

# *Physarum* nitric oxide synthases: genomic structures and enzymology of recombinant proteins

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*Physarum polycephalum* expresses two closely related, calcium-independent NOSs (nitric oxide synthases). In our previous work, we showed that both NOSs are induced during starvation and apparently play a functional role in sporulation. In the present study, we characterized the genomic structures of both *Physarum* NOSs, expressed both enzymes recombinantly in bacteria and characterized their biochemical properties. Whereas the overall genomic organization of *Physarum* NOS genes is comparable with various animal NOSs, none of the exon–intron boundaries are conserved. Recombinant expression of clones with various

N-termini identified N-terminal amino acids essential for enzyme activity, but not required for haem binding or dimerization, and suggests the usage of non-AUG start codons for *Physarum* NOSs. Biochemical characterization of the two *Physarum* isoenzymes revealed different affinities for L-arginine, FMN and 6R-5,6,7,8-tetrahydro-L-biopterin.

**Key words:** arginine, flavin, haem, nitric oxide synthase (NOS), *Physarum polycephalum*, 6R-5,6,7,8-tetrahydro-L-biopterin (H<sub>4</sub>-bip).

## INTRODUCTION

Nitric oxide, a signalling and cytotoxic/cytoprotective agent, plays a key role in regulating the nervous, immune and cardiovascular systems of animals. It is synthesized by NOS (nitric oxide synthase; EC 1.14.13.39), which occurs as three isoforms and genes, i.e. NOS1, NOS2 and NOS3, also termed nNOS (neuronal NOS), iNOS (inducible NOS) and eNOS (endothelial NOS) respectively. In addition, a number of tissue- and development-specific splice variants encoding variant NOS proteins have been identified [1–4]. All three NOS isoenzymes are part of a complex network regulating nitric oxide production in health and disease (for recent reviews, see [5–9]).

NOSs oxidize L-arginine to L-citrulline and nitric oxide in an NADPH-dependent reaction via forming N<sup>ω</sup>-hydroxy-L-arginine as an enzyme-bound intermediate [10,11]. The enzymes consist of an N-terminal oxygenase domain binding haem and a C-terminal reductase domain binding FMN, FAD and NADPH. The two domains are linked through a calmodulin-binding motif. For activity, NOSs are required to form a homodimer leading to the formation of high-affinity binding sites for H<sub>4</sub>-bip (6R-5,6,7,8-tetrahydro-L-biopterin) and L-arginine in each oxygenase domain, thus allowing for the electron transfer between flavin and haem groups (reviewed in [12]). Detailed biochemical and crystallographic studies revealed structural features of the three isoenzymes underlying the observed differences in dimer formation and stability caused by L-arginine and H<sub>4</sub>-bip [13]. In addition to stabilizing the NOS dimer and affecting the haem spin state as well as the affinity of NOS for its substrate, H<sub>4</sub>-bip also serves as an electron/proton donor for nitric oxide formation, thereby giving rise to an H<sub>4</sub>-bip radical [14–16]. Thus the role of H<sub>4</sub>-bip in NOS is unique as compared with aromatic amino acid hydroxylases, the classical H<sub>4</sub>-bip-dependent enzymes, where the

cofactor activates oxygen by transferring two electrons, thereby being oxidized and enzymatically recycled after each catalytic turnover (reviewed in [14,15]). Under conditions of limited H<sub>4</sub>-bip availability, NADPH oxidation becomes uncoupled from nitric oxide synthesis, thus leading to O<sub>2</sub><sup>−</sup>/H<sub>2</sub>O<sub>2</sub> production [11], a process involved in certain clinical settings such as endothelial dysfunction [15].

So far, the first and only NOS isolated and cloned from a non-animal species was found in *Physarum polycephalum* [17,18]. While many bacteria contain proteins with sequences similar to mammalian NOS oxygenase domains, these are not full-length NOSs but lack the reductase domain essential for nitric oxide production (for a review, see [11]). A defined functional role for these bacterial NOS oxygenase-like proteins, which are often termed bacterial NOS, is the nitration of tryptophan [19]. *P. polycephalum* is a unicellular model organism expressing two NOS isoforms (A and B) that share 82% amino acid identity and are expressed in parallel in the organism throughout various developmental stages. Starvation strongly induces expression of both NOSs, which appears to be a prerequisite to obtain sporulation competence, a process that also involves nitric oxide-sensitive guanylate cyclase [18]. The overall sequence identity to mammalian NOSs is less than 39%. Nevertheless, binding motifs for flavins, NADPH, H<sub>4</sub>-bip, calmodulin and caveolin are highly conserved in *Physarum* NOSs and both proteins resemble mammalian iNOS lacking the spacer sequence conferring calcium dependence [18].

In the present study, we characterized the genomic structures of both *Physarum* NOSs and set up bacterial overexpression of recombinant proteins. This allowed purification and biochemical characterization of *Physarum* NOSs as well as identification of the minimal N-terminal sequence being essential for activity.

Abbreviations used: NOS, nitric oxide synthase; iNOS, inducible NOS; TB, Terrific broth; DTE, dithioerythritol; H<sub>4</sub>-bip, 6R-5,6,7,8-tetrahydro-L-biopterin; LB, Luria–Bertani; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported will appear in GenBank<sup>®</sup>, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers DQ835529, DQ845107, DQ835525, DQ835527, DQ835526 and DQ825528.

## EXPERIMENTAL

### Cultivation of *P. polycephalum*

Strain M<sub>3</sub>b, a Wis 1 isolate used initially for purifying and cloning of *Physarum* NOS, was grown in a semi-defined medium as detailed previously [18]. The apogamic haploid strain LU352 was kindly provided by Professor Dr Wolfgang Marwan (Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany) and grown as described in [20]. Amoebae were generated and grown as described in [21].

### RNA isolation and quantitative PCR

RNA from LU352 amoebae and starved macroplasmidia was isolated using the RNeasy plant mini kit (Qiagen). *Physarum* NOS form A and B mRNAs were quantified using Taqman technology. Sequences for probes and primers were for NOS form A: 5'-ACGCGCACACAGCCAAGAAACG-3' (probe), 5'-CATCCCCGAAACTGTTGCTC-3' (forward primer), 5'-GCA-GTCCGTGGTAGCAACCT-3' (reverse primer); for NOS form B: 5'-CGCAGCGTGCTATCAGCCAGA-3' (probe), 5'-CCAG-AAGTACTACTATCCATCAGAAATC-3' (forward primer), 5'-GCGGCAATCAGATGTGGATA-3' (reverse primer). For a reference, 19 S RNA was quantified as described in [18].

### DNA isolation

DNA from frozen M<sub>3</sub>b microplasmidia suspended in lysis buffer was isolated using the DNeasy plant maxi kit (Qiagen) according to the manufacturer's instructions and precipitated using sodium acetate and ethanol. DNA was further purified using the UltraPure reagent (Gibco) and precipitated once more with sodium acetate and ethanol.

### PCR, genome walking, cloning and library screening

Genomic structures of *Physarum* NOSs were elucidated using a combination of various techniques. First, PCR primer pairs close to exon/exon boundaries of mammalian NOSs were synthesized using *Physarum* NOS cDNA sequences [18] [GenBank<sup>®</sup> Nucleotide Sequence Database accession numbers AF145041 (form A) and AF145040 (form B)]. Those regions that could not be identified by this approach were identified by genome walking using the Universal GenomeWalker kit from Clontech according to the manufacturer's instructions. Herculase Hotstart DNA polymerase (Stratagene) generating an A-overhang was used for PCR and genome walking, and the generated products were then cloned into TOPO TA cloning vector (Invitrogen). Finally, most gaps could be closed by screening a genomic library [produced by Professor Tim Burland (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, U.S.A.) and kindly provided by Professor Jonatha Gott (Center for RNA Molecular Biology, Case Western Reserve University, Cleveland, OH, U.S.A.)] using standard procedures. The library had been prepared from the LU352 strain by the customer service of Stratagene by using the LambdaZap vector.

### Sequencing and data analysis

Sequencing of clones and plasmids was done by the custom service of Microsynth (Balgach, Switzerland). Sequence data were analysed using the Wisconsin Sequence Analysis Package version 10.3 by the Genetics Computer Group (Accelrys, Cambridge, U.K.).

### Plasmids for the overexpression of *Physarum* NOSs

*Physarum* calmodulin cDNA (GenBank<sup>®</sup> accession number AB022702) was amplified using PCR and cloned into site 2 of the pET-Duet1 vector (Novagen) using EcoRV/XhoI restriction sites. *Physarum* NOS form A cDNA (AF145041, cDNA library clone) was then cloned into site 1 of the pET-Duet1 vector using SalI/NotI restriction digestion. This sequence included 104 amino acids upstream of the putative start methionine [18], 11 amino acids stemming from the original Bluescript SK<sup>-</sup> vector, and 25 amino acids from the pET-Duet1 vector including a His tag. Alternatively, *Physarum* NOS form B (AF145040) was cloned into the second cloning site of the pET-Duet1 vector using SacI/NotI restriction digestion. This cDNA started at the putative start methionine [18] and also contained an N-terminal His tag. Various N-termini of *Physarum* NOS form A were generated by the introduction of an additional SalI site at the desired position (QuikChange<sup>®</sup> II kit; Stratagene), cutting by SalI and re-ligating. Mutations were introduced by site-directed mutagenesis (QuikChange<sup>®</sup> II kit). For elongating the N-terminus of *Physarum* NOS form B, 155 bp of the genomic sequence 5' to the putative start ATG were introduced using the Infusion cloning kit (Clontech). This region was confirmed to be identical with the cDNA 5'-region generated by RACE (rapid amplification of cDNA ends) PCR (see below). This clone was further modified by introducing an additional SacI restriction site at the desired position (QuikChange<sup>®</sup> II kit), cutting by SacI and re-ligating, thus leading to recombinant proteins with various N-termini. In addition, we introduced an N-terminal StrepTag [22] using the QuikChange<sup>®</sup> II protocol into one form of physnosa (A1) and its almost inactive mutant (A1LD) to allow the preparation of homogeneous, active proteins.

### 5' Elongation of cDNA clones

In order to elongate 5' sequences of the previously published *Physarum* NOS form A and B cDNA clones [18], the SMART RACE cDNA amplification kit (Clontech) was used.

### Bacterial overexpression of *Physarum* NOSs and preparation of homogenates

For overexpression, pET-Duet1 expression plasmids (see above), grown in NovaBlue (Novagen) cells and purified by the SNAP midi prep plasmid preparation kit (Invitrogen), were transformed into TUNER DE3 cells (Novagen). Bacterial starter cultures (5 ml) from single colonies were grown in LB (Luria-Bertani) broth (Difco) with ampicillin (50 µg/ml) at 37 °C and 220 rev./min to an attenuation at 600 (*D*<sub>600</sub>) of 0.8, and then pelleted and diluted in 15 ml of fresh TB (Terrific broth) without ampicillin but supplemented with 1.5 % (v/v) ethanol in order to increase recovery of soluble protein by induction of a heat-shock response [23]. Aliquots for determining NOS activity at zero time were taken and cultures were supplemented with 450 µM 5-aminolaevulinic acid (Sigma A-7793) and 3 µM riboflavin (Sigma R-4500), in order to provide sufficient haem and flavins, and 50 µM IPTG (isopropyl β-D-thiogalactoside). Bacteria were grown for a further 24 h at 20 °C at 220 rev./min in the dark for NOS expression. For purification, this protocol was scaled up to 30 ml starter cultures and 400 ml expression cultures. TB was used instead of LB for expression cultures. Bacteria from these cultures were resuspended in 40 ml of buffer A [50 mM Tris/HCl, pH 8.0, containing 10 % (v/v) glycerol, 25 µM FAD, 25 µM FMN, 50 µM L-arginine, 5 µM H<sub>4</sub>-bip (Schircks Laboratories, Jona, Switzerland), 5 mM DTE (dithioerythritol), 1 mM PMSF and 0.2 mg/ml lysozyme]. After incubation for 20 min at 25 °C

at 200 rev./min, cells were homogenized using a French press [20 000 lbf/in<sup>2</sup> (1 lbf/in<sup>2</sup> = 6.9 kPa) twice] and mixed with 110 ml of buffer B [50 mM Tris/HCl, pH 8.0, containing 10% (v/v) glycerol, 100  $\mu$ M L-arginine, 10  $\mu$ M H<sub>4</sub>-bip and 5 mM DTE].

### Purification of recombinant *Physarum* NOSs

All steps were carried out at 4 °C and all elution buffers contained 100  $\mu$ M L-arginine and 10  $\mu$ M H<sub>4</sub>-bip. Protein was precipitated using 35.6 g of ammonium sulfate (45% saturation) and stirring for 1 h followed by centrifugation at 48 000 g for 20 min. The pellet was then resuspended in 20 ml of buffer B by stirring for 1 h and undissolved material was collected by centrifugation at 48 000 g for 20 min. The supernatant was dialysed twice for 2 h against 1 litre of buffer B using a 50K membrane (Pierce). The dialysate was adjusted to 0.15 M NaCl and then incubated (at 4 °C) with 2',5'-ADP-Sepharose 4B (Amersham Biosciences) for 1 h using a rocking mixer at a ratio of 1 part dialysate protein to 2 parts ADP-Sepharose (w/w). After washing twice with 10 ml of buffer B containing 0.15 M NaCl and washing once with 10 ml of buffer B, protein was eluted from ADP-Sepharose with 1 ml of buffer B containing 10 mM NADPH. Protein determination at various steps was performed using the Bradford Protein Assay reagent from Bio-Rad. To obtain homogeneous active protein, the dimeric fractions of NOS were then collected by gel filtration on a Superose 12 10/300 GL column (GE Healthcare), and were finally purified using the StrepTag affinity protocol [22].

### Native *Physarum* NOS

Native *Physarum* NOS was purified from microplasmidia as described in [18].

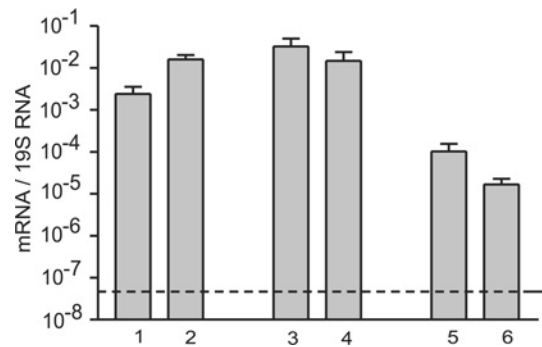
### NOS assay

Radiometric citrulline NOS assay was carried out as described previously [17] using buffer B (see above) supplemented with 2 mM NADPH as the assay buffer. Briefly, bacterial homogenates (bacteria from 2 ml of culture were diluted in 700  $\mu$ l of buffer A, incubated for 10 min at 25 °C and 1000 rev./min in an Eppendorf thermomixer and finally mixed for 10 s with an Ultra-Turrax homogenizer) or partially purified NOS (5–20  $\mu$ l per assay corresponding to 10–15  $\mu$ g of protein in the case of bacterial homogenates or ~1  $\mu$ g of protein when using purified enzyme), were incubated in assay buffer containing 40 000 c.p.m. of HPLC-purified <sup>3</sup>H-L-arginine (Amersham Biosciences) in a final volume of 200  $\mu$ l for 10 min at 25 °C on an Eppendorf thermomixer. The reaction was then stopped, separated on Dowex 50 W columns and the flow-through was subjected to scintillation counting. In some assays, concentrations of cofactors or substrate were varied. IC<sub>50</sub>, EC<sub>50</sub> and K<sub>M</sub> values were calculated with SigmaPlot 9.0 software (Systat Software).

For comparison of nitric oxide formation with NADPH consumption, a kinetic microtitre-plate assay based on measurement of nitric oxide formation by binding to myoglobin (monitored by UV absorption at 405 nm) combined with measurement of NADPH consumption (monitored at 340 nm) was performed at 25 °C as described in [24], using a PowerWaveX thermostatically controlled kinetic microplate reader (BioTek Instruments, Winooski, VT, U.S.A.).

### Gel electrophoresis

Proteins were separated on SDS/polyacrylamide gels. Gels were stained using Coomassie Blue or silver stain according to standard procedures and scanned using an ImageScanner (Amersham Biosciences). For quantification, gels were stained with Deep Purple



**Figure 1** Quantification of *Physarum* NOS form A and form B mRNA levels

Total RNA was isolated from starved macroplasmidia of the diploid M<sub>3</sub>b and the haploid LU352 strain as well as from LU352 amoebae, and the two NOS forms were quantified by Tagman technology in relation to 19 S RNA. The means  $\pm$  S.D. for three independent experiments are shown. The dashed line indicates the detection limit of the method. Bar 1, M<sub>3</sub>b macroplasmidia, *Physarum* NOS form A; bar 2, M<sub>3</sub>b macroplasmidia, *Physarum* NOS form B; bar 3, LU352 macroplasmidia, *Physarum* NOS form A; bar 4, LU352 macroplasmidia, *Physarum* NOS form B; bar 5, LU 352 amoebae, *Physarum* NOS form A; bar 6, LU352 amoebae, *Physarum* NOS form B.

Total Protein Stain (Amersham Biosciences) and fluorescence was scanned by a Typhoon 9410 scanner (Amersham Biosciences) at 532 nm and evaluated using ImageQuant software (Amersham Biosciences).

### Determination of haem content by HPLC

Haem content of pure forms A1 and A1LD was checked by HPLC according to the method of Bonkovsky et al. [25] by reversed-phase HPLC separation of porphyrins and UV detection at 405 nm. The suitability of the application of this method for the analysis of protein-associated haem was confirmed with equine myoglobin (Serva, Heidelberg, Germany) yielding  $0.9 \pm 0.1$  haem per myoglobin.

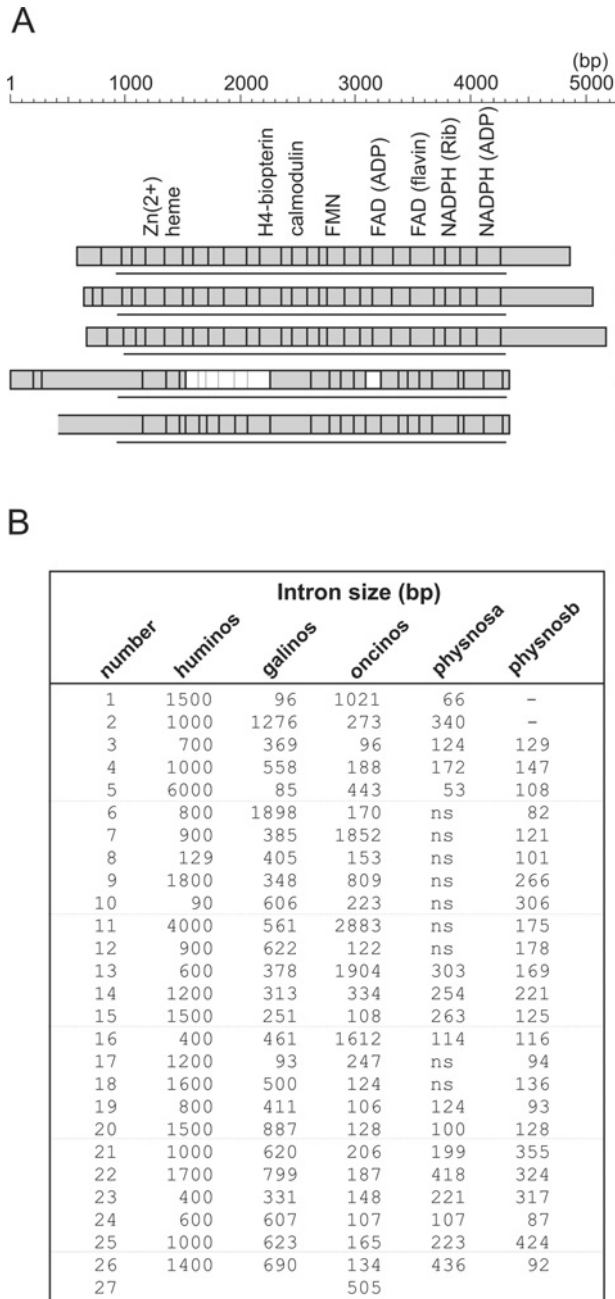
## RESULTS

### Expression of *Physarum* NOS form A and B mRNA

We first wanted to clarify whether the two highly related *Physarum* NOS isoforms [18] are the products of two genes or of two alleles of the same gene, as was shown for the *Physarum hapP* gene [26]. For this purpose, we used the haploid *Physarum* strain LU352 and checked for the expression of *Physarum* NOS form A and B mRNA. Both mRNAs were expressed in LU352 macroplasmidia starved for 5 days in a way comparable with the diploid M<sub>3</sub>b strain, which we used in our initial studies [17,18]. Moreover, LU352 amoebae also expressed both mRNAs (Figure 1).

### Genomic structures of both *Physarum* NOSs

We then studied the genomic structures of both *Physarum* NOSs. As can be seen from Figure 2(A), 25 exons could be identified for *Physarum* NOS form B. Form A has two additional 5' exons as was determined by extending the cDNA into the 5' direction, a strategy that was not successful for the form B cDNA where only a shorter 5' extension could be achieved starting within an exon (Figure 2A). On the other hand, the exact number of exons for NOS form A could not be determined since some internal genomic regions were inaccessible to sequencing (Figure 2A). However, since all of the 21 exon/intron boundaries that could be determined were conserved between the two *Physarum* NOSs, it is conceivable that the lacking 5 boundaries



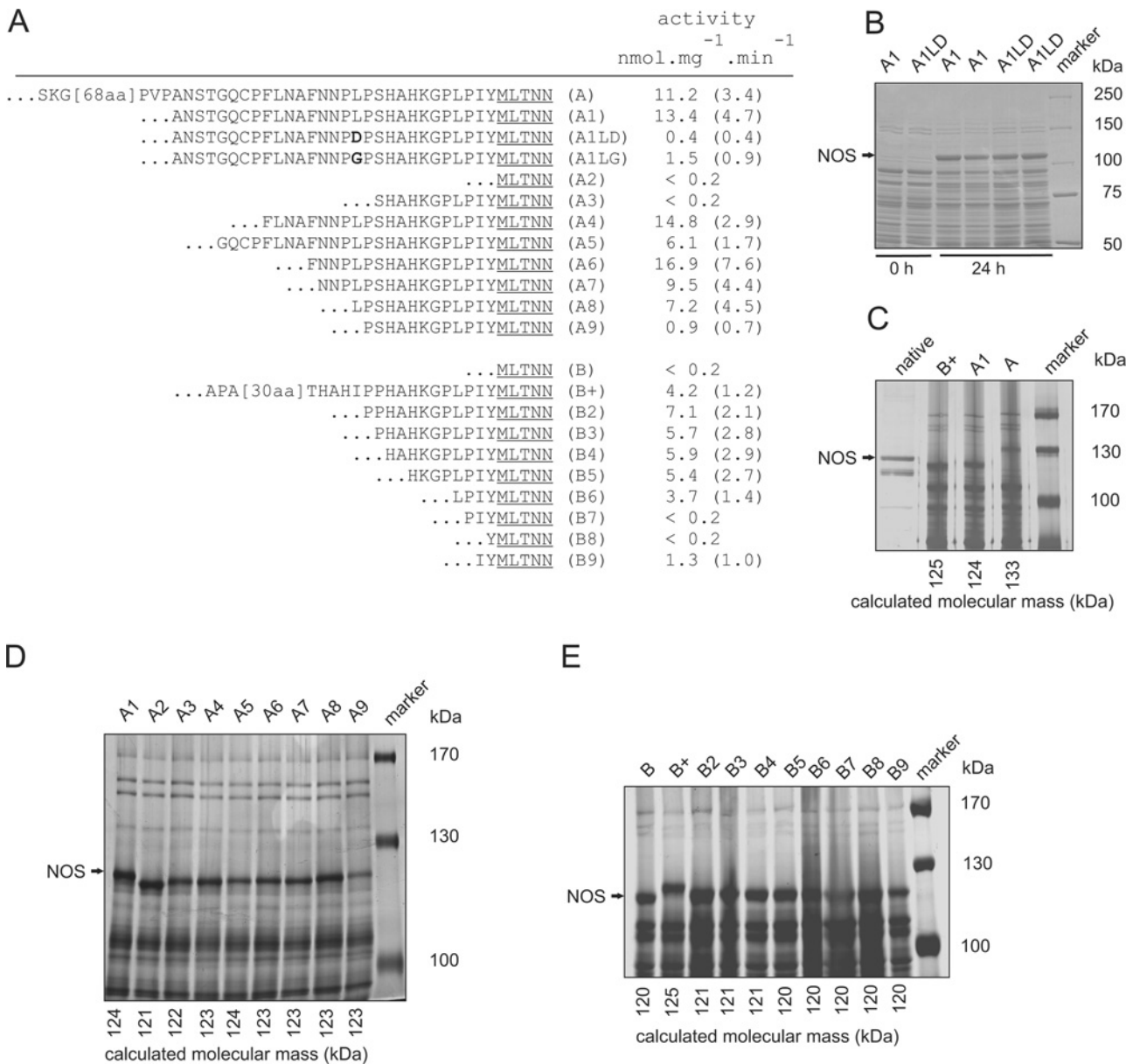
**Figure 2** Genomic organization of *Physarum* NOSs

(A) Exons in cDNAs of human iNOS (bar 1, 27 exons; GenBank® accession numbers for genomic sequence: NT\_010799.14; cDNA: NM\_000625), chicken iNOS (bar 2, 28 exons; genomic: NW\_060634.1[Gga19\_WGA443\_1; cDNA: NM\_204961), trout iNOS (bar 3, 27 exons; genomic: AJ295231; cDNA: AJ295230), *Physarum* NOS form A [bar 4, 27 exons deduced from the comparison of forms A and B; see the main text for details; genomic: DQ835529 (5'-region, 6 exons), DQ845107 (internal region, 5 exons), DQ835525 (3'-region, 9 exons); cDNA: DQ835526] and form B (bar 5, 27 exons deduced from the comparison of forms A and B; see the main text for details; genomic: DQ835527; cDNA: DQ835528). Lines below each bar indicate the translated region of the cDNA. Positions of various binding sites are marked as have been published previously [43–47]. For *Physarum* NOS form A, some internal exon/intron boundaries could not be determined by sequencing but were deduced from form B. This region is shown in white and the five deduced exon/exon boundaries are indicated by a thin line (bar 4). Also, exon 18 could not be fully sequenced. For *Physarum* NOS form B, the 5'-region could only be partially extended and starts within an exon (bar 5, indicated by interrupting the margin at the 5'-end), which was assigned number 3 as deduced from homology to NOS form A. (B) Intron sizes (bp) [GenBank® accession numbers for genomic and cDNA sequences are given under (A)] from iNOS genes of human (huminos), chicken (galinos), trout (oncinos) and the two *Physarum* NOSs (physnosa; physnosb). ns, not sequenced; –, not identified.

are also conserved. We therefore concluded that the two genes consist of 27 exons. For comparison, the gene structures of iNOS from human, chicken and trout are similarly organized into 27, 28 and 27 exons respectively (Figure 2A). However, none of the exon/intron boundaries are conserved between *Physarum* and the animal iNOSs (Figure 2A). Moreover, the size of introns in the *Physarum* genes is considerably smaller compared with animal NOSs, particularly the human iNOS gene (Figure 2B). As in other animal iNOSs, the reading frame of the *Physarum* NOSs starts in exon 3 (Figure 2A). The current version of the two gene structures was submitted to GenBank® Nucleotide Sequence Database (DQ835529, DQ845107 and DQ835525 for NOS form A and DQ835527 for NOS form B). The extended cDNA sequences were also submitted: DQ835526 for NOS form A and DQ825528 for NOS form B. The final gene structures will become available soon through the ongoing *Physarum* genome project (<http://www.genome.gov/12511858> and <http://genome.wustl.edu/genome.cgi?GENOME=Physarum%20polycephalum>).

### Recombinant overexpression of *Physarum* NOSs in *Escherichia coli*

Our next goal was to set up efficient recombinant overexpression of *Physarum* NOSs in *E. coli*. In our previous work, we could functionally express *Physarum* NOS form A in a baculovirus system [18], but expression levels were low and did not allow purification of recombinant protein. It is known that high-level expression of mouse iNOS requires co-expression of calmodulin [27]. We therefore cloned *Physarum* calmodulin and co-expressed it together with *Physarum* NOS forms A and B respectively using the pET-Duet1 vector. In contrast with the original *Physarum* NOS form A cDNA library clone (AF145041) that still contained 104 additional amino acids 5' to the putative start methionine [18] and a variant A1, which contained only 29 amino acids 5' to the putative start, the clone starting at the predicted methionine (A2) had no enzyme activity (Figure 3A), although it expressed the same amount of protein (Figure 3D). Elimination of vector-derived sequences including the His tag resulted in disappearance of both protein and activity, presumably due to proteolytic degradation (results not shown). Systematically shortening the N-terminus 5' to the assumed start methionine then revealed that 13 amino acids were sufficient to achieve a clone with enzyme activity (variant A8), which dropped dramatically when one amino acid (leucine) was deleted (variant A9) and became undetectable when deleting a further amino acid (variant A3) (Figure 3A). Exchanging this leucine residue in A1 for aspartic acid or for glycine (variants A1LD and A1LG) destroyed enzyme activity almost completely (Figure 3A), whereas it did not affect the amount of overexpressed protein (Figure 3B), and did not affect either the haem content of homogeneously purified proteins (see below) or the monomer/dimer ratio on gel filtration (results not shown). Expressing *Physarum* NOS form B using the predicted start methionine (clone B) also resulted in an inactive protein, whereas addition of 50 amino acids 5' to this methionine (clone B+) yielded active protein. Varying this N-terminus showed that only four additional amino acids were sufficient for full activity of *Physarum* NOS form B (Figure 3A). As in form A, removal of a hydrophobic amino acid (leucine for truncation from B6 to B7 and isoleucine for truncation from B9 to B8) leads to a decrease in activity, although the amount of NOS protein in inactive clones is comparable with that of active clones (Figure 3E). These results clearly indicated that the N-terminus of both *Physarum* NOSs as predicted previously [18] may be too short. In line with this, comparison of the proteins expressed from the A and B clones and their variants with native *Physarum* NOS showed that the molecular masses of several of these proteins were smaller than



**Figure 3 Overexpression of recombinant *Physarum* NOSs**

(A) N-termini of various clones of *Physarum* NOS form A and form B and their activity in whole homogenates. Values are means for five to ten independent experiments ( $\pm$  S.D). The previously predicted start of translation is underlined [18]. All overexpressed proteins start with a His tag sequence (MGSSHHHHHSQDPNSSARLQVD), indicated by an ellipsis. Clone A has an additional GIDKLDIEFRSKG sequence from the Bluescript SK<sup>-</sup> vector inserted between the His tag and the *Physarum* NOS sequence. aa, amino acids. (B) Overexpression of A1 and its A1LD mutant. Homogenates (15  $\mu$ g) harvested at times 0 and 24 h after induction were separated on SDS/6% polyacrylamide gels and stained with Coomassie Blue. For the induced cultures of A1 and A1LD, samples from two different expressions are shown. (C) Comparison of overexpressed *Physarum* NOS from clones A, A1 and B+ (3  $\mu$ g of homogenate protein per lane) with native NOS purified from *Physarum* microplasmidia (0.3  $\mu$ g per lane) [18]. Bands were detected by silver staining. Sequencing demonstrated that the second band in native *Physarum* NOS is a proteolytic fragment of the first one, and that both bands contained a mixture of *Physarum* NOS form A and *Physarum* NOS form B [18]. (D) Silver-stained gel of various A clones (homogenates, 3  $\mu$ g of protein per lane). (E) Silver-stained gel of various B clones (homogenates, 3  $\mu$ g per lane). Molecular masses (kDa) of several recombinant proteins were calculated for proteins containing the His tag and are given below the lanes of (C-E).

the native protein, which is a mixture of both isoforms [18] (Figure 3C).

Checking the elongated *Physarum* NOS cDNAs showed that form A cDNA (DQ835526) contained a stop codon 516 bp upstream of the predicted start ATG but no additional methionine codon, whereas the form B cDNA (DQ835528) had no stop codon over the whole cloned 740 bp region 5' to the putative start codon but encoded for an additional methionine 69 amino acids upstream of the predicted start [18] (Figure 4A). Starting with

this methionine, the predicted form B protein (Figure 4B) is still smaller than the native *Physarum* NOS (Figure 3C). Searching the cDNA sequence upstream of the putative start codon showed that both *Physarum* NOSs have several putative non-AUG start codons [28,29]. An isoleucine residue conserved in both *Physarum* NOSs is found 88 (form A) or 90 (form B) amino acids upstream (Figure 4A). However, the Kozak sequence for these codons is less optimal [30] than that of other nearby residues (Figure 4B): for *Physarum* NOS form A cDNA, this is the isoleucine codon

## A

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physnosa ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ 0
physnosb YPIMPNTPHA APHDSPASVL PRSSPRTTTP SPPPIPIRPH PITPPWPKRE NALSSLPKRE 60

physnosa ~~~~~~*TS KKKSSAQFPV LPTSFPILPG GESKALTSPR PSTSSNTTSS FPILPNSNAE 52
phasnosb SAFHETSGIT RRKDTPSTPT HNSNFPILP. .STRAITHPP TPPADTPAT QTTPPAQNLP 118
                                     ↓-97           ↓-88

physnosa P.SKSAYPV. .LPNGETSK GSARPAIPTL P...TSFPIL PS..ADSSHA PARTTPRDVA 103
physnosb PLNFSGLPAS DESASSETSG ITRRNHSPST PAHFSNYPI PSSRASTPTT STTTTANPPP 178
                                     ↑-108           ↑-90           ↑-69

physnosa PAHGTNYPIL PSASQELVPA APSHSHAAPN PAHAPHPVPA NSTGQCPFLN AFNNP.... 158
physnosb PSTAAHPPTN PTLATEI..S APAQN...PN PFHAPHPASS LSSGQCPFLN AFSTDTTHAH 233

physnosa LPSHAHKGPL PIYMLTNN 176
physnosb IPPHAHKGPL PIYMLTNN 251

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## B

Physarum NOS form	putative start	amino acid position	nucleotide position	number of amino acids	molecular mass (Da)	Kozak sequence
A	Met	1 (72)	844	1055*	118180, 89*	AUCUACaugU
A	Ile	-88 (84)	580	1143	127231, 88	UUC <b>CC</b> CaucC
A	Ile	-97 (75)	553	1152	128185, 98	CCUG <b>CC</b> CaucC
B	Met	1 (247)	740	1046*	117566, 52*	AUCUACaugC
B	Met	-69 (178)	533	1115	124814, 32	CCU <b>CC</b> CaugC
B	Ile	-90 (157)	491	1136	126827, 70	UAU <b>CC</b> GaucC
B	Ile	-108 (139)	416	1154	128891, 94	UCC <b>GG</b> CauuA

**Figure 4** Putative translation start sites in *Physarum* NOSs

(A) Amino acid sequence predicted from 5' extended cDNA clones of *Physarum* NOS form A (physnosa, GenBank® accession number DQ835526) and form B (physnosb, DQ835528). Conserved residues are shown in boldface. The previously predicted start of the protein [18] is framed. An additional methionine in form B is marked by a grey arrow, and putative alternative starts of the reading frames are indicated by black arrows. Numbers in italics refer to amino acid positions related to the previously predicted reading frames [18], and normal numbers refer to amino acids deduced from the 5' extended cDNA clones (DQ835526 and DQ835528). The asterisk denotes the stop found in the *Physarum* NOS form A cDNA. (B) Molecular mass for *Physarum* NOSs with various starts and the Kozak sequences of their putative start codons. Putative start codons are written in lower-case letters, whereas the context for translation is given in upper-case letters. The nucleotide positions as well as the amino acid positions given in parentheses refer to GenBank® sequences DQ835526 and DQ835528. Amino acid positions not in brackets refer to the previously predicted reading frame [18] starting with methionine 1. The asterisk indicates previously published data [18].

(ATT) at amino acid position -97; for *Physarum* NOS form B, an alternative start codon (ATT) with a nearly optimal context is encoding isoleucine in position -108. These sites yield proteins of nearly identical molecular mass (Figure 4B) that are comparable with that of native NOS (Figure 3C).

#### Enzymatic properties of recombinant *Physarum* NOSs

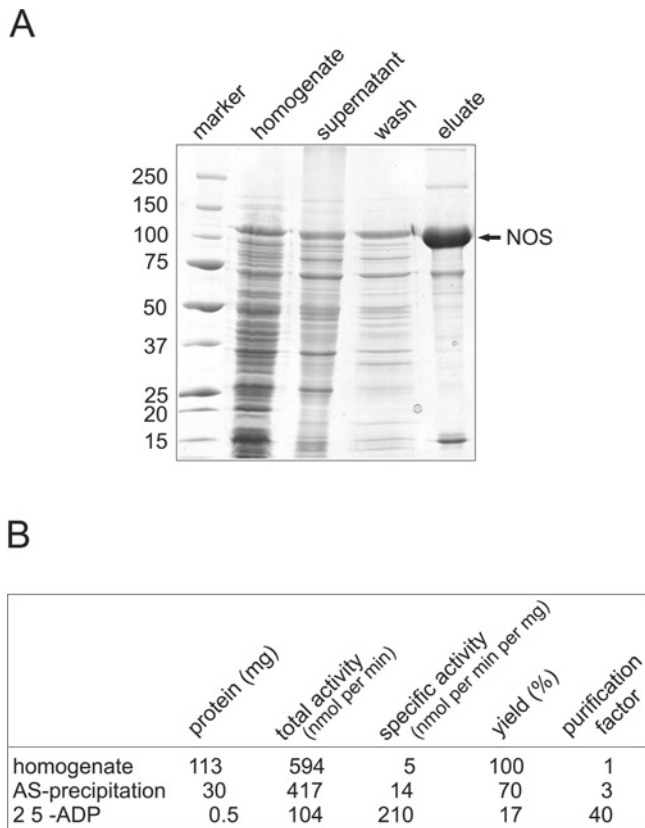
We then characterized enzymatic properties of recombinant *Physarum* NOSs. For this purpose, we used proteins expressed from the A1 and B+ clone partially purified by ammonium sulfate precipitation and subsequent ADP-Sepharose-affinity chromatography. This procedure yielded NOS with approx. 80% purity (cf. Figure 5A, last lane). The specific activity was  $244 \pm 47$  nmol · mg<sup>-1</sup> · min<sup>-1</sup> (form A, mean for three purifications ± S.D.) and  $253 \pm 47$  nmol · mg<sup>-1</sup> · min<sup>-1</sup> (form B, mean for four purifications ± S.D.). Figure 5 shows a typical purification of A1 recombinant protein. While both *Physarum* NOSs were comparably sensitive to NOS inhibitors and similarly dependent on NADPH

and FAD, and had similar dimer/monomer ratios of approx. 1:1 on gel filtration (Figures 6A and 6B), the  $K_M$  for L-arginine and the  $EC_{50}$  for FMN and H<sub>4</sub>-bip were clearly higher for NOS form A than for the form B protein (Table 1). A combined nitric oxide formation (determined by binding to myoglobin) and NADPH molecules consumption assay [24] showed partial decoupling of the enzyme preparations, with  $5.1 \pm 1.6$  NADPH molecules consumed in the formation of 1 nitric oxide molecule by *Physarum* NOS A and  $5.9 \pm 1.5$  NADPH molecules consumed per nitric oxide molecule in *Physarum* NOS B (mean ± S.D. for three determinations).

#### Haem content of homogeneous *Physarum* NOSs A1 and A1LD

To check whether or not the mutation of a residue in the N-terminus that led to a pronounced decrease in activity also affected haem binding and hence haem content of the active enzyme, we introduced into forms A1 and A1LD (see Figure 3A) a StrepTag, and further purified the tagged proteins by additional gel filtration, collection of the dimeric fractions and final purification





**Figure 5** Partial purification of recombinant *Physarum* NOS form A (clone A1)

(A) Coomassie-Blue-stained SDS/polyacrylamide gel (6–17% gradient) showing partial purification of recombinant *Physarum* NOS form A clone A1. The amount of total protein loaded per lane was 20  $\mu$ g. Homogenate, whole bacterial homogenate; supernatant, supernatant after loading the 2',5'-ADP-Sepharose 4B column; wash, first wash of 2',5'-ADP-Sepharose 4B with buffer B; eluate, protein eluted from 2',5'-ADP-Sepharose 4B with buffer B containing 10 mM NADPH. The ammonium sulfate precipitation step is not shown. (B) Activities, yield and purification factor of *Physarum* NOS form A clone A1 protein purified from 400 ml of bacterial culture (homogenate) by ammonium sulfate precipitation (AS precipitation) and 2',5'-ADP-Sepharose 4B affinity chromatography (2.5-ADP).

**Table 1** Enzymatic properties of recombinant *Physarum* NOSs

Partially purified recombinant *Physarum* NOS form A (clone A1) and form B (clone B+), each of them with an activity of approx. 250 nmol  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> (see text for data), were used for measuring substrate, cofactor and inhibitor effects. L-NIL, L-N6(1-iminoethyl)-lysine; L-NNA, N<sup>o</sup>-nitro-L-arginine; L-NMMA, N<sup>o</sup>-methyl-L-arginine. Values are means  $\pm$  S.D. for three to five independent experiments. Values in boldface are significantly different (Student's *t* test) between NOS form A and form B:  $K_M$  L-arginine,  $P < 0.001$ ; EC<sub>50</sub> H<sub>4</sub>-bip,  $P < 0.001$ ; EC<sub>50</sub> FMN,  $P < 0.02$ .

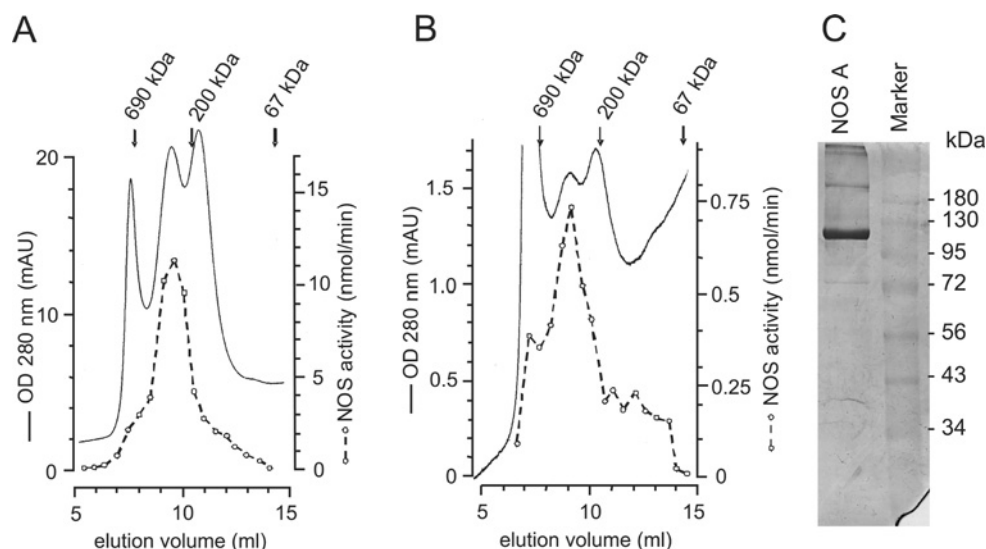
	Concentration ( $\mu$ M)	
	<i>Physarum</i> NOS A	<i>Physarum</i> NOS B
$K_M$ L-arginine	<b>66.7 <math>\pm</math> 14.5</b>	<b>26.8 <math>\pm</math> 9.7</b>
EC <sub>50</sub> H <sub>4</sub> -bip	<b>7.9 <math>\pm</math> 1.2</b>	<b>0.7 <math>\pm</math> 0.1</b>
EC <sub>50</sub> NADPH	57.2 $\pm$ 6.5	60.3 $\pm$ 13.8
EC <sub>50</sub> FAD	3.9 $\pm$ 1.6	2.1 $\pm$ 1.6
EC <sub>50</sub> FMN	<b>1.7 <math>\pm</math> 0.6</b>	<b>0.2 <math>\pm</math> 0.2</b>
IC <sub>50</sub> L-NIL	81.3 $\pm$ 7.8	76.7 $\pm$ 4.3
IC <sub>50</sub> L-NNA	25.7 $\pm$ 10.7	39.8 $\pm$ 20.3
IC <sub>50</sub> L-NMMA	45.2 $\pm$ 23.5	44.3 $\pm$ 19.2

using the StrepTag affinity column. This yielded a homogeneous protein (>95% SDS gel silver-stained; Figure 6C) free of *E. coli* impurities (minor impurities also contain the StrepTag as checked by Western blotting). Pure A1 (three preparations) had a specific activity of 585  $\pm$  144 nmol  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>, and a haem content of 0.41  $\pm$  0.14 haem per subunit, whereas pure A1LD (two preparations) displayed less than one-tenth of the activity (51  $\pm$  10 nmol  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>), but had a haem content (0.44  $\pm$  0.09 per subunit) comparable with A1 (Table 2).

## DISCUSSION

*P. polycephalum*, a member of the class/superclass Myxogastriidae (also termed myxomycetes or true slime molds), is the only so far identified non-animal species known to express NOS with the characteristics of mammalian iNOS [17]. Previously, we showed that this enzyme is induced by starvation of macroplasmidia and plays a crucial role in sporulation [18]. Cloning experiments indicated that *Physarum* contains two highly related calcium-independent NOSs (forms A and B, 82% amino acid identity) that are expressed in parallel throughout various developmental stages and have a similar molecular mass, as was not only deduced from the cDNA sequences but was underlined by the fact that purified native *Physarum* NOS was a mixture of both proteins that were not separated by SDS/PAGE [18]. While parallel expression of calcium-dependent and calcium-independent NOS isoforms within a certain cell type is common in animal species, the parallel occurrence of two versions of calcium-independent NOS has not been reported, at least to our knowledge. The previously used *Physarum* strain was the diploid M<sub>3</sub>b isolate. Thus it was possible that the two different cDNAs and proteins could stem from two alleles of the same gene, as it was described for the plasmodium-specific *hapP* mRNA that encodes proteins of unknown function differing by 9.6% in their predicted amino acid sequence [26]. We therefore checked for expression of *Physarum* NOS form A and form B in the haploid *Physarum* strain LU352 [23], the strain now being used for sequencing the *Physarum* genome (<http://www.genome.gov/12511858> and <http://genome.wustl.edu/genome.cgi?GENOME=Physarum%20polycephalum>). Quantifying *Physarum* NOS form A and form B expression in starved macroplasmidia of both strains showed comparable mRNA levels. Moreover, both mRNAs were also expressed in LU352 amoebae. Macroplasmidia of diploid strains develop after fusion of genetically different amoebae, but in the apogamic haploid strains like LU352 no such fusion is required [21,30]. Therefore expression of both NOS mRNAs in LU352 amoebae underlined the assumption that the two *Physarum* NOS forms stem from two genes rather than from two alleles of the same gene.

Studying the organization of these two genes revealed the high similarity of the overall structure of the *Physarum* NOS genes to those of NOS genes from higher animals including mammals. Intron size of *Physarum* NOSs is smaller than that of animal NOSs, which is in agreement with the smaller genome size of *Physarum* (300 Mb [30a,30b]) as compared with human (3038 Mb) or chicken (1200 Mb; <http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi>) [31]. None of the intron positions is conserved between *Physarum* and animal NOS genes, which is in line with the idea that introns are transposable elements that are acquired or also deleted during evolution (reviewed in [32]). Transposition of introns occurs very infrequently during evolution; for example, most orthologous genes between mouse and human, species that diverged at least 50 million years ago, have the same intron positions [32]. This gives some idea of the evolutionary distance between *Physarum* and higher animals and their early



**Figure 6** Gel filtration of purified *Physarum* NOSs

*Physarum* NOS isoforms (clones A1 and B+ respectively) carrying an N-terminal Strep tag were expressed together with *Physarum* calmodulin in *E. coli* using the pETDuet1 vector (see the Experimental section for details). A 200  $\mu$ l portion of purified protein was then loaded on to a Superose 12 10/300 GL gel filtration column and eluted with buffer B containing 0.15 M NaCl. Fractions were collected and NOS activity was determined by the radiometric citrulline assay. The solid line shows UV absorption (OD) at 280 nm, the dashed line shows NOS activity. Arrows indicate the elution volume of protein standards for gel filtration (thyroglobulin, 690 kDa;  $\beta$ -amylase, 200 kDa; albumin, 67 kDa). (A) *Physarum* NOS A; (B) *Physarum* NOS B; (C) silver-stained SDS gel showing the purity of Strep-tagged *Physarum* NOS A.

**Table 2** Original data for haem determination of pure *Physarum* NOS preparations

*Physarum* NOS clones A1 and A1LD containing an N-terminal Strep tag were co-expressed with *Physarum* calmodulin in *E. coli* with pETDuet1 and purified by ammonium sulfate fractionation, ADP-Sepharose-affinity chromatography, gel filtration and Strep-tag-affinity chromatography. Haem was determined in purified fractions by reversed-phase HPLC with UV detection. NOS activity was determined by the radiometric citrulline assay. Protein was determined by the Bradford assay using BSA as the standard. MM, calculated molecular mass. See the Experimental section for details of the method.

Sample	Batch	Haem ( $\mu$ M)	Protein (mg/ml)	MM (kDa)	Haem/subunit	NOS activity (nmol $\cdot$ mg <sup>-1</sup> $\cdot$ min <sup>-1</sup> )
NOSA1	1	2.57	1.30	126	0.25	768
NOSA1	2	7.47	2.32	126	0.41	417
NOSA1	3	4.71	1.02	126	0.58	569
NOSA1-LD	1	5.01	1.80	126	0.35	41
NOSA1-LD	2	4.16	1.00	126	0.52	61

divergence from a common ancestor (see below) where already some ancestral NOS had evolved. Once the final *Physarum* genome sequence is available, these speculations can be studied in more detail and it will also be possible to clarify whether the two *Physarum* NOS genes stem from single gene duplication or other evolutionary events.

Some further interesting aspects with regard to evolution become evident here. According to molecular phylogenetic data, *Physarum* is most closely related to the cellular slime moulds, the *Dictyostelidae* [33,34]. Together with other amoebae and slime mould classes, they are grouped as Amoebozoa, one of eight major groups composing the 'crown' of the phylogenetic tree of eukaryotes [33,35], thus indicating that these organisms cannot be considered as 'low' stages on the path to higher animals and fungi but have developed independently from a more ancient, not yet defined, root [35]. However, a more refined analysis of evolutionary relationships between *Physarum* and *Dictyostelium*, another model organism for cell biology, has to await the final *Physarum* genome. In any case, our results on NOS as well as previous observations concerning pteridine biosynthesis (see below) underline the diversity of *Physarum* and *Dictyostelium* despite the striking similarities of their life cycle, the occurrence

of mobile and stationary developmental stages, their habitat and their way of feeding as well as the fact that their protein sequences usually group on a common branch in phylogenetic analysis. First of all, *Dictyostelium discoideum* does not contain any gene similar to full-length NOS (as checked by BLAST-P search of the *Dictyostelium* genome with the *Physarum* NOSs; results not shown), whereas *Physarum* contains two genes with exon numbers comparable with those of higher animals (see Figure 2) and an even intron distribution characteristic of higher eukaryotes [36]. Secondly, *Dictyostelium* does not produce H<sub>4</sub>-bip like *Physarum* [17] but its stereoisomer tetrahydrodictyopterine [37], a pterin that cannot serve as a cofactor for mammalian NOS [38] or *Physarum* NOS (E. R. Werner, G. Golderer, P. Gröbner and G. Werner-Felmayer, unpublished work). Moreover, the genomic structures of GTP cyclohydrolase I, the first enzyme of H<sub>4</sub>-bip biosynthesis, differ significantly between the two species [39]: in accordance with its comparatively small genome of 34 Mb (<http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi>), *Dictyostelium* has only one 109 bp intron [40], whose location is conserved in *Physarum*, whereas the *Physarum* gene consists of 7 exons and thus resembles animal GTP cyclohydrolase I genes with some intron positions conserved in *Drosophila* or human. In addition,



alternative splicing of GTP cyclohydrolase I occurs in *Physarum* at a similar position to human [39]. Taken together, these findings suggest that *Physarum* may be more closely related to animals than *Dictyostelium* with respect of H<sub>4</sub>-bip and nitric oxide synthesis, and it underlines the value of *Physarum* as a model system for studying certain aspects of animal cell biology. In line with our observations, the recent analysis of *Physarum* expressed sequence tags [41] has revealed 895 genes of *Physarum* with similarities in other databases but not in the *Dictyostelium* protein database. In addition, approx. 9% of all detected genes are alternatively spliced in *Physarum*, whereas only approx. 0.2% of genes are alternatively spliced in *Dictyostelium* [41].

In the course of setting up recombinant expression of both *Physarum* NOSs in bacteria, we found that the previously predicted reading frame [18] was too short at its N-terminus. We concluded this not only from lack of activity of His-tagged expression constructs, but also from the smaller size of the recombinant proteins as compared with the native NOS purified from *Physarum* microplasmidia (Figure 3C). Addition of only a few amino acids (13 for NOS form A and four for NOS form B) to the N-terminus as deduced from the cDNAs led to expression of functional proteins. Producing 5'-extended cDNA sequences for both NOSs and checking for other possible translation starts revealed that only NOS form B had an upstream methionine within reasonable distance, whereas for NOS form A we found a stop codon and no additional AUG codons between this stop and the previously predicted start. Thus at least NOS form A appears to use a non-AUG start codon [29] and this may also be the case for NOS form B. The molecular masses of the predicted NOSs starting from these alternative start codons (both encoding isoleucine) compare much better with native NOS than those of the proteins starting with the first encoded AUG codon. It should be noted that none of the possible AUG codons have an optimal context, whereas the predicted alternative non-AUG codons, particularly of NOS form B, fit much better to this rule [29]. Future studies will clarify the exact start of *Physarum* NOS proteins, but at least NOS form A and presumably also form B appear to belong to the growing group of mRNAs with non-AUG translation initiation [29]. Sometimes, as was shown for the mammalian translational regulator EIF4G2 (eukaryotic translation initiation factor 4 $\gamma$ 2) [28], usage of a non-AUG translation initiation site is even conserved evolutionarily. However, there is no report that any of the animal NOS mRNAs starts translation at a non-AUG codon.

Although the two isoforms of *Physarum* NOS are highly similar, we found distinct differences in the enzymology of the recombinant proteins expressed in *E. coli*. *Physarum* NOS form B has a significantly higher affinity towards L-arginine, H<sub>4</sub>-bip and FMN than *Physarum* NOS form A. The purified recombinant proteins of form A and B contained about the same amount of dimer and monomer, and were partly decoupled in that they consumed more than 5 NADPH molecules per nitric oxide molecule formed rather than 1.5 NADPH molecules per nitric oxide molecule for the fully coupled mammalian enzyme [11].

We were surprised to find the requirement of N-terminal amino acid residues upstream of the first AUG codon since this is a region with low homology with other NOSs, and a region that iNOS oxygenase domains apparently do not require for function [42]. In iNOS oxygenase domain crystals, this region is disordered [43] so that no structural information on this region is available to date. After we had determined the minimum amount of residues required to yield a functional recombinant protein, we mutated the amino acid on the truncation of which the activity was lost, a leucine residue, in a larger construct to investigate whether we simply required a certain length of the protein, or whether specific residues were needed. Activity was lost on mutation of

this leucine residue to aspartate or glycine, without affecting the monomer/dimer ratio and the haem content of homogeneously purified proteins. We assume that specific residues in this region are possibly required for interactions between oxygenase and reductase domains in the dimer of the enzyme, for which hydrophobic forces might be crucial.

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