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Short title: *Physarum* nitric oxide synthases

SYNOPSIS.
Physarum polycephalum expresses two closely related, calcium-independent nitric oxide synthases. In previous work, we showed that both nitric oxide synthases are induced during starvation and apparently play a functional role for sporulation. Here, we characterized the genomic structures of both *Physarum* nitric oxide synthases, expressed both enzymes recombinantly in bacteria, and characterized their biochemical properties. Whereas the overall genomic organization of *Physarum* nitric oxide synthase genes is comparable to various animal nitric oxide synthases, 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 heme binding or dimerization, and suggests the usage of non-AUG start codons for *Physarum* nitric oxide synthases. Biochemical characterization of the two *Physarum* isoenzymes revealed different affinities for L-arginine, flavine mononucleotide and tetrahydrobiopterin.

Keywords. Physarum polycephalum, nitric oxide synthase, tetrahydrobiopterin, arginine, flavin, heme

Abbreviations. NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; e-NOS, endothelial NOS; TB, Terrific Broth; IPTG, isopropyl thiogalactoside; DTE, dithio-erythritol; PMSF, phenylmethylsulphonylfluoride; H_4-bip, tetrahydrobiopterin; L-NIL, L-N6(1-iminoethyl)-lysine; L-NNA, N^{ω}-nitro-L-arginine; L-NMMA, N^{ω}-methyl-L-arginine;

INTRODUCTION

Nitric oxide (NO), a signalling and cytotoxic/cytoprotective agent, plays a key role in regulating the nervous, immune and cardiovascular systems of animals. It is synthesized by nitric oxide synthase (NOS, E.C. 1.14.13.39) which occurs as 3 isoforms and genes, i.e. NOS1, NOS2 and NOS3, also termed neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS), 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

EXPERIMENTAL

Cultivation of *Physarum polycephalum*

Strain M_3b, a Wis 1 isolate used initially for purifying and cloning of *Physarum* NOS, was grown in a semi-defined medium as detailed before [18]. The apogamic haploid strain LU352 was kindly provided by Wolfgang Marwan, University of Magdeburg, Germany, and grown as described [20]. Amoebae were generated and grown as described [21].

RNA isolation and quantitative PCR

RNA from LU352 amoebae and starved macroplasmodia was isolated using the RNeasy plant mini kit (Qiagen). *Physarum* NOS forms A and B mRNAs were quantified using Taqman technology. Sequences for probes and primers were for NOS form A: 5'ACGCGCACACAGCCAAGAAACG3' (probe), 5'CATCCCCGAAACTGTTGCTC3' (forward primer), 5'GCAGTCCGTGGTAG CAACCT3' (reverse primer); for NOS form B: 5'CGCAGC GTGCTATCAGCCCAGA3' (probe), 5'CCAGAAGTACACTATTCCAT CAGAAATC3' (forward primer), 5'GCGGC AATCAGATGTGGATA3' (reverse primer). For a reference, 19 S RNA was quantified as described [18].

DNA Isolation

DNA from frozen M_3b microplasmodia 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), 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 accession no. AF145041 (form A) and AF145040 (form B)). 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 the TOPO TA cloning vector (Invitrogen). Finally, most gaps could be closed by screening a genomic library (produced by Tim Burland and kindly provided by Jonatha Gott, Case Western Reserve University, Cleveland, Ohio) using standard procedures. The library had been prepared from the LU352 strain by Stratagene custom service using the LambdaZap vector.

Sequencing and data analysis

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

Plasmids for overexpression of *Physarum* NOSs

Physarum calmodulin cDNA (GenBank accession no. 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 up-stream of the putative start methionine [18], 11 amino acids stemming from the original Bluescript SK^- 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 introduction of an additional SalI site at the desired position (Quikchange II kit, Stratagene), cutting by SalI and re-ligating. Mutations were introduced by site-directed mutagenesis (Quikchange 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 to the cDNA 5'-region generated by RACE-PCR (see below). This clone was further modified introducing an additional SacI restriction site at the desired position (Quikchange 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 II protocol into one form of physnosa (A1) and its almost inactive mutant (A1LD) to allow preparation of homogenous, active proteins.

5' Elongation of cDNA clones

In order to elongate 5' sequences of the previously published *Physarum* NOS form A and form 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 S.N.A.P. midi prep plasmid preparation kit (Invitrogen) were transformed into TUNER DE3 cells (Novagen). Bacterial starter cultures (5 ml) from single colonies were grown in Luria Bertani Broth (LB, Difco) with ampicillin (50 µg/ml) to an OD_{600} of 0.8 at 37 °C and 220 rpm, then pelleted and diluted in 15 ml of fresh TB 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 time zero were taken and cultures were supplemented with 450 µM 5-aminolevulinic acid (Sigma A-7793) and 3 µM riboflavin (Sigma R-4500) in order to provide sufficient heme and flavins and 50 µM isopropyl thiogalactoside (IPTG). Bacteria were grown for further 24 h at 20 °C at 220 rpm in the dark for NOS expression. For purification, this protocol was up-scaled to 30 ml starter cultures and 400 ml expression cultures. TB (terrific broth) was used instead of LB for expression cultures. Bacteria from these cultures were resuspended in 40 ml 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 tetrahydrobiopterin (6*R*-5,6,7,8-tetrahydro-L-biopterin, Schircks Laboratories, Jona, Switzerland), 5 mM dithio-erythritol (DTE), 1 mM phenylmethyl-sulphonylfluoride (PMSF), 0.2 mg/ml lysozyme). After incubation for 20 min at 25 °C at 200 rpm, cells were homogenized using a French press (20.000 psi, 2 times), and mixed with 110 ml buffer B (50 mM Tris/HCl, pH 8.0, containing 10 % (v/v) glycerol, 100 µM L-arginine, 10 µM tetrahydrobiopterin, 5 mM dithio-erythritol (DTE)).

Purification of recombinant Physarum NOSs

All steps were carried out at 4 ºC and all elution buffers contained 100 µM L-arginine and 10 µM tetrahydrobiopterin. Protein was precipitated using 35.6 g ammonium sulphate (45% of saturation) and stirring for 1 h followed by centrifugation at 48.000 x 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 x g for 20 min. The supernatant was dialysed for 2 times 2 h versus 1 L of buffer C (buffer B without flavins) using a 50 K membrane (Pierce). The dialysate was adjusted to 0.15 M NaCl and then incubated with 2', 5' ADP-sepharose 4B (Amersham Pharmacia) for 1 h using a rocking mixer at a ratio of 1 part dialysate-protein to 2 parts ADP-sepharose (w/w). After washing 2 times with 10 ml buffer B containing 0.15 M NaCl and 1 time with 10 ml buffer B, protein was eluted from ADP-sepharose with 1 ml

buffer B containing 10 mM NADPH. Protein determination at various steps was done using the Bradford Protein Assay reagent from Bio-Rad. To obtain homogenous active protein, the dimeric fractions of NOS were then collected by gel filtration on Superose12 10/300 GL (GE Healthcare), and were finally purified using the StrepTag affinity protocol [22].

Native *Physarum* NOS

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

NOS assay

Radiometric citrulline NOS assay was carried out as described before [17] using buffer B (see above) supplemented with 2 mM NADPH as assay buffer. Briefly, bacterial homogenates (bacteria from 2 ml of culture were diluted in 700 µl of buffer A, incubated for 10 min at 25 °C and 1000 rpm in an Eppendorf thermomixer and finally mixed for 10 sec with an Ultra-Turrax) or partially purified NOS (5-20 µl/assay corresponding to 10 - 15 µg of protein in the case of bacterial homogenates or ~1 µg of protein when using purified enzyme) were incubated in assay buffer containing 40000 cpm of HPLC-purified ^{3}H-L-arginine (Amersham) in a final volume of 200 µl for 10 min at 25 °C on an Eppendorf thermomixer. The reaction was then stopped, separated over Dowex 50 W columns and the flow-through was subjected to scintillation counting. In some assays, concentrations of cofactors or substrate were varied. IC_{50}, EC_{50} and K_M values were calculated with the SigmaPlot 9.0 software (Systat Software Inc.).

For comparison of NO formation with NADPH consumption, a kinetic microtiter-plate assay based on measurement of NO 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 25ºC as described [24], using a PowerWaveX thermostatted kinetic microplate reader (Bio-Tek Instruments,,Winooski, VT, U.S.A.).

Gel electrophoresis

Proteins were separated over SDS polyacrylamide gels. Gels were stained by Coomassie Blue or silver stain according to standard procedures and scanned using an ImageScanner (Amersham Biosciences). For quantification, gels were stained with Deep Purple Total Protein Stain (Amersham Biosciences) and fluorescence was scanned by a Typhoon 9410 scanner (Amersham Biosciences) at 532 nm and evaluated using the ImageQuant software (Amersham Biosciences).

Determination of heme content by HPLC

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

RESULTS

Expression of *Physarum* NOS forms 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 like it was shown for the *Physarum hapP* gene [26]. For this purpose, we used the haploid *Physarum* strain LU352 and checked for expression of *Physarum* NOS forms A and B mRNA. Both mRNAs were expressed in LU352 macroplasmodia starved for 5 days in a way comparable to the diploid

M_3b strain which we used in our initial studies [17, 18]. Moreover, LU352 amoebae also expressed both mRNAs (Fig. 1).

Genomic structures of both *Physarum* NOSs

We then studied the genomic structures of both *Physarum* NOSs. As can be seen from Fig. 2A, 25 exons could be identified for *Physarum* NOS form B. Form A has two additional 5' exons as was determined extending the cDNA into the 5' direction, a strategy which was not successful for the form B cDNA where only a shorter 5' extension could be achieved starting within an exon (Fig. 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 (Fig. 2A). However, since all the 21 exon/intron boundaries that could be determined were conserved between the two *Physarum* NOSs, it is conceivable that also the lacking 5 boundaries are 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 (Fig. 2A). However, none of the exon/intron boundaries are conserved between *Physarum* and the animal iNOSs (Fig. 2A). Moreover, the size of introns in the *Physarum* genes is considerably smaller as compared to animal NOSs, particularly the human iNOS gene (Fig. 2B). As in other animal iNOSs, the reading frame of the *Physarum* NOSs starts in exon 3 (Fig. 2A). The current version of the two gene structures was submitted to GenBank (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 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 coexpression of calmodulin [27]. We therefore cloned *Physarum* calmodulin and coexpressed it together with *Physarum* NOS form A and B, respectively, using the pET-Duet1 vector. In contrast to the original *Physarum* NOS form A cDNA library clone (AF145041) which 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 (Fig. 3A), although it expressed the same amount of protein (Fig. 3D). Elimination of vector-derived sequences including the His-Tag resulted in disappearance of both, protein and activity, presumably due to proteolytic degradation (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 cut off (variant A9) and became undetectable when deleting a further amino acid (variant A3) (Fig. 3A). Exchanging this leucine residue in A1 for aspartic acid or for glycine (variants A1LD and A1LG) destroyed enzyme activity almost completely (Fig. 3A), while it did not affect the amount of overexpressed protein (Fig.3B), and did not affect the heme content of homogenously purifed proteins (see below), nor the monomer-dimer ratio on gel filtration (not shown). Expressing *Physarum* NOS form B using the predicted start methionine (clone B) also resulted in an inactive protein while addition of 50 amino acids 5' to this methionine (clone B+) yielded active protein. Varying this N-terminus showed that only 4 additional amino acids were sufficient for full activity of *Physarum* NOS form B (Fig. 3A). Like in form A, removal of a

hydrophobic amino acid (leucine for truncation from B6 to B7, isoleucine for truncation from B9 to B8) leads to the drop in activity, although the amount of NOS protein in inactive clones is comparable to that of active clones (Fig. 3E). These results clearly indicated that the N-terminus of both *Physarum* NOSs as predicted before [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 the native protein which is a mixture of both isoforms [18] (Fig. 3C).

Checking the elongated *Physarum* NOS cDNAs showed that form A cDNA (DQ835526) contained a stop codon 516 bp up-stream 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 up-stream of the predicted start [18] (Fig. 4A). Starting with this methionine, the predicted form B protein (Fig. 4B) is still smaller than the native *Physarum* NOS (Fig. 3C). Searching the cDNA sequence up-stream of the putative start codon showed that both *Physarum* NOSs have several putative non-AUG start codons [28, 29]. An isoleucine conserved in both *Physarum* NOSs is found 88 (form A) or 90 (form B) amino acids upstream (Fig. 4A). However, the Kozak sequence for these codons is less optimal [30] than that of other nearby residues (Fig. 4B): for *Physarum* NOS form A cDNA this is the isoleucine codon (ATT) at amino acid position -97; for *Physarum* NOS form B, an alternative start codon (ATT) with a nearly optimal context is encoding for isoleucine in position -108. These sites yield proteins of nearly identical molecular mass (Fig. 4B) which are comparable to that of native NOS (Fig. 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 sulphate precipitation and subsequent ADP-sepharose affinity chromatography. This procedure yielded NOS with about 80 % purity, as determined by scanning bands stained with a fluorescence dye (see Experimental Procedures, data not shown, compare Fig. 5A, last lane). The specific activity was 244 ± 47 $nmol.mg^{-1}.min^{-1}$ (form A, mean of 3 purifications ± SD) and 253 ± 47 $nmol.mg^{-1}.min^{-1}$ (form B, mean of 4 purifications ± SD). Fig. 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 about 1:1 on gel filtration (Figure 6 A, B), the K_M for L-arginine and the EC_{50} for FMN and tetrahydrobiopterin was clearly higher for NOS form A than for the form B protein (Table 1). A combined NO formation (determined by binding to myoglobin) and NADPH consumption assay [24] showed partial decoupling of the enzyme preparations, with 5.1 ± 1.6 NADPH consumed in the formation of 1 NO molecule by *Physarum* NOS A and 5.9 ± 1.5 NADPH consumed per NO in *Physarum* NOS B (mean ± SD of three determinations).

Heme content of homogenous *Physarum* NOSs A1 and A1LD

To check whether or not the mutation of a residue in the N-terminus that led to a pronounced drop in activity also affected heme binding and hence heme content of the active enzyme, we introduced into forms A1 and A1 LD (see Fig. 3A) a StrepTag, and further purified the tagged proteins by additional gel filtration, collection of the dimeric fractions and final purification using the StrepTag affinity column. This yielded a homogenous protein (> 95 % SDS gel silver stained, Fig. 6C) free of *E.coli* impurities (minor impurities also contain the StrepTag as checked by Western blotting). Pure A1 (3 preparations) had a specific activity of 585 ± 144 $nmol.mg^{-1}.min^{-1}$, and a heme content of 0.41 ± 0.14 heme per subunit, whereas

pure A1LD (2 preparations) displayed less than a tenth of the activity (51 ± 10 nmol.mg^{-1}.min^{-1}), but had a heme content (0.44 ± 0.09 per subunit) comparable to A1 (Table 2).

DISCUSSION

Physarum polycephalum, a member of the class/superclass Myxogastridae (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 macroplasmodia 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 gel electrophoresis [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 was never reported, at least to our knowledge. The previously used *Physarum* strain was the diploid M_3b 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 which 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 macroplasmodia of both strains showed comparable mRNA levels. Moreover, both mRNAs were also expressed in LU352 amoebae. Macroplasmodia 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 a 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, personal communication of Gérard Pierron, Villejuif, France) as compared to 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 aquired 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 divergence from a common ancestor (see below) where already some ancestral NOS had evolved. Once the final *Physarum* genome sequence will be 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 molds, the *Dictyostelidae* [33-34]. Together with other amoebae and slime mold 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 data 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, not shown), while *Physarum* contains two genes with exon numbers comparable to those of higher animals (see Fig. 2) and an even intron distribution as it is characteristic for higher eukaryotes [36]. Secondly, *Dictyostelium* does not produce tetrahydrobiopterin like *Physarum* [17] but its steroisomer tetrahydrodictyopterin [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 data). Moreover, the genomic structures of GTP cyclohydrolase I, the first enzyme of tetrahydrobiopterin 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 tetrahydrobiopterin and NO 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, about 9 % of all detected genes are alternatively spliced in *Physarum*, whereas only about 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 to the native NOS purified from *Physarum* microplasmodia (Fig. 3C). Addition of only a few amino acids (13 for NOS form A and 4 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 up-stream 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 for isoleucine) compare much better to 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, like was shown for the mammalian translational regulator NAT1 [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 recombinat proteins expressed in *E. coli*. *Physarum* NOS form B has a significantly higher affinity towards L-arginine, tetrahydrobiopterin 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 NAPDH per NO formed rather than 1.5 NADPH per NO as 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 to other NOSs, and a region which 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 of this region is available to date. After we had determined the minimal amount of residues required to yield a functional recombinant protein, we mutated the amino acid upon the truncation of which the activity was lost, a leucine, 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 upon mutation of this leucine to aspartate or glycine, without affecting the monomer-dimer ratio and the heme content of homogenously 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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REFERENCES

1 Forstermann, U., Boissel, J. P. and Kleinert, H. (1998) Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). FASEB J., **12,** 773-790.

2 Eissa, N. T., Yuan, J. W., Haggerty, C. M., Choo, E. K., Palmer, C. D. and Moss, J. (1998) Cloning and characterization of human inducible nitric oxide synthase splice variants: a domain, encoded by exons 8 and 9, is critical for dimerization. Proc.Natl.Acad.Sci.U.S.A, **95,** 7625-7630.

3 Tiscornia, A. C., Cayota, A., Landoni, A. I., Brito, C., Oppezzo, P., Vuillier, F., Robello, C., Dighiero, G., Gabus, R. and Pritsch, O. (2004) Post-transcriptional regulation of inducible nitric oxide synthase in chronic lymphocytic leukemia B cells in pro- and antiapoptotic culture conditions. Leukemia, **18,** 48-56.

4 Bros, M., Boissel, J. P., Godtel-Armbrust, U. and Forstermann, U. (2006) Transcription of human neuronal nitric oxide synthase mRNAs derived from different first exons is partly controlled by exon 1-specific promoter sequences. Genomics, **87,** 463-473.

5 Guix, F. X., Uribesalgo, I., Coma, M. and Munoz, F. J. (2005) The physiology and pathophysiology of nitric oxide in the brain. Prog.Neurobiol., **76,** 126-152.

6 Mungrue, I. N. and Bredt, D. S. (2004) nNOS at a glance: implications for brain and brawn. J.Cell Sci., **117,** 2627-2629.

7 Bronte, V. and Zanovello, P. (2005) Regulation of immune responses by L-arginine metabolism. Nat.Rev.Immunol., **5,** 641-654.

8 Belge, C., Massion, P. B., Pelat, M. and Balligand, J. L. (2005) Nitric oxide and the heart: update on new paradigms. Ann.N.Y.Acad.Sci., **1047,** 173-182.

9 Danson, E. J. and Paterson, D. J. (2005) Cardiac neurobiology of nitric oxide synthases. Ann.N.Y.Acad.Sci., **1047,** 183-196.

10 Griffith, O. W. and Stuehr, D. J. (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu.Rev.Physiol, **57,** 707-736.

11 Gorren, A. C. and Mayer, B. (2007) Nitric-oxide synthase: a cytochrome P450 family foster child. Biochim.Biophys.Acta, **1770,** 432-445.

12 Stuehr, D. J., Santolini, J., Wang, Z. Q., Wei, C. C. and Adak, S. (2004) Update on mechanism and catalytic regulation in the NO synthases. J.Biol.Chem, **279,** 36167-36170.

13 Panda, K., Rosenfeld, R. J., Ghosh, S., Meade, A. L., Getzoff, E. D. and Stuehr, D. J. (2002) Distinct dimer interaction and regulation in nitric-oxide synthase types I, II, and III. J.Biol.Chem, **277,** 31020-31030.

14 Wei, C. C., Crane, B. R. and Stuehr, D. J. (2003) Tetrahydrobiopterin radical enzymology. Chem Rev., **103,** 2365-2383.

15 Werner, E. R., Gorren, A. C., Heller, R., Werner-Felmayer, G. and Mayer, B. (2003) Tetrahydrobiopterin and nitric oxide: mechanistic and pharmacological aspects. Exp.Biol.Med.(Maywood.), **228,** 1291-1302.

16 Gorren, A. C., Sorlie, M., Andersson, K. K., Marchal, S., Lange, R. and Mayer, B. (2005) Tetrahydrobiopterin as combined electron/proton donor in nitric oxide biosynthesis: cryogenic UV-Vis and EPR detection of reaction intermediates. Methods Enzymol., **396,** 456-466.

17 Werner-Felmayer, G., Golderer, G., Werner, E. R., Grobner, P. and Wachter, H. (1994) Pteridine biosynthesis and nitric oxide synthase in Physarum polycephalum. Biochem.J., **304 (Pt 1),** 105-111.

18 Golderer, G., Werner, E. R., Leitner, S., Grobner, P. and Werner-Felmayer, G. (2001) Nitric oxide synthase is induced in sporulation of Physarum polycephalum. Genes Dev., **15,** 1299-1309.

19 Buddha, M. R., Tao, T., Parry, R. J. and Crane, B. R. (2004) Regioselective nitration of tryptophan by a complex between bacterial nitric-oxide synthase and tryptophanyl-tRNA synthetase. J.Biol.Chem., **279,** 49567-49570.

20 Daniel J.W. and Baldwin H.H. (1964) Methods of culture for plasmodial myxomycetes. In Methods of Cell Physiology, vol. 1 (Prescott D.M. Ed.), pp. 9-41. Academic Press, New York

21 Dee, J., Foxon, J. L. and Anderson, R. W. (1989) Growth, Development and Genetic-Characteristics of Physarum-Polycephalum Amebas Able to Grow in Liquid, Axenic Medium. Journal of General Microbiology, **135,** 1567-1588.

22 Schmidt, T. G. and Skerra, A. (2007) The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. Nat.Protoc., **2,** 1528-1535.

23 Barroso, J. F., Elholm, M. and Flatmark, T. (2003) Tight binding of deoxyribonucleotide triphosphates to human thymidine kinase 2 expressed in Escherichia coli. Purification and partial characterization of its dimeric and tetrameric forms. Biochemistry, **42,** 15158-15169.

24 Gross, S. S. (1996) Microtiter plate assay for determining kinetics of nitric oxide synthesis. Methods Enzymol., **268,** 159-168.

25 Bonkovsky, H. L., Wood, S. G., Howell, S. K., Sinclair, P. R., Lincoln, B., Healey, J. F. and Sinclair, J. F. (1986) High-performance liquid chromatographic separation and quantitation of tetrapyrroles from biological materials. Anal.Biochem., **155,** 56-64.

26 Lepine, G., Laroche, A., Lemieux, G. and Pallotta, D. (1995) The two alleles of the hapP gene in Physarum polycephalum code for different proteins. Biochim.Biophys.Acta, **1264,** 271-274.

27 Wu, C., Zhang, J., Abu-Soud, H., Ghosh, D. K. and Stuehr, D. J. (1996) High-level expression of mouse inducible nitric oxide synthase in Escherichia coli requires coexpression with calmodulin. Biochem.Biophys.Res.Commun., **222,** 439-444.

28 Takahashi, K., Maruyama, M., Tokuzawa, Y., Murakami, M., Oda, Y., Yoshikane, N., Makabe, K. W., Ichisaka, T. and Yamanaka, S. (2005) Evolutionarily conserved non-AUG translation initiation in NAT1/p97/DAP5 (EIF4G2). Genomics, **85,** 360-371.

29 Kozak, M. (2005) Regulation of translation via mRNA structure in prokaryotes and eukaryotes. Gene, **361,** 13-37.

30 Bailey, J., Anderson, R. W. and Dee, J. (1987) Growth and Development in Relation to the Cell-Cycle in Physarum-Polycephalum. Protoplasma, **141,** 101-111.

31 Charlesworth, B. and Barton, N. (2004) Genome size: does bigger mean worse? Curr.Biol., **14,** R233-R235

32 Fedorova, L. and Fedorov, A. (2003) Introns in gene evolution. Genetica, **118,** 123-131.

33 Baldauf, S. L. and Doolittle, W. F. (1997) Origin and evolution of the slime molds (Mycetozoa). Proc.Natl.Acad.Sci.U.S.A, **94,** 12007-12012.

34 Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. and Doolittle, W. F. (2000) A kingdom-level phylogeny of eukaryotes based on combined protein data. Science, **290,** 972-977.

35 Baldauf, S. L. (2003) The deep roots of eukaryotes. Science, **300,** 1703-1706.

36 Mourier, T. and Jeffares, D. C. (2003) Eukaryotic intron loss. Science, **300,** 1393

37 Klein, R., Thiery, R. and Tatischeff, I. (1990) Dictyopterin, 6-(D-threo-1,2-dihydroxypropyl)-pterin, a new natural isomer of L-biopterin. Isolation from vegetative cells of Dictyostelium discoideum and identification. Eur.J.Biochem., **187,** 665-669.

38 Werner, E. R., Pitters, E., Schmidt, K., Wachter, H., Werner-Felmayer, G. and Mayer, B. (1996) Identification of the 4-amino analogue of tetrahydrobiopterin as a dihydropteridine reductase inhibitor and a potent pteridine antagonist of rat neuronal nitric oxide synthase. Biochem.J., **320 (Pt 1),** 193-196.

39 Golderer, G., Werner, E. R., Heufler, C., Strohmaier, W., Grobner, P. and Werner-Felmayer, G. (2001) GTP cyclohydrolase I mRNA: novel splice variants in the slime mould Physarum polycephalum and in human monocytes (THP-1) indicate conservation of mRNA processing. Biochem.J., **355,** 499-507.

40 Witter, K., Cahill, D. J., Werner, T., Ziegler, I., Rodl, W., Bacher, A. and Gutlich, M. (1996) Molecular cloning of a cDNA coding for GTP cyclohydrolase I from Dictyostelium discoideum. Biochem.J., **319 (Pt 1),** 27-32.

41 Gloeckner, G., Golderer, G., Werner-Felmayer, G., Meyer, S. and Marwan, W. (2008) A first glimpse at the transcriptome of Physarum polycephalum. BMC.Genomics, **9,** 6, doi:10.1186/1471-2164-9-6

42 Ghosh, D. K., Crane, B. R., Ghosh, S., Wolan, D., Gachhui, R., Crooks, C., Presta, A., Tainer, J. A., Getzoff, E. D. and Stuehr, D. J. (1999) Inducible nitric oxide synthase: role of the N-terminal beta-hairpin hook and pterin-binding segment in dimerization and tetrahydrobiopterin interaction. EMBO J., **18,** 6260-6270.

43 Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J. and Tainer, J. A. (1998) Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. Science, **279,** 2121-2126.

44 Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. and Snyder, S. H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature, **351,** 714-718.

45 Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S. and Poulos, T. L. (1998) Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. Cell, **95,** 939-950.

46 Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K. and Weber, P. C. (1999) Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. Nat.Struct.Biol., **6,** 233-242.

47 Garcia-Cardena, G., Martasek, P., Masters, B. S., Skidd, P. M., Couet, J., Li, S., Lisanti, M. P. and Sessa, W. C. (1997) Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. J.Biol.Chem, **272,** 25437-25440.

Table 1. **Enzymatic properties of recombinant *Physarum* NOSs.** Partially purified recombinant *Physarum* NOS form A (clone A1) and form B (clone B+) with an activity of about 250 nmol.mg^{-1}.min^{-1} each (see text for data) was used for measuring substrate, cofactor and inhibitor effects. H_4-bip, tetrahydrobiopterin; L-NIL, L-N6(1-iminoethyl)-lysine; L-NNA, N^{ω}-nitro-L-arginine; L-NMMA, N^{ω}-methyl-L-arginine. Values are means of 3 to 5 independent experiments ± SD. Values in bold are significantly different (Student's t-test) between NOS form A and form B: K_M L-arginine, $P < 0.001$; EC_{50} tetrahydrobiopterin, $P < 0.001$; EC_{50} FMN, $P < 0.02$.

concentration (µM)	*Physarum* NOS A	*Physarum* NOS B
K_M L-arginine	**66.7 ± 14.5**	**26.8 ± 9.7**
EC_{50} H_4-bip	**7.9 ± 1.2**	**0.7 ± 0.1**
EC_{50} NADPH	57.2 ± 6.5	60.3 ± 13.8
EC_{50} FAD	3.9 ± 1.6	2.1 ± 1.6
EC_{50} FMN	**1.7 ± 0.6**	**0.2 ± 0.2**
IC_{50} L-NIL	81.3 ± 7.8	76.7 ± 4.3
IC_{50} L-NNA	25.7 ± 10.7	39.8 ± 20.3
IC_{50} L-NMMA	45.2 ± 23.5	44.3 ± 19.2

Table 2 Original data of heme determination of pure *Physarum* NOS preparations. Physarum NOS clones A1 and A1LD containing an N-terminal Strep tag, were coexpressed with *Physarum* calmodulin in *E.coli* with pETDuet1 and purified by ammonium sulphate fractionation, ADP-sepharose affinity chromatography, gel filtration and Strep-tag affinity chromatography. Heme 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 bovine serum albumin as standard. MM (calculated molecular mass). See Materials and Methods section for method details.

Sample	Batch	Heme (µM)	Protein (mg/ml)	MM (kDa)	Heme/ subunit	NOS activity (nmol/mg.min)
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

Figure 1. Quantification of *Physarum* NOS form A and form B mRNA levels. Total RNA was isolated from starved macroplasmodia of the diploid M_3b and the haploid LU352 strain as well as from LU352 amoebae and the two NOS forms were quantified by Taqman technology in relation to 19 S RNA. The mean of 3 independent experiments ± SD is shown. The dashed line indicates the detection limit of the method. **1**: M_3b macroplasmodia, *Physarum* NOS form A; **2**: M_3b macroplasmodia, *Physarum* NOS form B; **3**: LU352 macroplasmodia, *Physarum* NOS form A; **4**: LU352 macroplasmodia, *Physarum* NOS form B; **5**: LU 352 amoebae, *Physarum* NOS form A; **6**: LU352 amoebae, *Physarum* NOS form B.

Figure 2. Genomic organization of *Physarum* NOSs. *A*, Exons in cDNAs of human iNOS (bar 1, 27 exons; GenBank accession 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; 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; genomic: DQ835527; cDNA: DQ835528). Lines below each bar indicate the translated region of the cDNA. Positions of various binding sites are marked as published before [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 5 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 accessions 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.

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 raw homogenates. Values are means of 5-10 independent experiments ± SD in brackets. The previously predicted start of translation is underlined [18]. All overexpressed proteins start with a His-tag sequence (MGSSHHHHHHSQDPNSSSAR LQVD), indicated by 3 dots. Clone A has an additional GIDKLDIEFRSKG sequence from the Bluescript SK^- vector inserted between the His-tag and the *Physarum* NOS sequence. aa, amino acids. ***B,*** Overexpression of A1 and its A1LD mutant. Raw homogenates (15 µg) harvested at times 0 and 24 h after induction were separated over 6 % SDS 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 µg protein of raw homogenates per lane) with native NOS purified from *Physarum* microplasmodia (0.3 µg/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 (raw homogenates, 3 µg of protein/lane). ***E***, Silver stained gel of various B clones (raw homogenates, 3 µg/lane). Molecular masses (kDa) of several recombinant proteins were calculated for proteins containing the His-tag and are given below the lanes of panels *C-E*.

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 DQ835526) and form B (physnosb, DQ835528). Conserved residues are shown in bold. The previously predicted start of the protein [18] is framed. An additional methionine in form B is

marked by a grey arrow, putative alternative starts of the reading frames are indicated by black arrows. Italic numbers refer to amino acid positions related to the previously predicted reading frames [18], 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 small letters whereas the context for translation is given in large letters. The nucleotide positions as well as the amino acid positions given in brackets 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].

Figure 5. Partial purification of recombinant *Physarum* NOS form A (clone A1). ***A,*** 6-17 % gradient, Coommassie-Blue-stained SDS polyacrylamide gel showing partial purification of recombinant *Physarum* NOS form A clone A1. 20 µg of total protein were loaded per lane; homogenate, raw 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-sulphate precipitation step is not shown. ***B***, Activities, yield and purification factor of *Physarum* NOS form A clone A1 protein purified from 400 ml bacterial culture (homogenate) by ammonium-sulphate precipitation (AS-precipitation) and 2',5' ADP sepharose 4B affinity chromatography (2'5'-ADP).

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 Materials and Methods for details). 200 µl of purified protein was then loaded onto a Superose 12 10/300 GL gel filtration column, eluted with buffer B containing 0.15 M NaCl. Fractions were collected and NOS activity was determined by the radiometric citrulline assay. The full line shows UV absorption at 280 nm, the dashed line shows NOS activity. Arrows indicate the elution volume of protein standards for gel filtration (thyreoglobulin, 690 kDa; ß-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.

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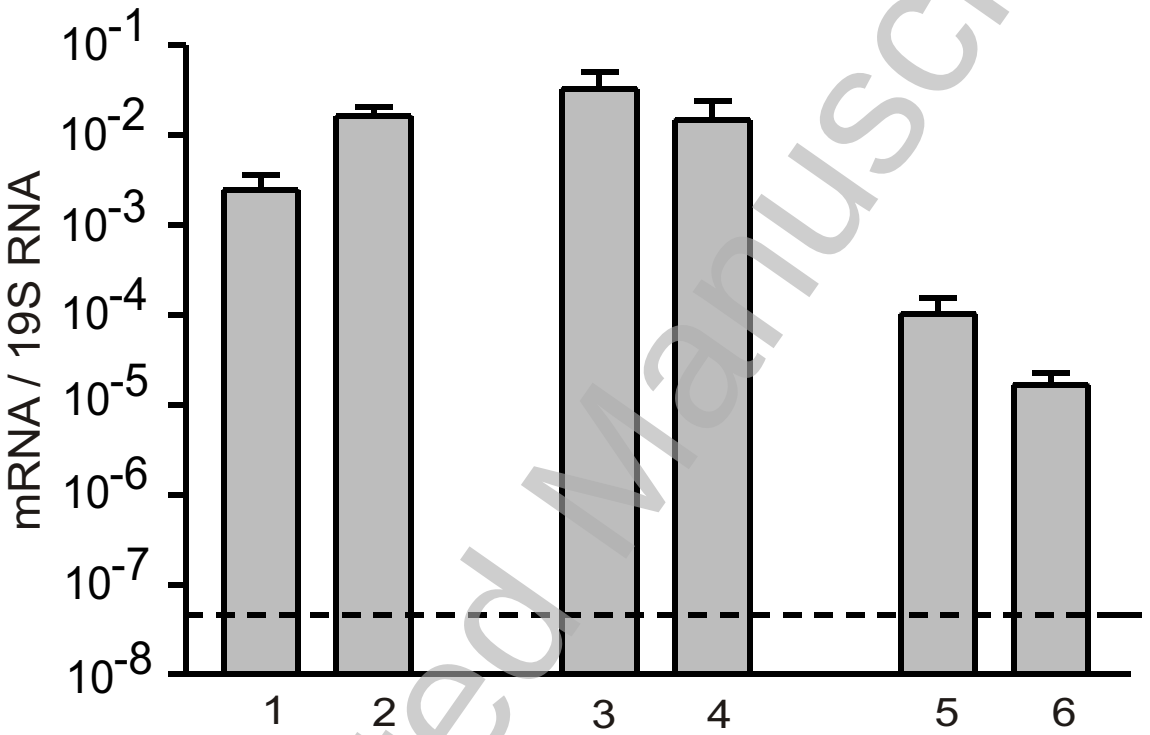

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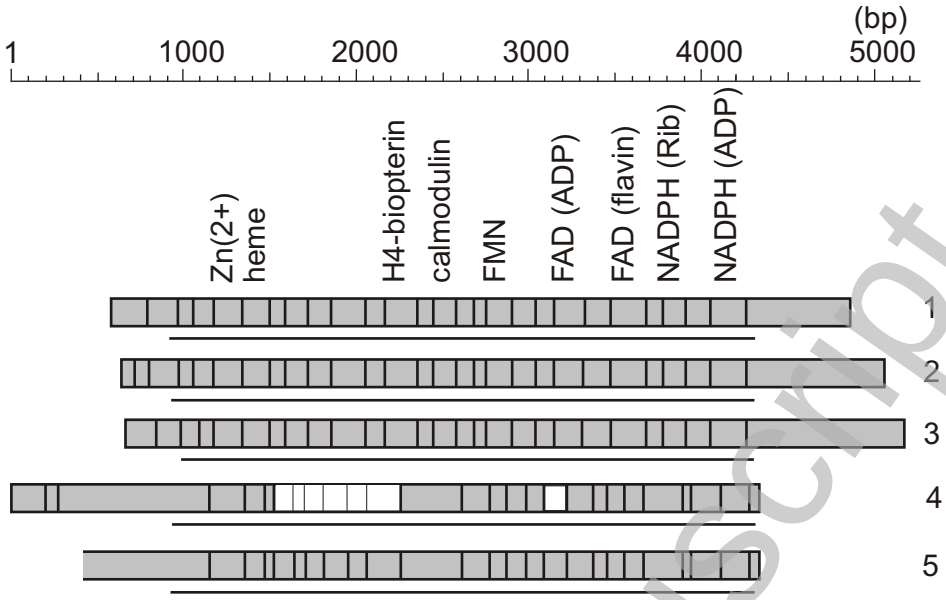
A



B

number	Intron size (bp) huminos	galinos	oncinos	physnosa	physnosb
1	1500	96	1021	66	-
2	1000	1276	273	340	-
3	700	369	96	124	129
4	1000	558	188	172	147
5	6000	85	443	53	108
6	800	1898	170	ns	82
7	900	385	1852	ns	121
8	129	405	153	ns	101
9	1800	348	809	ns	266
10	90	606	223	ns	306
11	4000	561	2883	ns	175
12	900	622	122	ns	178
13	600	378	1904	303	169
14	1200	313	334	254	221
15	1500	251	108	263	125
16	400	461	1612	114	116
17	1200	93	247	ns	94
18	1600	500	124	ns	136
19	800	411	106	124	93
20	1500	887	128	100	128
21	1000	620	206	199	355
22	1700	799	187	418	324
23	400	331	148	221	317
24	600	607	107	107	87
25	1000	623	165	223	424
26	1400	690	134	436	92
27			505		

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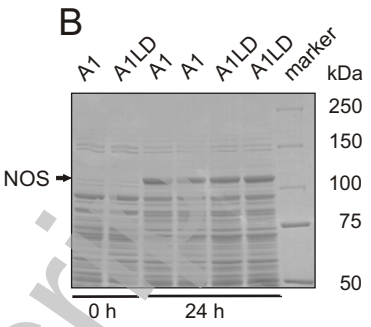
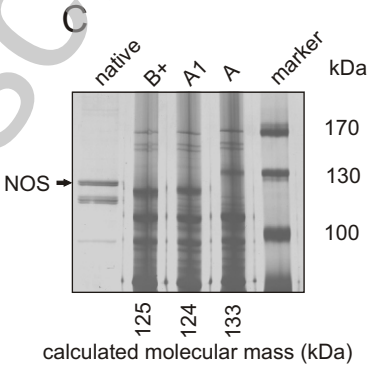
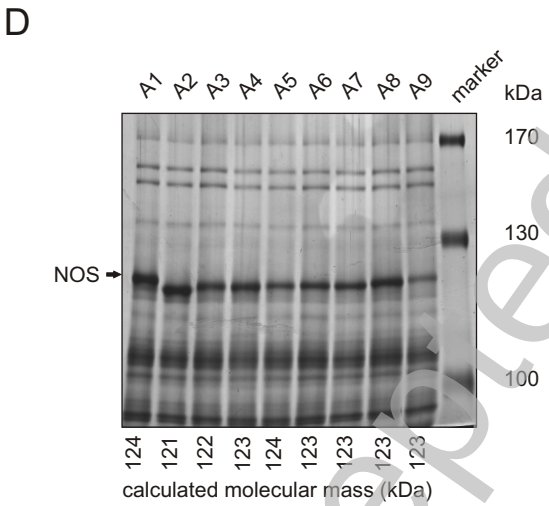
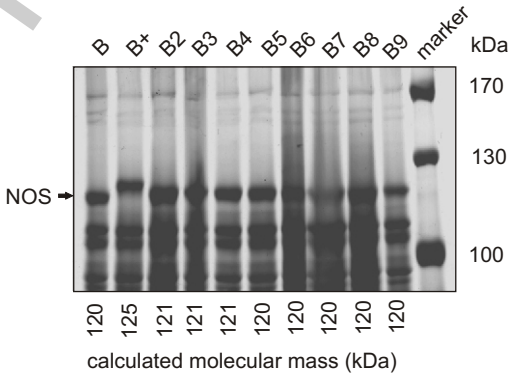
A

		activity nmol.mg^{-1}.min^{-1}
...SKG[68aa]PVPANSTGQCPFLNAFNNPLPSHAHKGPLPIYMLTNN	(A)	11.2 (3.4)
...ANSTGQCPFLNAFNNPLPSHAHKGPLPIYMLTNN	(A1)	13.4 (4.7)
...ANSTGQCPFLNAFNNP**D**PSHAHKGPLPIYMLTNN	(A1LD)	0.4 (0.4)
...ANSTGQCPFLNAFNNP**G**PSHAHKGPLPIYMLTNN	(A1LG)	1.5 (0.9)
...MLTNN	(A2)	< 0.2
...SHAHKGPLPIYMLTNN	(A3)	< 0.2
...FLNAFNNPLPSHAHKGPLPIYMLTNN	(A4)	14.8 (2.9)
...GQCPFLNAFNNPLPSHAHKGPLPIYMLTNN	(A5)	6.1 (1.7)
...FNNPLPSHAHKGPLPIYMLTNN	(A6)	16.9 (7.6)
...NNPLPSHAHKGPLPIYMLTNN	(A7)	9.5 (4.4)
...LPSHAHKGPLPIYMLTNN	(A8)	7.2 (4.5)
...PSHAHKGPLPIYMLTNN	(A9)	0.9 (0.7)
...MLTNN	(B)	< 0.2
...APA[30aa]THAHIPPHAHKGPLPIYMLTNN	(B+)	4.2 (1.2)
...PPHAHKGPLPIYMLTNN	(B2)	7.1 (2.1)
...PHAHKGPLPIYMLTNN	(B3)	5.7 (2.8)
...HAHKGPLPIYMLTNN	(B4)	5.9 (2.9)
...HKGPLPIYMLTNN	(B5)	5.4 (2.7)
...LPIYMLTNN	(B6)	3.7 (1.4)
...PIYMLTNN	(B7)	< 0.2
...YMLTNN	(B8)	< 0.2
...IYMLTNN	(B9)	1.3 (1.0)









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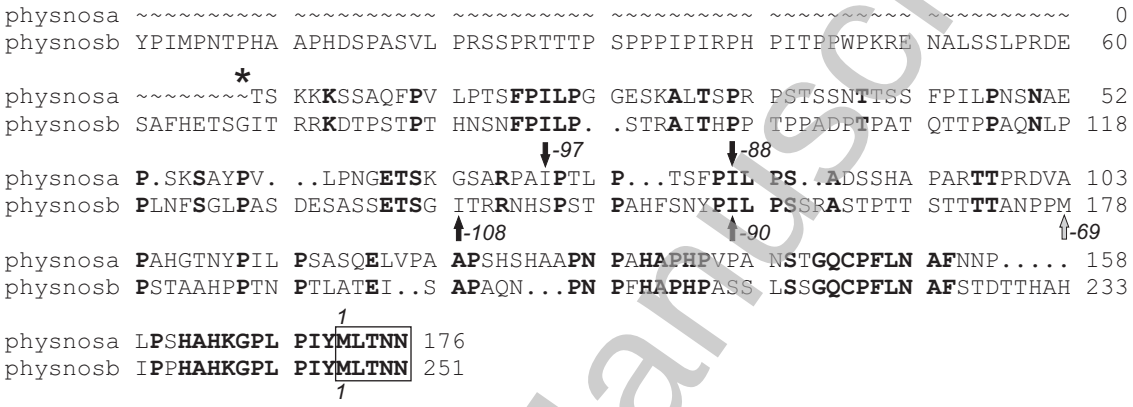
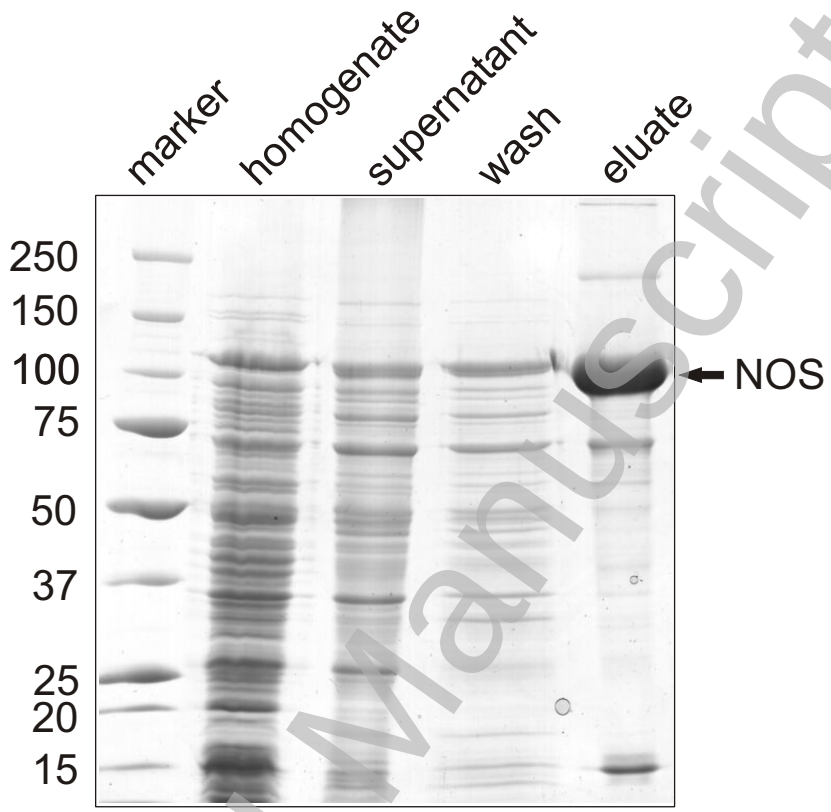


B

Physarum NOSf orm	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	UUCCCCaucC
A	Ile	-97 (75)	553	1152	128185,98	CCUGCCaucC
B	Met	1 (247)	740	1046*	117566,52*	AUCUACaugC
B	Met	-69 (178)	533	1115	124814,32	CCUCCCaugC
B	Ile	-90 (157)	491	1136	126827,70	UAUCCGaucC
B	Ile	-108 (139)	416	1154	128891,94	UCCGGCauuA

A



B

	protein (mg)	total activity (nmol per min)	specific activity (nmol per min per mg)	yield (%)	purification factor
homogenate	113	594	5	100	1
AS-precipitation	30	417	14	70	3
2'5'-ADP	0.5	104	210	17	40

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Messner et al., Figure 6