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# Site and mechanism of uncoupling of nitric-oxide synthase: Uncoupling by monomerization and other misconceptions

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ABSTRACT

Nitric oxide synthase (NOS) catalyzes the transformation of L-arginine, molecular oxygen ($O_2$), and NADPH-derived electrons to nitric oxide (NO) and L-citrulline. Under some conditions, however, NOS catalyzes the reduction of $O_2$ to superoxide ($O_2^-$) instead, a phenomenon that is generally referred to as uncoupling. In principle, both the heme in the oxygenase domain and the flavins in the reductase domain could catalyze $O_2^-$ formation. In the former case the oxyferrous ($Fe(II)O_2$) complex that is formed as an intermediate during catalysis would dissociate to heme and $O_2^-$; in the latter case the reduced flavins would reduce $O_2$ to $O_2^-$. The NOS cofactor tetrahydrobiopterin (BH4) is indispensable for coupled catalysis. In the case of uncoupling at the heme this is explained by the essential role of BH4 as an electron donor to the oxyferrous complex; in the case of uncoupling at the flavins it is assumed that the absence of BH4 results in NOS monomerization, with the monomers incapable to sustain NO synthesis but still able to support uncoupled catalysis. In spite of little supporting evidence, uncoupling at the reductase after NOS monomerization appears to be the predominant hypothesis at present. To set the record straight we extended prior studies by determining under which conditions uncoupling of the neuronal and endothelial isoforms (nNOS and eNOS) occurred and if a correlation exists between uncoupling and the monomer/dimer equilibrium. We determined the rates of coupled/uncoupled catalysis by measuring NADPH oxidation spectrophotometrically at 340 nm and citrulline synthesis as the formation of [$^3$H]-citrulline from [$^3$H]-Arg. The monomer/dimer equilibrium was determined by FPLC and, for comparison, by low-temperature polyacrylamide gel electrophoresis. Uncoupling occurred in the absence of Arg and/or BH4, but not in the absence of $Ca^{2+}$ or calmodulin (CaM). Since omission of $Ca^{2+}$/CaM will completely block heme reduction while still allowing substantial FMN reduction, this argues against uncoupling by the reductase domain. In the presence of heme-directed NOS inhibitors uncoupling occurred to the extent that these compound allowed heme reduction, again arguing in favor of uncoupling at the heme. The monomer/dimer equilibrium showed no correlation with uncoupling. We conclude that uncoupling by BH4 deficiency takes place exclusively at the heme, with virtually no contribution from the flavins and no role for NOS monomerization.

## 1. Introduction

Nitric oxide (NO) is produced in the human body from L-arginine (Arg) by the enzyme nitric-oxide synthase (NOS[1], EC 1.14.13.39, reviewed in Refs. [1,2]). There are three isoforms of NOS, two of which, neuronal and endothelial NOS (nNOS and eNOS, respectively), are constitutively expressed, whereas the third one, inducible NOS (iNOS), is expressed in response to cytokines. NO formation occurs in two steps in which Arg is first transformed to *N*-hydroxy-L-arginine (NHA), which is subsequently converted to citrulline (Citr) and NO. Both steps consume molecular oxygen ($O_2$) and NADPH-derived electrons (Eq. (1)).

$$\text{Arg} + 1.5\ \text{NADPH} + O_2 \rightarrow\rightarrow \text{Citr} + 1.5\ \text{NADP}^+ + 1.5\ \text{H}^+ + \text{NO} + \text{H}_2\text{O} \quad (1)$$

NOS operates as an obligate homodimer, with each monomer consisting of a reductase and an oxygenase domain. NOS employs several prosthetic groups and cofactors to produce NO. Catalysis takes place at a cytochrome P450-type heme in the oxygenase domain in the immediate

*Abbreviations:* NOS, nitric-oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; NHA, *N*-hydroxy-L-arginine; Citr, citrulline; CaM, calmodulin; BH4, tetrahydrobiopterin; [$^3$H]-Arg, L-[2,3,4,5-$^3$H]arginine hydrochloride; TEA, triethanolamine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 2-ME, 2-mercaptoethanol; DTPA, diethylene triamine pentaacetic acid; LT-PAGE, low-temperature polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; NNA, *N*-nitro-L-arginine; NMA, *N*-methyl-L-arginine

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vicinity of the substrate-binding site. The heme also binds $O_2$, whereas NADPH binds to the reductase domain, where two flavin moieties (one FAD and one FMN) shuttle electrons from NADPH to the heme in the oxygenase domain. However, interdomain electron transfer only takes place when the enzyme has bound calmodulin (CaM). This, in turn, requires CaM to bind $Ca^{2+}$, which renders the two constitutive isoforms $Ca^{2+}$-sensitive, whereas iNOS is active at basal $Ca^{2+}$ concentrations.

The reaction cycle differs between the first and second half reaction (from Arg to NHA and from NHA to Citr and NO, respectively), but the initial steps in the cycles are identical. In both cases the reaction starts by reduction of the ferric heme by the reductase domain. The reduced heme then binds $O_2$ yielding an oxyferrous complex. This species must be reduced by a further electron, which formally yields a ferrous superoxy heme that will be rapidly protonated. From this point onward the reaction cycles may diverge between the first and second half reaction. The second electron that reduces the oxyferrous complex eventually derives from NADPH and thus from the reductase domain. However, since interdomain electron transfer is rather slow and the oxyferrous complex rather unstable, NOS uses tetrahydrobiopterin (BH4), which is bound near the heme in the oxygenase domain, as a rapid auxiliary electron donor.

The overall reaction (Eq. (1)) predicts that 1.5 mol of NADPH will be oxidized for each mol of NO formed, and this stoichiometry is indeed observed under optimal experimental conditions. However, when BH4 is lacking, transfer of the second electron is too slow and the oxyferrous complex will dissociate to ferric heme and superoxide (Eq. (2)). Similarly, in the absence of Arg NO formation is impossible and $O_2^-$, or possibly $H_2O_2$, will be formed instead.

$$NADPH + 2\ O_2 \rightarrow NADP^+ + H^+ + 2\ O_2^- \quad (2)$$

The diversion of NADPH oxidation from NO generation to formation of $O_2^-$ and $H_2O_2$, which results in increased $NADP^+$/citrulline stoichiometries, is called uncoupling [1,3]. Principally, there are three sites in NOS that might be capable of producing $O_2^-$. In addition to release of $O_2^-$ from the oxyferrous complex, as described above, uncoupling may ensue when $O_2$ is reduced directly by the reduced flavin cofactors in the reductase domain. This could happen when electron transfer to the heme is blocked, for instance in the absence of CaM/$Ca^{2+}$, in the presence of NOS inhibitors that lower the heme redox potential, or by monomerization of the enzyme.

It has been known for quite some time that BH4 is essential to prevent NOS uncoupling [4,5]. In case of uncoupling at the heme, this is explained by the obligate role of BH4 as a donor of the second electron [6,7]. For uncoupling by the reductase domain, it is usually assumed that the absence of BH4, which is known to stimulate enzyme dimerization, results in monomerization and thus in NOS uncoupling (see for instance Refs. [8–11]). Despite little supporting evidence, uncoupling by NOS monomerization appears to be the prevalent hypothesis (an extended but far from exhaustive list of references to papers propagating this view is provided in the Supplementary Material).

In the present study we aim to settle this matter by careful determination of the rates of NADPH oxidation and citrulline formation and of the monomer/dimer equilibrium under a range of conditions. The results indicate that uncoupling occurs primarily, if not exclusively, at the oxygenase domain. Furthermore, we find no correlation between uncoupling and the monomer/dimer equilibrium. On the basis of these results we conclude that uncoupling by $O_2$ reduction at the flavins in the reductase domain is negligible and that the NOS monomer is essentially inactive.

## 2. Materials & methods

### 2.1. Materials

L-[2,3,4,5-$^3$H]Arginine hydrochloride ([$^3$H]-Arg, 57 Ci/mmol) was from American Radiolabeled Chemicals Inc., purchased through Humos Diagnostic GmbH (Maria Enzersdorf, Austria). BH4 was from Dr. B. Schircks Laboratories (Jona, Switzerland). Stock solutions of BH4 were prepared in 10 mM HCl. General materials for molecular biology were from New England Biolabs GmbH (Frankfurt/Main, Germany), Fisher Scientific (Vienna, Austria), and Qiagen GmbH (Holden, Germany). Zeocin and the EasySelect *Pichia* Expression Kit were from Invitrogen Fisher Scientific (Vienna, Austria). NADPH was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Other chemicals were from Sigma-Aldrich/Merck (Vienna, Austria) or from Carl Roth GmbH through Lactan GmbH (Graz, Austria). Recombinant human nNOS and eNOS were expressed in and purified from *Pichia pastoris* as described [12,13].

### 2.2. Determinations of NOS activity

Coupled NOS activity was determined as the formation of [$^3$H]-L-citrulline from [$^3$H]-Arg [14]. Unless indicated otherwise, 50 nM purified nNOS or eNOS was incubated for 5 min in 0.25 mL of 50 mM triethanolamine/HCl (TEA, pH 7.4) containing 0.1 mM [$^3$H]-Arg (~60000 cpm), 0.2 mM NADPH, 10 μM BH4, 0.5 mM $CaCl_2$, 10 μg/mL CaM, 0.2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 2.4 mM 2-mercaptoethanol (2-ME) at 37 °C, followed by separation and detection of [$^3$H]-citrulline. Blank values were determined in the absence of enzyme.

1NADPH oxidation (the sum of coupled and uncoupled catalysis) was determined by UV/Vis absorption spectroscopy in a Hewlett-Packard 8452A diode array spectrophotometer at 340 nm ($\varepsilon$ = 6.22 $mM^{-1}cm^{-1}$) and 37 °C as described elsewhere [4]. Unless indicated otherwise, samples containing 50 nM nNOS or eNOS, 0.1 mM Arg, 10 μM BH4, 0.5 mM $CaCl_2$, 10 μg/mL CaM, 0.2 mM CHAPS, and 2.4 mM 2-ME in 50 mM TEA (pH 7.4) were incubated at 37 °C. The reaction was initiated by the addition of 0.2 mM NADPH and monitored for 5 min. Averaged apparent absorbances between 600 and 800 nm were subtracted from the values at 340 nm to correct for baseline drift. Observed rates were corrected by subtraction of blank rates obtained in the absence of enzyme.

### 2.3. FPLC gel filtration chromatography

NOS dimerization was analyzed by gel filtration with a Superose 6 HR 10/30 column under the control of an ÄKTA chromatography system at 10 °C as described with minor modifications [13]. The flow rate was set to 0.3 mL $min^{-1}$ and the elution buffer consisted of 20 mM TEA (pH 7.4), 150 mM NaCl, 5% (v/v) glycerol, and 0.5 mM diethylene triamine pentaacetic acid (DTPA). Purified nNOS or eNOS (1.6 μM) was incubated for 2 min in 0.5 mL of 0.06 M TEA (pH 7.4), containing 0.1 mM Arg, 10 μM BH4, 0.5 mM $CaCl_2$, 10 μg/mL CaM, 0.2 mM CHAPS, 2.4 mM 2-ME, ~0.1 M NaCl, ~3% glycerol, and ~0.3 mM DTPA at 37 °C. Subsequently, 250 μL aliquots were injected and monitored by UV/Vis absorption at 280 nm.

### 2.4. Low-temperature polyacrylamide gel electrophoresis (LT-PAGE)

Dimerization was also analyzed by LT-PAGE [15]. Purified nNOS or eNOS (5 μg/mL) was incubated for 15 min on ice in 0.04 mL of 50 mM TEA (pH 7.4) containing 0.1 mM Arg, 10 μM BH4, 0.5 mM $CaCl_2$, 10 μg/mL CaM, 0.2 mM CHAPS, and 2.4 mM 2-ME. After addition of 0.01 mL chilled 0.325 M Tris (pH 6.8), containing 10% (w/v) sodium dodecyl sulfate (SDS), 25% (v/v) 2-ME, 50% (w/v) glycerol, and 0.05% (w/v) bromophenol blue, samples containing 5 μg nNOS or eNOS were subjected to SDS-PAGE for 150 min at 100 V on 5% SDS gels (1.5 mm), using the Mini-Protean II System from Bio-Rad (Vienna, Austria). Gels and buffers were equilibrated at 4 °C and the buffer tank was cooled during electrophoresis in an ice bath. Molecular weight markers (PageRuler™ Prestained Protein Ladder 10–180 kDa) were from ThermoFisher Scientific, Vienna, Austria). Coomassie Brilliant Blue stained gels were analyzed densitometrically with ImageJ.

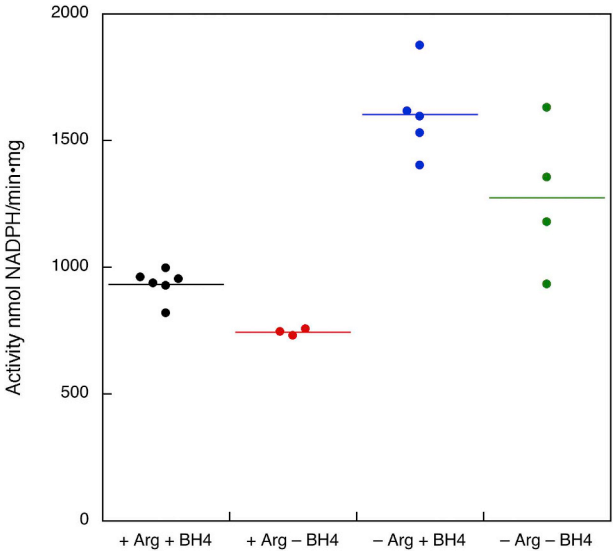


**Fig. 1.** Effects of Arg and BH4 on the rate of NADPH oxidation by nNOS.
nNOS-catalyzed NADPH oxidation rates were measured at 340 nm. Experimental conditions: 50 nM nNOS, 0.2 mM NADPH, 0.5 mM $CaCl_2$, 10 μg/mL CaM, 0.2 mM CHAPS, and 2.4 mM 2-ME in 50 mM TEA (pH 7.4) with 0.1 mM Arg and 10 μM BH4 as indicated at 37 °C.

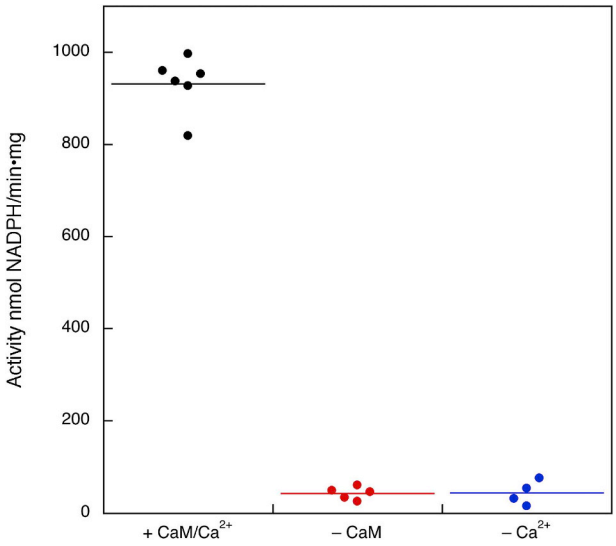


**Fig. 2.** Effects of CaM and $Ca^{2+}$ on the rate of NADPH oxidation by nNOS.
nNOS-catalyzed NADPH oxidation rates were measured at 340 nm. Experimental conditions: 50 nM nNOS, 0.1 mM Arg, 0.2 mM NADPH, 10 μM BH4, 0.2 mM CHAPS, and 2.4 mM 2-ME in 50 mM TEA (pH 7.4) with 0.5 mM $CaCl_2$ and 10 μg/mL CaM as indicated at 37 °C.

## 3. Results

As shown in Fig. 1, omission of BH4 and/or Arg did not block NOS-catalyzed NADPH oxidation, in line with previous reports [4,16]. Since in the absence of either compound NO generation is blocked completely [results not shown and refs. [4,17,18]), the observed activities can be ascribed to the reduction $O_2$ to $O_2^-$ and possibly, in the presence of BH4, directly to $H_2O_2$ [19,20]. In other words, in the absence of BH4 and/or Arg NOS became completely uncoupled with undiminished turnover rate. By contrast and in line with prior observations [4,5,21,22], NADPH oxidation was blocked almost completely when $Ca^{2+}$ or CaM was omitted (Fig. 2), indicating that under these conditions the reductase domain did not produce $O_2^-$, which argues against the flavins as the site(s) of NOS uncoupling.

Since CaM binding is not only necessary for interdomain electron transfer but stimulates interflavin electron transfer as well [22,23], it is conceivable that uncoupling by FMN does occur in the presence of $Ca^{2+}$/CaM. Therefore, we measured NADPH oxidation in the presence of several NOS inhibitors that are directed towards the oxygenase domain. In the presence of *N*-nitro-L-arginine (NNA) NADPH oxidation was again almost totally blocked, with approx. 10% activity remaining (Fig. 3). Inhibition of NADPH oxidation and NOS heme reduction by NNA were also reported previously [5,17] and ascribed to a large decrease in the redox potential of the heme caused by the NNA nitro group [16]. Since NNA is not expected to affect the reductase domain, this observation suggests that the contribution of the reductase domain to NOS uncoupling amounts to 10% or less. For comparison, we also determined NADPH oxidation in the presence of *N*-methyl-L-arginine (NMA), which only moderately decreased NADPH oxidation (Fig. 3), in line with prior observations [5,17]. Like NNA, NMA inhibits NO synthesis by competition with Arg for its binding site but, unlike NNA, it does not strongly affect the NOS redox potential and thus does not prevent heme reduction [16]. Consequently, the small effect of this compound on NADPH oxidation again suggests that uncoupling occurs at the heme.

In the presence of imidazole, which inhibits NO synthesis by directly binding to the heme [24], NADPH oxidation was once more strongly, though not completely, inhibited (Fig. 3). Incomplete inhibition of nNOS by saturating imidazole concentrations has been reported before

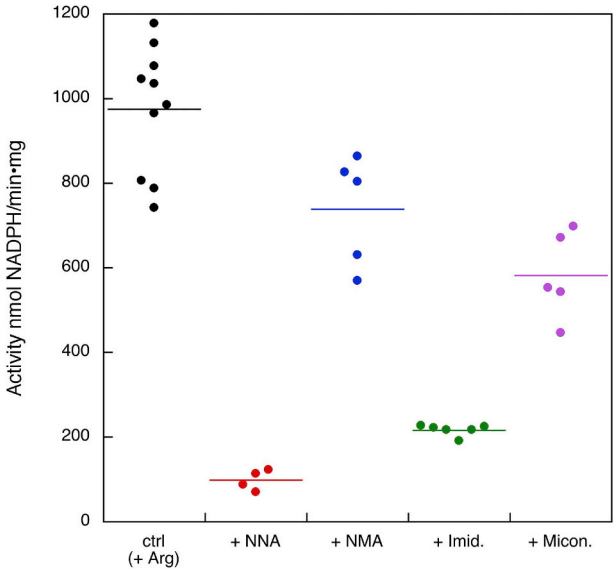


**Fig. 3.** Effects of oxygenase directed inhibitors on the rate of NADPH oxidation by nNOS.
nNOS-catalyzed NADPH oxidation rates were measured at 340 nm. Experimental conditions: 50 nM nNOS, 0.2 mM NADPH, 10 μM BH4, 0.5 mM $CaCl_2$, 10 μg/mL CaM, 0.2 mM CHAPS, and 2.4 mM 2-ME in 50 mM TEA (pH 7.4) with 0.1 mM Arg, 0.1 mM NNA, 1 mM NMA, 5 mM imidazole (Imid.), or 0.1 mM miconazole (Micon.) as indicated at 37 °C.

[25] and may be due to enzyme heterogeneity, with a fraction of the enzyme being insensitive to imidazole [26]. Consequently, the present observations are in good agreement with NOS uncoupling being completely or almost completely occurring at the heme.

Since in the literature NOS uncoupling is often associated with NOS monomerization, we also determined the effect on NADPH oxidation of miconazole, which acts at the heme but has also been reported to induce monomerization [27,28]. In agreement with a prior report [29] and with its binding to the heme, miconazole at the highest attainable concentration caused (partial) inhibition of NADPH oxidation.

In Fig. 4 citrulline production and NADPH oxidation are compared

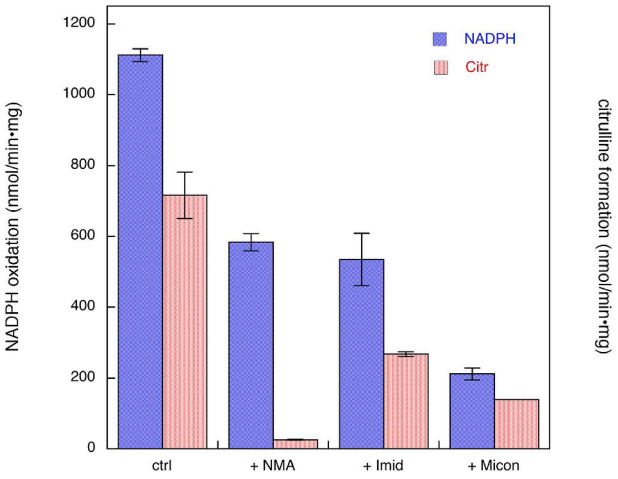


**Fig. 4.** Comparison between NADPH oxidation and citrulline formation by nNOS.

nNOS-catalyzed NADPH oxidation rates were measured at 340 nm; citrulline formation rate was measured as the conversion of [$^3$H]-Arg to [$^3$H]-L-citrulline. Experimental conditions: 50 nM nNOS, 0.1 mM Arg, 0.2 mM NADPH, 10 µM BH4, 0.5 mM $CaCl_2$, 10 µg/mL CaM, 0.2 mM CHAPS, and 2.4 mM 2-ME in 50 mM TEA (pH 7.4) in the absence (ctrl) or presence of 1 mM NMA, 5 mM imidazole (Imid.), or 0.1 mM miconazole (Micon.) as indicated at 37 °C.

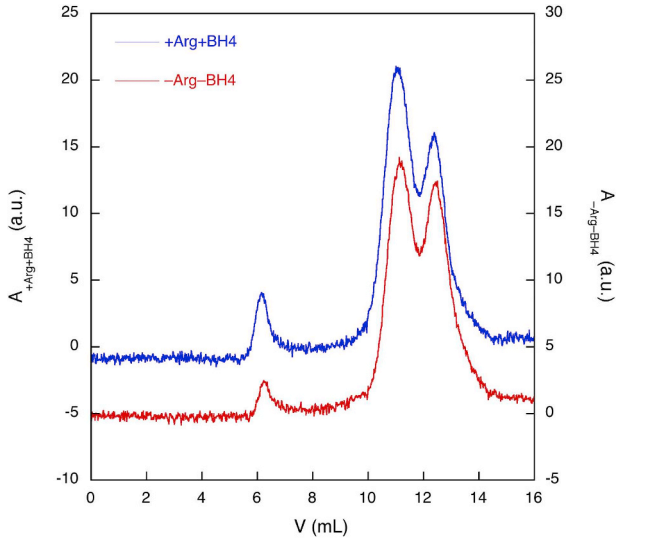


**Fig. 5.** Effect of Arg and BH4 on the dimer content of nNOS as measured by FPLC.

NOS dimerization was analyzed by gel filtration with a Superose 6 HR 10/30 column under the control of an ÄKTA chromatography system at 10 °C. Experimental conditions: 20 mM TEA (pH 7.4), containing 0.5 mM $CaCl_2$, 10 µg/mL CaM, 0.2 mM CHAPS, and 2.4 mM 2-ME at 37 °C in the absence of presence of 0.1 mM Arg and 10 µM BH4 as indicated.

for several inhibitors. Under standard assay conditions we observed a NADPH-to-citrulline stoichiometry of 1.55 ± 0.14 in good agreement with theory. In the presence of 1 mM NMA NADPH oxidation was decreased by ~50%, whereas citrulline formation was blocked by more than 95%, indicative of strong uncoupling occurring at the heme. In the presence of imidazole and miconazole inhibition of NADPH oxidation and citrulline formation were both incomplete, with both activities decreasing to similar extent (NADPH-to-citrulline stoichiometry 2.00 ± 0.28 and 1.52 ± 0.12 for imidazole and miconazole, respectively), suggesting minor uncoupling under these conditions, i.e. by the reductase domain.

To assess the effect of the aggregation state of NOS on uncoupling we determined the monomer/dimer equilibrium by FPLC. As illustrated in Fig. 5 and Table 1, we obtained very similar mixtures of monomers and dimers under all conditions. Notably, even miconazole, which was reported to inhibit NOS by enzyme monomerization, hardly affected the monomer/dimer equilibrium. Significantly, since the enzymes used in this study were expressed in and purified from *P. pastoris*, which does not contain BH4, they were pterin-free. The relative insensitivity of the monomer/dimer equilibrium, at least in the case of the constitutive isoforms, to the presence of substrates, cofactors, and inhibitors has been noted before [30,31]. By contrast, when the enzyme quaternary state was studied by LT-PAGE, larger effects of substrates, cofactors and inhibitors were observed (Fig. 6 and Table 1), suggesting that the binding of these substances increases the resistance of the dimer to SDS, in line with prior reports [15]. However, as illustrated by Fig. 7, in neither case a correlation between the monomeric enzyme fraction and the rate of uncoupled catalysis exists, at variance with the notion that uncoupling is caused by NOS monomerization.

Uncoupling is far more prominent for nNOS than for the other two isoforms [2]. However, since the majority of literature on the topic concerns eNOS, we repeated some of the experiments with that isoform as well. Omission of calmodulin again caused almost complete inhibition of NADPH oxidation, but similar to prior observations [12,32], omission of BH4 and Arg resulted in a 70 ± 7% decrease of NADPH oxidation, with the remaining activity only partly (62 ± 32%) inhibited by NNA (Suppl. Fig. S1). The corresponding citrulline assay yielded an activity of 202 ± 41 nmol/mg/min, whereas no citrulline was formed in the absence of CaM or BH4 or in the presence of NNA (not shown). As measured by HPLC, eNOS remained completely dimeric

**Table 1**
Comparison of dimer contents determined by FPLC and LT-PAGE.

| conditions | dimer content[a] (FPLC) | SDS-resistant dimer content[b] (LT-PAGE) |
|---|---|---|
| standard | 57.2 ± 2.9 | 32.3 ± 2.3 |
| – $Ca^{2+}$ | 58.6 | 31.8 ± 2.3 |
| – CaM | 57.1 | 32.4 ± 2.0 |
| – BH4 | – | 5.4 ± 3.6 |
| – Arg | – | 29.6 ± 3.6 |
| – Arg – BH4 | 56.7 ± 6.6 | 4.0 ± 6.6 |
| + NMA | 57.2 ± 4.5 | 31.7 ± 1.3 |
| +NNA | 58.1 | 32.6 ± 1.0 |
| + Imidazole | 58.4 | 31.8 ± 1.9 |
| + Miconazole | 58.5 ± 1.2 | 2.4 ± 3.3 |

[a] Dimer contents (in percent) as presented by FPLC gel filtration were estimated from the heights of the dimer and monomer peaks ($n$ = 1–2).

[b] Dimer contents as presented by LT-PAGE were estimated with ImageJ ($n$ = 2).

under all conditions (not shown), whereas LT-PAGE yielded 87 ± 3% and 47.0 ± 1.5% dimeric NOS in the presence and absence of Arg and BH4, respectively (Suppl. Figs. S2 & S3).

## 4. Discussion

To date there are two main hypotheses to explain the mechanism of NOS uncoupling and the part that BH4 plays in its prevention. After the role of BH4 as an electron donor to the oxyferrous complex that is formed as an intermediate in the catalytic cycle had been elucidated, it was proposed that uncoupling occurs by release of $O_2^-$ form the Fe(II) $O_2$ complex, regenerating ferric heme in the process [2,6,7,33,34]. Alternatively, it has been suggested that the absence of BH4 may result in NOS monomerization and that the NOS monomer, unable to sustain interdomain electron transfer, produces $O_2^-$ from the reaction of one or both of the reduced flavins with $O_2$ [8–11]. Over the years the latter alternative appears to have attained textbook status, with numerous research papers as well as reviews unquestioningly assuming a direct link between NOS monomerization and uncoupling. Consequently, the monomer/dimer equilibrium is often determined, customarily by LT-

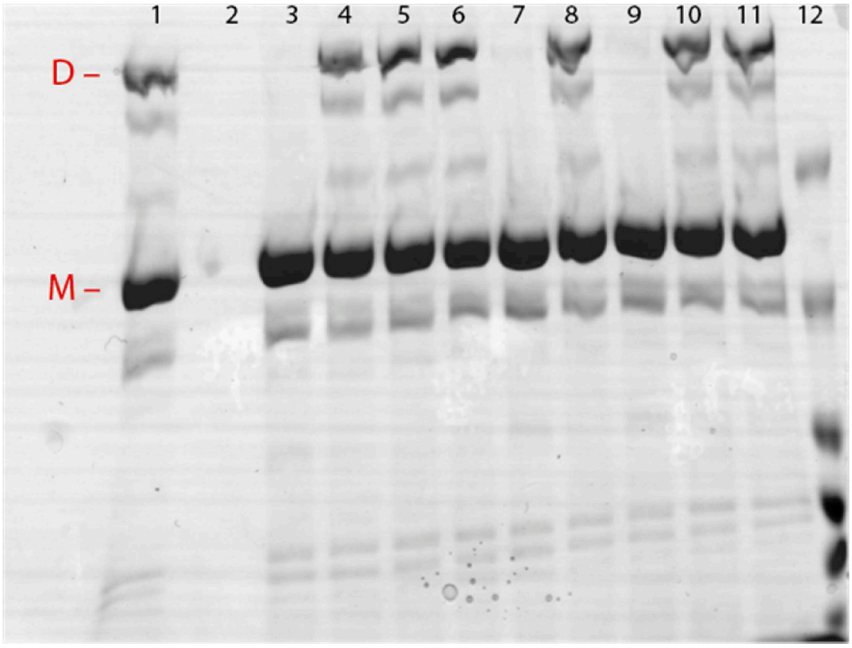


**Fig. 6.** Effect of Arg and BH4 on the SDS-resistant dimer content of nNOS as measured by LT-PAGE.
Dimerization was analyzed by LT-PAGE. Experimental conditions: Purified 5 μg/mL nNOS in 50 mM TEA (pH 7.4) containing 0.1 mM Arg, 10 μM BH4, 0.5 mM $CaCl_2$, 10 μg/mL CaM, 0.2 mM CHAPS, and 2.4 mM 2-ME (Ctrl). Lane 1: - $Ca^{2+}$; lane 2: empty; lane 3: + miconazole (0.1 mM); lane 4: + imidazole (1 mM); lane 5: + NNA (0.1 mM); lane 6: + NMA (0.1 mM); lane 7: - BH4; lane 8: - Arg; lane 9: - Arg – BH4; lane 10: - CaM; lane 11: Ctrl; lane 12: molecular weight markers with approximate weights, from top to bottom: 180, 130, 100, 70, 55, and 40 kDa. Positions of the dimer (D) and monomer (M) are indicated on the gel.

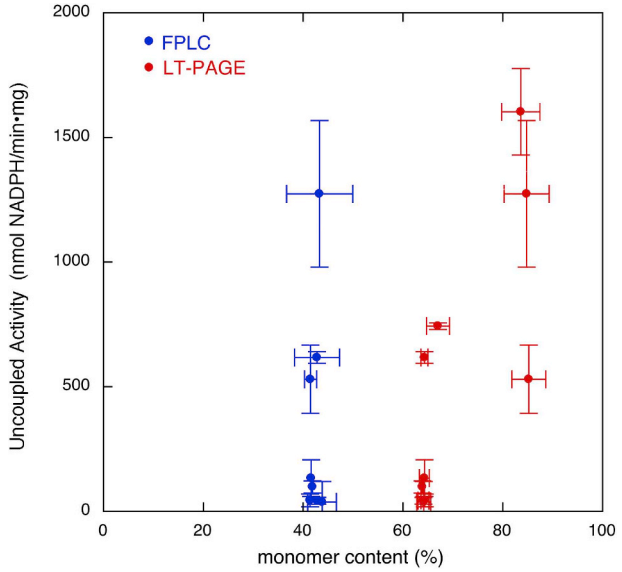


**Fig. 7.** (No) Correlation between uncoupling and monomer content.
The uncoupled activity of nNOS, calculated as $v = v_{NADPH} – 1.5 * v_{citr}$, is plotted against the monomeric fraction as determined by FPLC (blue dots) and LT-PAGE (red dots). Data are presented ± SD.

PAGE, as a measure of the coupling status of NOS (see for instance the list of references in the Supplementary Material). Whereas the prevailing opinion thus assumes that $O_2^-$ formation by the flavins in the reductase domain of monomeric NOS is the central pathway of NOS uncoupling, a large body of evidence arguing against that has in fact been gathered over the years. In an attempt to set the record straight we repeated and extended a number of these experiments.

### 4.1. Contribution of the reductase domain to uncoupling

Whereas it was initially taken for granted that uncoupling is heme-catalyzed, this changed when a number of studies that actually measured $O_2^-$ formation, usually by spin trapping, suggested reductase-mediated uncoupling in some instances. Eventually, for nNOS most studies concluded that $O_2^-$ production occurred at the heme [35–38] with the exception of one study that applied a hard to interpret adrenochrome assay [39] and another that based its conclusion of equal rates of $O_2^-$ generation by both domains on partial inhibition of $O_2^-$ formation by (unsaturating) concentrations of cyanide and imidazole [40]. For eNOS a similar conclusion of heme-mediated $O_2^-$ production was reached in most studies [41–43] apart from one [44] that based its conclusion on the lack of inhibiton by L-nitroarginine methyl ester (L-NAME). For iNOS, in contrast, $O_2^-$ was reported to originate from the reductase domain, in one study [45] based on the absence of an effect of cyanide at a concentration that almost certainly was too low to affect iNOS catalysis [46] and in another study based on the lack of an effect of imidazole [47].

Importantly, virtually no uncoupling occurs in the absence of CaM/$Ca^{2+}$ (Fig. 1; refs. [4,17,18]). Since CaM is absolutely required for interdomain electron transfer but not for electron transfer within the reductase, this strongly suggests that uncoupling mainly occurs in the oxygenase domain. One might argue that CaM binding also stimulates electron transfer inside the reductase domain and that cytochrome *c* reduction, as a measure of electron transfer from NADPH to FMN, is strongly stimulated by CaM/$Ca^{2+}$ as well [22,23]. However, it should be realized that the electron transfer steps within the reductase domain are generally much faster than interdomain electron transfer from FMN to the heme and, indeed, the rate of cytochrome *c* reduction in the absence of CaM/$Ca^{2+}$ is in the same range as the rate of NO formation in the presence of CaM/$Ca^{2+}$ (see for instance Ref. [48]). Consequently, the (almost) complete absence of uncoupled catalysis in the absence of CaM/$Ca^{2+}$ argues strongly against a prominent role for FMN as the site of uncoupling. Furthermore, uncoupling in the presence of heme-directed inhibitors closely correlates with the extent to which these compounds allowed heme reduction (Figs. 1 and 3; refs. [4,5,14,16,17]), again providing strong arguments for the heme as the main site of uncoupling.

Analysis of experimentally observed rates of the relevant reactions, i.e. of formation of $O_2^-$ by dissociation of $Fe(II)O_2/Fe(III)O_2^-$ heme and by reduction of $O_2$ by reduced FAD or FMN (Eqs. (3) and (4), respectively, with $Fl_{red}$ and $Fl_{sq}$ representing the fully reduced and semiquinone flavin redox states) also strongly points to the former reaction (Eq. (3)) as the only relevant source of uncoupling. Estimated rates of $O_2^-$ formation by the heme, as gathered from the decay of the nNOS oxyferrous complex, varied depending on the temperature, the isoform, and the presence of Arg or BH4 [49–54], but were generally at

least as fast as and often much faster than the corresponding enzyme turnover numbers for all three isoforms. Consequently, $O_2^-$ formation by the heme is sufficiently fast to enable uncoupled NADPH oxidation to occur at the same rate as coupled turnover. The rate of $O_2$ reduction by the flavins, on the other hand, as estimated from the aerobic stability of the fully reduced reductase domain [55–59], is only in the range of 0.03–0.13 $s^{-1}$, which is at least one order of magnitude slower than the rate of $O_2^-$ formation at the heme, and clearly too slow to cause substantial uncoupling.

$$Fe(II)\text{-}O_2 \leftrightarrow Fe(III)\text{-}O_2^- \rightarrow Fe(III) + O_2^- \quad (3)$$

$$Fl_{red} + O_2 \rightarrow Fl_{sq} + O_2^- \quad (4)$$

Apparent discrepancies with some of the earlier reports are caused by a too strong reliance on the spin-trapping results without considering the corresponding NADPH oxidation, which was either not measured or not taken into account. In the case of iNOS, in particular, even if the observed $O_2^-$ production occurs at the flavins, it should be borne in mind that omission of Arg or BH4 lowers the NADPH oxidation rate by ~80–90%, while in the absence of both less than 5% of the rate of coupled catalysis persists [16,17]. Taken together, we conclude that NOS uncoupling mainly affects the neuronal isoform in a reaction that takes place at the heme, while iNOS and eNOS show only minor uncoupling to which the reductase domain may (iNOS) or may not (eNOS) contribute.

It should be noted that this conclusion is valid for uncoupling caused by actions at the heme such as those involving BH4, Arg, and heme directed inhibitors, and does not necessarily apply to other modes of uncoupling reported in recent years [60–63]. Since several studies have demonstrated that the resistance of the flavins to autoxidation can be manipulated by mutations in the reductase domain (see for instance Refs. [56,57,59]), it is conceivable that certain post-translational modifications or protein-protein interactions induce uncoupling by the flavins. Probably the best-characterized example is the partial uncoupling reported for GSSG-glutathionylated eNOS [62]. Glutathionylation of eNOS by GSSG, which targets two cysteines in the reductase domain, resulted in uncoupling that was not blocked by L-NAME. By contrast, glutaredoxin-1-mediated glutathionylation, which targets a different cysteine in the oxygenase domain, did not cause uncoupling but blocked NADPH oxidation [63], suggesting an inhibitory effect on interdomain electron transfer.

### 4.2. Role of the dimer/monomer equilibrium

Since BH4 is known to strengthen the NOS dimer and to prevent uncoupling [3], at some stage a direct link was suggested between these two phenomena. One study that may have been instrumental in this phenomenon reported that eNOS uncoupling by peroxynitrite is caused by disruption of the zinc-thiolate site followed by disulfide formation at the dimer interface [64], although in that paper it was clearly stated that this did not result in NOS monomerization but in destabilization of the SDS-resistant dimer. Moreover, the central conclusions of that study have been called into question more recently [65,66]. Nevertheless, determination of the monomer/dimer equilibrium by LT-PAGE, has been established as a convenient way to monitor NOS uncoupling intracellularly. However, LT-PAGE does not measure the monomer/dimer equilibrium, but illustrates how Arg, BH4, and other substrates, cofactors, and inhibitors render the NOS dimer astonishingly resistant to the harsh conditions of PAGE in the presence of SDS [15]. As shown in the present study, the actual dimer content, at least of the constitutive isoforms, is much greater and hardly affected by absence or presence of substrates, cofactors, and inhibitors. Moreover, uncoupling occurs in the presence of substrate analogs like NMA and pteridines like dihydrobiopterin, even though these have similar dimer-stabilizing effects as Arg and BH4 [3,67].

More recently, a (minor) role for the eNOS monomer in uncoupled catalysis was claimed on the basis of cellular experiments with a novel intracellular NOS dimerization sensor and mutants that are defective in BH4 binding [68]. Specifically, in this first study that did not rely on LT-PAGE to determine intracellular NOS dimerization, BH-deficient eNOS mutants were found to exhibit both uncoupling and monomerization. However, we would like to point out that the underlying results merely reflect the well-established correlations between BH4 content and (un) coupling on the one hand and dimer stability on the other; they do not necessarily imply a causal relationship between uncoupling and monomerization. Significantly, whereas in that study the dimer contents of the purified eNOS mutants were not characterized, studies with the corresponding purified iNOS and nNOS mutants showed no correlation between monomer content and the strong uncoupling exhibited by these enzymes [69].

As for the potential activity of the monomer, early studies clearly demonstrated that NOS monomers preserve the ability to reduce artificial electron acceptors like cytochrome *c* or potassium ferricyanide, but exhibit very low (≤3% compared to the dimer) NADPH oxidation rates [70,71]. More recently, a study with eNOS in which the zinc-binding cysteines were mutated also showed that monomerization does not results in uncoupling but in almost complete inhibition: cf. the rate of NO formation of wild-type eNOS of ~90 nmol/min mg in Fig. 8A of ref. [72] with the rate of $O_2^-$ formation of wild-type eNOS of 3.8 nmol/min mg and the even lower $O_2^-$ formation rates of the Cys-mutants of ~0.3–2.7 nmol/min mg in Fig. 8B of ref. [72]. Taking into account the different stoichiometries of uncoupled and coupled catalysis (3 $e^-$/NO and 1 $e^-/O_2^-$), those data indicate that the mostly monomeric Cys-mutants are inhibited by ≥ 99%. In line with the obvious lack of activity of monomeric NOS, the present study showed that there is no correlation between uncoupling and the monomer fraction.

A final strong argument against the involvement of both the monomer and the reductase domain in uncoupling is provided by observations with single site-mutants of nNOS in which the cysteine ligand of the heme (C415) was converted to histidine or alanine [73]. These mutants, which lack the heme and, consequently, are monomeric, are unable to oxidize NADPH unless an artificial electron acceptor like cytochrome *c* is present.

In summary, we conclude that NOS uncoupling, at least when it is induced by the absence of BH4 or substrate or by the presence of inhibitory pteridines or substrate analogs, takes place (almost) exclusively at the heme site in the oxygenase domain and does not involve the monomer.

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## Appendix A. Supplementary data

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