalum* (Werner-Felmayer et al., 1994), and by us for *Euglena gracilis* and the fungi *N. crassa* and *P. blakesleeanus* (Maier and Ninnemann, 1995a).

Measurement of citrulline formation from ³H-labeled Arg showed an NOS-like activity present in the fungi *N. crassa* and *P. blakesleeanus* (Ninnemann and Maier, 1996). Such NOS-like activities were also found in eubacteria (Chen and Rosazza, 1995; Chen et al., 1996; Morita et al., 1997; Son and Rosazza, 2000), the slime mold *Physarum polycephalum* (Werner-Felmayer et al., 1994), and in several species of higher plants (Cueto et al., 1996; Ninnemann and Maier, 1996; Delledonne et al., 1998; Durner et al., 1998; Barroso et al., 1999; Ribeiro et al., 1999). NO production may also result from nitrite reductase in bacteria (Chen et al., 1996) or from nitrate reductase in plants (Rockel et al., 1996; Wildt et al., 1997; Yamasaki et al., 1999; Yamasaki and Sakihama, 2000).

No NOS gene from higher plants or fungi has been cloned and no studies showed a dependence of NO or citrulline formation on BH₄. Calcium-independent NOS with biochemical features closely resembling those of mammalian-inducible NOS was purified from the slime mold *P. polycephalum* (Werner-Felmayer et al., 1994) and was recently cloned (Golderer et al., 2001).

In *N. crassa* and *P. blakesleeanus*, the synthesis of BH₄ is inhibited by 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of GTP-cyclohydrolase I, the first enzyme in folic acid and BH₄ biosynthesis (Maier and Ninnemann, 1995a). In both fungi, block-

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ing the synthesis of pteridines has a strong effect on vegetative spore development: In *N. crassa* the production of conidia is reduced, and in *P. blakesleeanus* BH₄ depletion affects photomorphogenesis of sporangiophores. In *P. blakesleeanus*, two types of sporangiophores exist: up to 10-cm-long macrosporangiophores with sporangia containing some 100,000 spores, and 100- μ m microsporangiophores with several hundred spores in each sporangium. The development of both types is differently controlled by blue light, macrosporangiophores being promoted by light and microsporangiophores photoinhibited (Corrochano and Cerdá-Olmedo, 1988; Corrochano and Cerdá-Olmedo, 1992). The development of macro- and microsporangiophores in *P. blakesleeanus* is controlled by different photoreceptors and different light signal transduction pathways (Corrochano et al., 1988; Flores et al., 1998). Inhibition of BH₄ synthesis prevents the blue light-enhanced development of macrosporangiophores and suspends the blue light-suppressed development of microsporangiophores partly (Maier and Ninnemann, 1995b). We found an NOS-like activity in mycelia and macrosporangiophores of *P. blakesleeanus*, which was higher in irradiated than in dark macrosporangiophores (Ninnemann and Maier, 1996).

In this study we investigated whether NO-releasing substances will substitute for NOS activity and substitute for the effect of light; then NOS inhibitors should reduce the effects of light on sporangiophorogenesis. We also investigated whether NOS activity can be stimulated by light *in vivo* and *in vitro* and whether this light activation depends on BH₄. We compared citrulline production and NO emission of developing fungi quantitatively.

RESULTS

Effect of an NO Generator and of NOS Inhibitors on Sporangiophore Photomorphogenesis

We investigated the effects of the NO-releasing substance sodium nitroprusside (SNP) and of the NOS inhibitor L-N^G-nitro-Arg methylester (L-NAME) on the development of macro- and microsporangiophores (Fig. 1) to test whether the inhibition of photostimulation of macrosporangiophore development

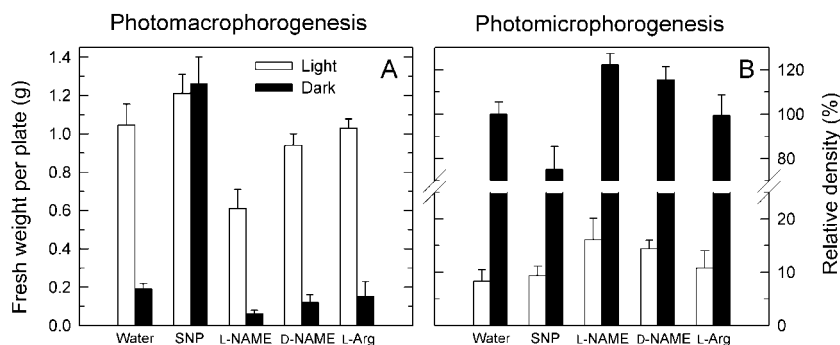
and photodepression of microsporangiophore density due to the lowered BH₄ content (Maier and Ninnemann, 1995b) is caused by a deficiency of NOS. In dark controls (water), 0.2 g of macrosporangiophores was produced (Fig. 1A) and irradiation caused a 5-fold increase in its yield. With NO-releasing SNP the macrosporangiophore yield in darkness reached the same levels as in light. SNP only weakly stimulated an additional growth of macrosporangiophores on irradiated mycelia, where its yield was nearly maximal. Thus, NO has the same effect as light for induction of macrosporangiophore development. Application of the inhibitor L-NAME reduced macrosporangiophore yield to 60% on irradiated mycelia and to 40% on dark ones. The stereoisomer D-NAME lowered sporangiophore yield under both conditions, but at a reduced extent than that by L-NAME. L-Arg had no effect.

The development of microsporangiophores was suppressed by blue light (Fig. 1B). In darkness the density of microsporangiophores reached several thousand per centimeter⁻²; on irradiated plates it was reduced by 90%. In light, SNP had no effect on microsporangiophores, but reduced them in darkness to 80% of the control levels. L-NAME enhanced microsporangiophores in the dark to 120% and doubled them on light-treated mycelia. D-NAME also enhanced them slightly, whereas L-Arg had no effect. L-N^G-monomethyl-Arg (L-NMMA) and D-NMMA had no effect on photomorphogenesis (data not shown).

Effect of NOS Inhibitors and Light on Citrulline-Forming Activity

We investigated the effect of NOS inhibitors (Fig. 2) and light (Fig. 3) on the enzyme activity of NOS, to compare the results of these photobiological experiments with the corresponding NOS activities. The BH₄-dependent NOS produce NO and citrulline from Arg and oxygen, which requires electron flow from NADPH via FAD, FMN, and heme to the substrates. We measured the formation of ³H-citrulline from ³H-Arg (Bredt and Snyder, 1990; Ninnemann and Maier, 1996). About 70% of citrulline-forming activity was NADPH independent (Fig. 2), presumably due

Figure 1. Effects of the NO generator SNP and the NOS inhibitor L-NAME on sporangiophore development in *P. blakesleeanus*. A, Effect on photomacrosporogenesis. B, Effect on photomicrosporogenesis. Water, D-NAME, and L-Arg were used as controls. Data were calculated from three independent experiments with five parallel plates in each treatment. Error bars: ses.



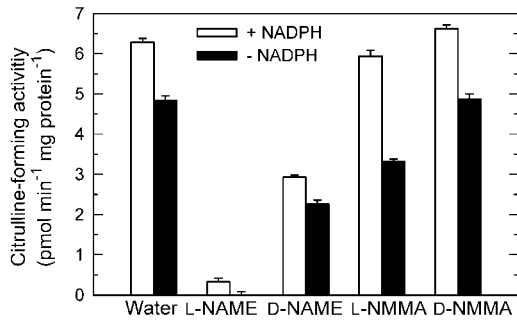


Figure 2. Inhibition of citrulline-forming activity in *P. blakesleeanus*. Activities were measured by conversion of ³H-Arg to ³H-citrulline at pH 7.0 with and without 1 mM NADPH in crude extracts prepared from irradiated submerged mycelia. Inhibitors and control substances were 5 mM. Error bars are SES ($n = 3$).

to alternative electron sources in crude extracts. This is a common observation for NOS activities in unpurified enzyme extracts from various sources. In extracts from irradiated mycelium the citrulline-forming activity reached 6 pmol min⁻¹ mg⁻¹ protein at pH 7. Removal of Ca²⁺ from the assay mixture had no effect on the activity (102% ± 4% of control levels). Citrulline formation was nearly completely inhibited by 5 mM L-NAME, 50% inhibited by 5 mM D-NAME, but only slightly inhibited by 5 mM L-NMMA and not by 5 mM D-NMMA. These inhibitory effects on enzyme activity was in correlation with the inhibitory effect of L-NAME *in vivo* on photomorphogenesis (Fig. 1) and with little effect of L-NMMA and D-NMMA.

Light enhanced the citrulline-forming activity two to three times in mycelia grown in liquid culture (Fig. 3A). At the end of the logarithmic phase the difference disappeared. The macrosporangioophores showed an enhanced citrulline-forming activity in light (Fig. 3B). The increase in light was highest in nearly ripe and declined in older macrosporangioophores. In young microsporangioophores the activity was high in darkness and declined rapidly with age (Fig. 3B). The data show that in developmentally important growth phases (young mycelia and sporangioophores) NOS activity was higher in light than in the dark. Irradiation of extracts from dark-grown mycelia showed no effect on the citrulline-forming activity (data not shown).

Light Stimulation of Citrulline-Forming Activity Is Dependent on BH₄ *in Vivo*

Exogenous BH₄ had only a small effect on the citrulline-forming activity *in vitro* (Fig. 4). Because the cofactor is firmly bound to NOS, it cannot be removed during desalting procedures. This behavior is also known from metazoan NOS, where BH₄ dependence could unequivocally be shown only after expression in BH₄-free *Escherichia coli* cells (Gorren et al., 1996). To investigate the effect of depletion of BH₄

in vivo on the fungal citrulline-forming activity we grew *P. blakesleeanus* on a medium containing 5 mM DAHP. In irradiated mycelia, the BH₄ content was reduced from 1.3 to 0.2 nmol g⁻¹, and in dark-grown mycelia from 1.2 to 0.1 nmol g⁻¹. We measured the citrulline-forming activity with and without BH₄ in the assay mixture (Fig. 4). DAHP treatment reduced the citrulline-forming activity of mycelia grown in light and darkness. *In vivo* inhibition of BH₄ synthesis by growing in DAHP blocked nearly all light-enhanced NOS activity.

Determination of NO in Citrulline-Forming Assays

During the NOS reaction, NO is formed from Arg in equimolar amounts with citrulline. Because the magnitude of the citrulline-forming activity was in the range of 1 to 10 pmol min⁻¹ mg⁻¹, one expects hundreds of picomoles of NO from 1 mg protein within 60 min. The produced NO was rapidly oxidized to nitrite within the assay mixture, which accumulated to concentrations of several hundred nanomolar. For measurement of nitrite we reduced it to NO in a stirring solution of KI/HCl and measured the released NO in the gas phase by chemiluminescence detection following the method described by Archer et al. (1995). Addition of nitrite to the enzyme extract and buffer solution before starting the incubation had shown that the nitrite was destroyed dur-

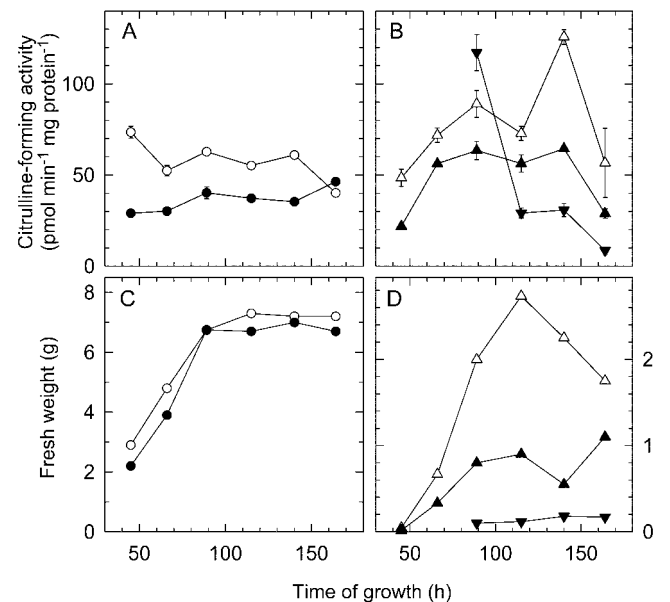


Figure 3. Light effect on citrulline-forming activity (measured at pH 8.3) during growth of mycelia in liquid culture (A) and during development of sporangioophores (B). The time course of growth of mycelia mass in one flask (C) and sporangioophore yield of one plate (D) are shown for comparison with the respective changes in citrulline-forming activity (A and B). Mycelia in light (○) or darkness (●); macrosporangioophores in light (△) or darkness (▲) and microsporangioophores in darkness (▼), they were too few in light for analysis. Error bars are SES ($n = 3$).

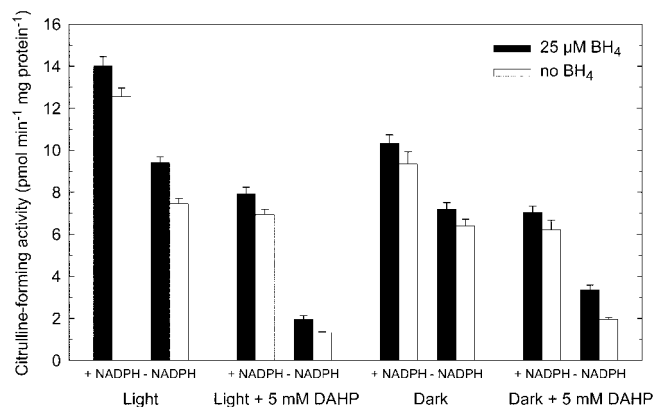


Figure 4. Effect of in vivo inhibition of BH₄ synthesis during growth (with/without 5 mM DAHP) and/or addition of BH₄ on mycelial citrulline formation. Mycelia of *P. blakesleeanus* were grown on DAHP (5 mM) in liquid culture in light or darkness. Activities were measured in enzyme extracts at pH 7 by conversion of ³H-Arg to ³H-citrulline with and without added NADPH to the reaction mixture. Error bars are SES ($n = 3$).

ing incubation with a rate of 2 to 10 pmol min⁻¹ mg⁻¹, a value comparable to the expected NO/nitrite formation. We found 0% to 5% of the expected NO formation in the enzyme assays as nitrite. Because of the variable rates of nitrite destruction it was difficult to compare samples with each other quantitatively. However, protein extracts of mycelia grown on DAHP, with BH₄ levels reduced to 15% of control levels, produced residual nitrite levels below the detection limit, whereas in assays performed with extracts of mycelium grown without inhibitor a small amount of nitrite was detected.

NO Emission of *Phycomyces blakesleeanus* Growing on Solid Medium

The chemiluminescence detector system is able to detect NO added to purified air down to a concentration of 10 pL L⁻¹ in the gas phase. The citrulline-forming assay suggested that the mycelium of *P. blakesleeanus* produces 1 to 10 pmol NO min⁻¹ mg⁻¹ extractable protein during development. We measured the NO emission of *P. blakesleeanus* at different ages (3 d old, 72–77 h, Fig. 5A; 5 d old, 121.5–126.5 h, Fig. 5B). The NO flux was about 2 to 2.5 pmol min⁻¹ in the dark at both ages. In 3-d-old, fungus NO emissions reached 11 pmol min⁻¹ within 40 min after starting irradiation, about five times the dark level, and dropped again to 6 pmol min⁻¹ during continued illumination within the next 2 h. Irradiated fungi grown on DAHP showed nearly no increase of NO emission when compared with the illuminated controls. Figure 5B shows an experiment with 5-d-old fungus. Illumination raised the NO emission by three times, whereas the DAHP-treated irradiated fungus emitted only 30% of the illuminated inhibitor-free mycelium. After ending the illumination, the NO emission reached dark levels within 1 h. Fungi,

which were 3 to 5 d old and grown on solid medium, contained 2 to 5 mg extractable protein in a growth area of 100 cm². Therefore, the NO flux can be estimated to originate from an NO-forming process with a minimum activity of 1 pmol min⁻¹ mg⁻¹ extractable protein in irradiated *P. blakesleeanus*.

DISCUSSION

The data of this study suggest that a fungal NOS is involved in sporangiophore development in *P. blakesleeanus*. The citrulline-forming activity of protein extracts from mycelium and the NO-forming activity in developing fungus on solid medium were in the same range of 1 to 10 pmol min⁻¹ mg⁻¹. Citrulline formation and NO emission were enhanced by light and inhibited at the same degree, if the fungi were grown on medium containing DAHP (Figs. 4 and 5). Addition of BH₄ to the citrulline-forming assay had only a small effect on the activity, presumably because the cofactor is firmly bound to the enzyme and cannot be removed by gel filtration. If grown in presence of DAHP, BH₄ levels were reduced to 15% of controls. Lowered BH₄ levels could cause an incorrect assembly of the NOS dimers and accumulation of inactive enzyme, which cannot be reactivated by addition of BH₄ to the citrulline-forming assay (Fig. 4). Measurement of NO in the assay mixture of the citrulline-forming assay was not possible quantitatively because the NO was rapidly

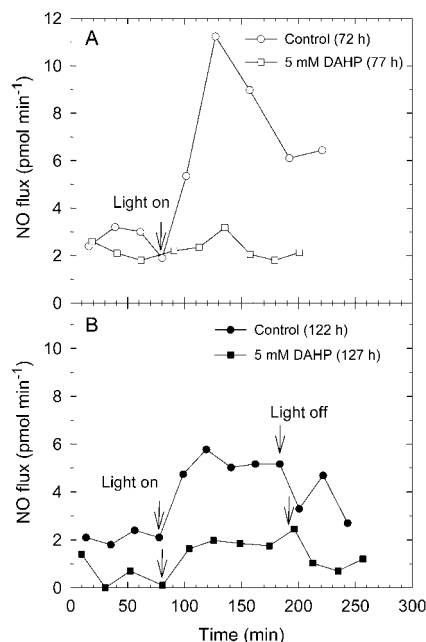


Figure 5. NO emission from controls and BH₄-depleted (5 mM inhibitor DAHP) mycelia growing on two plates with 14.5-cm diameter (A) and on two plates with 14.5- and 8.5-cm diameters (B). At the start of the respective experiments the mycelia had grown for 72 h (A, control), 77 h (A, DAHP), 122 h (B, control), and 127 h (B, DAHP). Start and stop of illumination are indicated by arrows.

oxidized to nitrite, which was further destroyed in an NADPH-enhanced reaction to unknown products. Measurement of residual nitrite showed that its production was dependent on BH_4 during growth of the fungus.

The NO emission was enhanced by light. This was confirmed by citrulline formation of protein extracts of mycelia and sporangiophores at different ages (Fig. 3) and by light-enhanced NO flux of developing fungi (Fig. 5). The time resolution of our NO emission measurements was 20 min. NO emission reached maximum activity after 40 min, adapted to somewhat reduced levels during continuous illumination, and dropped again to dark levels within 1 h after ending the illumination. The kinetics suggest an induction by enhancement of protein synthesis of NOS itself or of an activating factor, followed by adaptation after reaching maximum levels and down-regulation by reduced expression after ending of illumination. This behavior is typical for elements of signaling chains. Because the citrulline-forming activity was not activated by light in vitro in crude extracts, the photoactivation of NO emission presumably was established by a signal transduction chain originating in different still unknown flavoprotein photoreceptor(s) of *P. blakesleeanus*.

The citrulline-forming activity is not enhanced by Ca^{2+} . The inducible NOS of animals are also Ca^{2+} independent and are mainly controlled by transcriptional regulation. Also, the NOS of *P. polycephalum* is Ca^{2+} independent (Werner-Felmayer et al., 1994). A receptor for NO of fungi might be a soluble guanylate cyclase as in metazoans. An involvement of cyclic nucleotides in light signaling for transient increase of sporangiophore growth was suggested (Cohen, 1974; Cohen and Atkinson, 1978), but the findings could not be repeated (Leutwiler and Brandt, 1983). No investigations on the role of cyclic nucleotides in photomorphogenesis of sporangiophores were published.

Depletion of BH_4 during development by growth on DAHP inhibited light-enhanced growth of macrophores more than it released light-dependent suppression of microsporangiophore density (Maier and Ninnemann, 1995b). Also, NO donors and NOS inhibitors had a more pronounced effect on macrosporangiophores than on microphores (Fig. 1). These observations suggest that NOS affects the signaling events controlling macrophorogenesis more than those controlling microphorogenesis. The action spectra for the high and low fluence responses of photomorphogenesis and photomicrophorogenesis suggest different but related photoreceptors, perhaps even four different receptors (Corrochano et al., 1988). A recent study has shown that the kinase inhibitor staurosporine can replace the effect of light in photophorogenesis (Tsolakis et al., 1999), suggesting that a kinase is inhibited during light signaling in photophorogenesis.

MATERIALS AND METHODS

Strains and Media

Phycomyces blakesleeanus Burgeff wild type strain NRRL 1555(-) was grown on minimal medium (Thornton, 1973; Maier and Ninnemann, 1995b), containing 13.3 mM L-Asn and 0.5 μM thiamine. Liquid cultures were grown from 10^5 heat-activated spores (48°C, 15 min) in batches of 0.2 L in 2-L glass flasks with baffles for improved aeration at 20°C and 80 rpm agitation. Pteridine synthesis was inhibited by adding 5 mM sterile filtered DAHP (20-mM stock solution). Irradiation was by white fluorescence light at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Dark controls were wrapped in light-tight aluminum foil and black clothes. Mycelia were harvested under red safety light by filtration through a nylon mesh, washed with water, and frozen in liquid nitrogen and stored at -70°C.

Photomorphogenesis Experiments

Sporangiophore development (Fig. 1) was monitored as described (Corrochano and Cerdá-Olmedo, 1988, 1992; Corrochano et al., 1988; Maier and Ninnemann, 1995b). Spores were obtained from pooled stage V macrosporangia, suspended in water, and heat activated at 48°C for 15 min. 10^5 spores were mixed with 3 mL minimal agar (7 g L^{-1} agar, held at 50°C) and layered on a 9-cm plate containing 20 mL of solidified minimal agar (15 g L^{-1} agar). Plates were left unsealed and unsealed in the dark at 20°C and at saturated humidity. After 48 h, 200 μL of freshly prepared, sterile filtered solutions of SNP (1 mM), NOS inhibitors (5 mM), L-Arg (5 mM), or water were plated under red safety light in a sterile working bench on the surface of the agar. The mycelia had already germinated from the spores and were grown to a dense lawn of hyphae still not elevating above the agar surface. The plates were irradiated through a milk glass window for 6 h with diffuse white light from a 200-W halogen lamp mounted 1.5 m above the plates in a reflecting metal housing. The energy fluence rate was adjusted to 4 W m^{-2} at the agar surface, corresponding to a blue light energy fluence rate of 1 W m^{-2} (measured through a no. 5562 Corning broadband blue filter, maximum at 430 nm). This energy fluence rate corresponded to a blue light (430 nm) photon fluence rate of 4 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and is just below the saturation level for induction of macrophorogenesis (Corrochano et al., 1988; Corrochano and Cerdá-Olmedo, 1992; Maier and Ninnemann, 1995b). The temperature inside the plates was monitored and stable at 20°C. After illumination all plates were incubated for 90 h in the dark. Macrophores were harvested with forceps, frozen in liquid nitrogen, and weighed. For microphore analysis we photographed the agar surface at 20 \times magnification with a videocamera and counted microphores at 10 randomly chosen areas on each plate using image processing software (Sigmascan 2.0, SPSS Science, Erkrath, Germany). Reported values are means with SEs of three independent experiments, with five parallel series of irradiated and dark plates. Treatments were compared with ANOVA.

Analysis of Citrulline-Forming Activity

NOS activity (Figs. 2, 3, and 4) was measured by the conversion of $^3\text{H-Arg}$ to $^3\text{H-citrulline}$ (Bredt and Snyder, 1990; Hofmann and Schmidt, 1995; Ninnemann and Maier, 1996). For preparation of enzyme extracts, the frozen mycelia were powdered under liquid nitrogen, suspended in three volumes of 50 mM triethanolamine hydrochloride and 0.5 mM EDTA (pH 7.0) containing 1 μM leupeptin, 1 μM pepstatin A, 7 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol, and disrupted with a glass homogenizer and a Teflon pestle. The homogenates were cleared by centrifugation at 4°C (30 min, 48,000g, and 60 min, 85,000g). The endogenous Arg and soluble cofactors were removed by gel filtration using Sephadex G25 columns. The triplicate incubations were started by addition of 50 μL of the extract to the reaction mixture containing 0.05 μM calmodulin (bovine brain), 226 μM CaCl_2 , 477 μM MgCl_2 , 0.5 mM EDTA, 5 μM FAD, 5 μM FMN, 25 μM BH_4 , 100 μM L-Arg (including 37 kBq L-[2,3,4,5- ^3H]Arg), 3.5 mM dithiothreitol, and 1 mM NADPH in 50 mM triethanolamine-hydrochloride buffer (pH 7.0 or pH 8.3) and 10% (v/v) glycerol. After 30 min at 37°C the reaction was stopped with 0.89 mL 0.2 M sodium acetate, 2 mM EDTA, 2 mM EGTA, and 2 mM citrulline (pH 5.5). The incubation mixture was applied to 1-mL columns of Dowex AG50 WX-8 (H^+ form), and eluted with 1 mL of water. The cation-exchange resin binds all products that are positively charged at pH 5.5; the non-charged products like L-citrulline flow through. The produced L-[^3H]citrulline was quantified by liquid scintillation counting. Protein concentrations were determined by the Coomassie Blue dye assay using bovine serum albumin as a standard.

Pteridine isolation and quantification were performed as described previously (Maier and Ninnemann, 1995a).

Measurement of NO Emission from Developing Mycelia

NO emitted by developing mycelia was measured in the air by chemiluminescence detection. Plates with developing mycelium at different stages were transferred to a sealed glass vessel (6.2-L volume), closed at the bottom by a Teflon plate. Purified air was passed through the vessel with a constant rate of 2 L min^{-1} . Background concentration of NO in the inflowing air was less than 50 pL L^{-1} . The concentration of NO in the air was measured by chemiluminescence detection (CLD AL ppt 770, ECO-Physics, Dürnten, Switzerland; detection limit 20 pL L^{-1} ; 5-s time resolution). NO fluxes were calculated from the concentration differences between the inlet and outlet of the chamber (Wildt et al., 1997). Plates were placed in the chamber without lid. Because continuous NO emission of the mycelia in the dark was near the detection limit of the instrumental setting (reaching 50–100 pL L^{-1}), the gas flow was interrupted for 5 min to accumulate the produced NO in the air volume inside the vessel. After reestablishing the gas flow, the chamber was continuously flushed for 15 min until NO concentration in the outflowing air was constant again. We summed up the NO emitted during these 15 min

of flushing, which represents the NO emitted during a period of 20 min. During the 5 min of accumulation only a small amount of NO was destroyed by oxidation with oxygen. At a concentration of 500 pL L^{-1} , NO has a very slow reaction rate with oxygen in clean air (5×10^{-7} pL $\text{L}^{-1} \text{ s}^{-1}$ with $k = 1 \times 10^{-11} \text{ M}^2 \text{ s}^{-1}$; Fukuto, 1995). NO emission was followed continuously for 80 min in the dark. Then irradiation was started with white light by a halogen lamp at 250 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ for 100 min. Infrared light emitted by the lamp was absorbed by a heat reflection filter (filter type IR3, Tempax naturblank, OptoChem Glass-Coating, Stromberg, Germany). The light induced NO release by photolysis from nitrite contaminations in the growth media. The release of trace amounts of NO from nitrite by light (295–410 nm) is known from experiments in seawater (Zafariou et al., 1980). We eliminated the NO release caused by photolysis using a blue light filter absorbing any light below 450 nm (Plexiglas XT 35270, yellow, 3 mm, Röhm, Darmstadt, Germany). Application of the filter to illuminated cultures immediately stopped the photolytic NO release, whereas the enzymatic NO release continued. Fifty percent of the NO release from illuminated cultures and 75% of the NO release of fungi growing on media containing DAHP was of photolytic origin. The photolytic NO release was subtracted from the data.

Measurement of NO Emission from Enzyme Extracts

Initial measurements with the NO donor SNP added at different concentrations to the reaction mixture used in the citrulline-forming assay had shown that nearly all released NO is rapidly converted to nitrite. Because the low amounts of NOS activity deduced from the citrulline-forming assay (1–10 pmol $\text{min}^{-1} \text{ mg}^{-1}$) predicted only a nitrite accumulation of several 100 pmol in 1 mL-assays (up to 500 nm of nitrite), we had to use a very sensitive method for nitrite detection. We measured nitrite by a chemiluminescence assay using the experimental setting described above for NO measurement after nitrite was released from reaction mixtures by reduction to NO with potassium iodide in a stirred acidic solution (Archer et al., 1995). In this way, 20% to 40% of the produced nitrite could be released as NO from the reaction mixture and measured by the chemiluminescence detector. A plate (8.5-cm diameter) with 40 mL of 0.18 M HCl/0.5 M KI was placed into a glass vessel (0.74 L) connected to the chemiluminescence NO detector as described above. NOS assays were performed as described above for the citrulline-forming assay, but without addition of ^3H -labeled Arg. After 1 h of incubation the assays were frozen immediately in liquid nitrogen and stored at -80°C until measurement. Addition of nitrite to the assay mixture before starting the incubation had shown that nitrite is stable during storage. For the measurements the assay mixtures were thawed and immediately added to the stirring KI/HCl solution inside the glass vessel through a small inlet, opened only for a few seconds. The released NO was accumulated for 45 s and then released into the chemiluminescence detector by flushing with clean air at 2 L min^{-1} . After 5 to 15 min of continuous measurement the

NO emission reached background levels and the next sample could be added. Up to nine samples could be measured with one batch of KI/HCl without a decrease of the chemiluminescence signal. After renewal of the KI/HCl solution, the solution had to stir for 5 min to reach background levels, and a further nine samples could be measured. The detection limit was 50 pmol nitrite. The chemiluminescence signal was linear with nitrite amounts higher than 100 pmol.

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LITERATURE CITED

- Archer SL, Shultz PJ, Warren JB, Hampl V, Demaster EG (1995) Preparation of standards and measurement of nitric oxide, nitroxyl, and related oxidation products. *Methods* **7**: 21–34
- Barroso JB, Corpas FJ, Carreras A, Sandalio LM, Valderama R, Palma JM, Lupianez JA, del Rio LA (1999) Localization of nitric-oxide synthase in plant peroxisomes. *J Biol Chem* **274**: 36729–36733
- Bömmel HM, Reif A, Fröhlich LG, Frey A, Hofmann H, Marecak DM, Groehn V, Kotsonis P, La ML, Köster S et al. (1998) Anti-pterins as tools to characterize the function of tetrahydrobiopterin in NO synthase. *J Biol Chem* **273**: 33142–33149
- Bredt DS, Snyder SH (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci USA* **87**: 682–685
- Chen J-Y, Chang W-C, Chang T, Chang W-C, Liu M-Y, Payne WJ, LeGall J (1996) Cloning, characterization, and expression of the nitric oxide-generating nitrite reductase and of the blue copper protein genes of *Achromobacter cycloclastes*. *Biochem Biophys Res Commun* **219**: 423–428
- Chen YJ, Rosazza JPN (1995) A bacterial nitric oxide synthase from a *Nocardia* species. *Biochem Biophys Res Commun* **203**: 1251–1258
- Cohen RJ (1974) Cyclic AMP levels in *Phycomyces* during a response to light. *Nature* **251**: 144–146
- Cohen RJ, Atkinson MM (1978) Activation of *Phycomyces* adenosine 3',5'-monophosphate phosphodiesterase by blue light. *Biochem Biophys Res Commun* **83**: 616–621
- Corrochano LM, Cerdá-Olmedo E (1988) Photomorphogenesis in *Phycomyces*: dependence on environmental conditions. *Planta* **174**: 309–314
- Corrochano LM, Cerdá-Olmedo E (1992) Sex, light and carotenoids: the development of *Phycomyces*. *Trends Genet* **8**: 268–274
- Corrochano LM, Galland P, Lipson ED, Cerdá-Olmedo E (1988) Photomorphogenesis in *Phycomyces*: fluence-response curves and action spectra. *Planta* **174**: 315–320
- Crane BR, Arvai AS, Ghosh DK, Wu CQ, Getzoff ED, Stuehr DJ, Tainer JA (1998) Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science* **279**: 2121–2126
- Cueto M, Hernandez-Perera O, Martin R, Bentura ML, Rodrigo J, Lamas S, Golvano MP (1996) Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. *FEBS Lett* **398**: 159–164
- Delledonne M, Xia YJ, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**: 585–588
- Duch DS, Smith GK (1991) Biosynthesis and function of tetrahydrobiopterin. *J Nutr Biochem* **2**: 411–423
- Durner J, Wendehenne D, Klessig DF (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc Natl Acad Sci USA* **95**: 10328–10333
- Fischmann TO, Hruza A, Niu XD, Fossetta JD, Lunn CA, Dolphin E, Prongay AJ, Reichert P, Lundell DJ, Narula SK et al. (1999) Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat Struct Biol* **6**: 233–242
- Flores R, Cerdá-Olmedo E, Corrochano LM (1998) Separate sensory pathways for photomorphogenesis in *Phycomyces*. *Photochem Photobiol* **67**: 467–472
- Fukuto JM (1995) Chemistry of nitric oxide: biologically relevant aspects. In L Ignarro, F Murad, eds, *Nitric Oxide: Biochemistry, Molecular Biology and Therapeutic Implications*. Academic Press, San Diego, pp 1–15
- Golderer G, Werner ER, Leitner S, Gröbner P, Werner-Felmayer G (2001) Nitric oxide synthase is induced in sporulation of *Physarum polycephalum*. *Genes Dev* (in press)
- Gorren ACF, List BM, Schrammel A, Pitters E, Hemmens B, Werner ER, Schmidt K, Mayer B (1996) Tetrahydrobiopterin-free neuronal nitric oxide synthase: evidence for two identical highly anticooperative pteridine binding sites. *Biochemistry* **35**: 16735–16745
- Hofmann H, Schmidt HHHW (1995) Thiol dependence of nitric oxide synthase. *Biochemistry* **34**: 13443–13452
- Hurshman AR, Krebs C, Edmondson DE, Huynh BH, Marletta MA (1999) Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen. *Biochemistry* **38**: 15689–15696
- Lee SW, Lee HW, Chung HJ, Kim YA, Kim YJ, Hahn Y, Chung JH, Park YS (1999) Identification of the genes encoding enzymes involved in the early biosynthetic pathway of pteridines in *Synechocystis* sp. PCC 6803. *FEMS Microbiol Lett* **176**: 169–176
- Leutwiler LS, Brandt M (1983) Absence of significant light-induced changes in cAMP levels in sporangiophores of *Phycomyces blakesleeanus*. *J Bacteriol* **153**: 555–557
- Maier J, Ninnemann H (1995a) Biosynthesis of pteridines in *Neurospora crassa*, *Phycomyces blakesleeanus* and *Euglena gracilis*: detection and characterization of biosynthetic enzymes. *Photochem Photobiol* **61**: 43–53
- Maier J, Ninnemann H (1995b) Inhibition of light-dependent photomorphogenesis of sporangiophores

- from *Phycomyces blakesleeanus* by application of pteridine biosynthesis inhibitors. *Photochem Photobiol* **61**: 206–209
- Morita H, Yoshikawa H, Sakata R, Nagata Y, Tanaka H** (1997) Synthesis of nitric oxide from the two equivalent guanidino nitrogens of L-arginine by *Lactobacillus fermentum*. *J Bacteriol* **179**: 7812–7815
- Ninnemann H, Maier J** (1996) Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photocondiation of *Neurospora crassa*. *Photochem Photobiol* **61**: 393–398
- Raman CS, Li HY, Martasek P, Kral V, Masters BSS, Poulos TL** (1998) Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell* **95**: 939–950
- Ribeiro EA, Cunha FQ, Tamashiro WMSC, Martins IS** (1999) Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. *FEBS Lett* **445**: 283–286
- Riethmüller C, Gorren ACF, Pitters E, Hemmens B, Habisch HJ, Heales SJR, Schmidt K, Werner ER, Mayer B** (1999) Activation of neuronal nitric-oxide synthase by the 5-methyl analog of tetrahydrobiopterin: functional evidence against reductive oxygen activation by the pterin cofactor. *J Biol Chem* **274**: 16047–16051
- Rockel P, Rockel A, Wildt J, Segschneider H-J** (1996) Nitric oxide (NO) emission by higher plants. In O Van Cleemput, G Hofmann, A Vermoesen, eds, *Progress in Nitrogen Cycling Studies*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 603–606
- Son JK, Rosazza JPN** (2000) Cyclic guanosine-3',5'-monophosphate and biopteridine biosynthesis in *Nocardia* sp. *J Bacteriol* **182**: 3644–3648
- Thöny B, Auerbach G, Blau N** (2000) Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem J* **347**: 1–16
- Thornton RM** (1973) New photoresponses of *Phycomyces*. *Plant Physiol* **51**: 570–576
- Tsolakis G, Parashi E, Galland P, Kotzabasis K** (1999) Blue light signaling chains in *Phycomyces*: phototransduction of carotenogenesis and morphogenesis involves distinct protein kinase/phosphatase elements. *Fungal Genet Biol* **28**: 201–213
- Werner-Felmayer G, Golderer G, Werner ER, Gröbner P, Wachter H** (1994) Pteridine biosynthesis and nitric oxide synthase in *Physarum polycephalum*. *Biochem J* **304**: 105–111
- Wildt J, Kley D, Rockel A, Rockel P, Segschneider H-J** (1997) Emission of NO from several higher plant species. *J Geophys Res D* **102**: 5919–5927
- Yamasaki H, Sakihama Y** (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: *in vitro* evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett* **468**: 89–92
- Yamasaki H, Sakihama Y, Takahashi S** (1999) An alternative pathway for nitric oxide production in plants: new features of an old enzyme. *Trends Plant Sci* **4**: 128–129
- Zafariou O, McFarland M, Bromund R** (1980) Nitric oxide in seawater. *Science* **207**: 637–639