FOCUS PAPER

Mechanisms for nitric oxide synthesis in plants

Nigel M. Crawford*

Section of Cell and Developmental Biology, Division of Biological Sciences, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92037-0116, USA

Received 26 July 2005; Accepted 10 November 2005

Abstract

The discovery that nitric oxide (NO) acts as a signal fundamentally shifted our understanding of free radicals from toxic by-products of oxidative metabolism to key regulators of cellular functions. This discovery has led to intense investigation into the synthesis of NO in both animals and plants. Nitric oxide synthases (NOS) are the primary sources of NO in animals and are complex, highly regulated enzymes that oxidize arginine to NO and citrulline. Plant NO synthesis, however, appears more complex and includes both nitrite and arginine-dependent mechanisms. The components of the arginine pathway have been elusive as no known orthologues of animal NOS exist in plants. An Arabidopsis gene (AtNOS1) has been identified that is needed for NO synthesis in vivo and has biochemical properties similar to animal cNOS, yet it has no sequence similarity to any known animal NOS. An Atnos1 insertion mutant has been useful for genetic studies of NO regulation and for uncovering new roles for NO signalling. The elucidation of plant NO synthesis promises to yield novel mechanisms that may be applicable to animal systems.

Key words: Arabidopsis, AtNOS1, mitochondria, nitric oxide synthase.

Introduction

In the late 1980s, a paradigm shift occurred in our understanding of free radicals when it was discovered that nitric oxide was the signal released by endothelial cells in response to vasodilators such as acetylcholine or bradykinin (Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Palmer et al., 1987). Previously, free radicals and reactive oxygen species (ROS) were considered toxic by-products of metabolism dependent on oxygen-based respiration (Hensley and Floyd, 2002). Their pathophysiological properties were well known and, over time, the involvement of oxidative stress in ageing and age-related diseases was recognized (Finkel and Holbrook, 2000; Balaban et al., 2005). With the discovery of the endothelial-derived relaxation factor (EDRF), the idea that reactive intermediates and free radicals can serve as endogenous signals in cellular metabolism became evident.

Nitric oxide is a lipophilic molecule that diffuses through membranes. It readily reacts with superoxide anions, metalloenzymes, and oxygen, the latter producing nitrate and nitrite in aqueous environments. Based on these chemical properties, one might predict that NO plays a role in pathophysiological processes, but it would be difficult to conceive of a signalling role (Culotta and Koshland, 1992; Wink and Mitchell, 1998; Davis et al., 2001). Yet, NO functions in the control of such fundamental processes as respiration, programmed cell death, gene expression and mitochondrial biogenesis (Ignarro, 2000; Moncada and Erusalimsky, 2002; Brown, 2003) as well as cell motility, blood flow, and neural development in animals (Gibbs, 2003; Bicker, 2005; Singel and Stamler, 2005) and germination, defence responses, and flowering in plants (Lamattina et al., 2003; Neill et al., 2003; Wendehenne et al., 2004; Crawford and Guo, 2005; Delledonne, 2005; Lamotte et al., 2005; Simpson, 2005). The size and simplicity of NO belie the potency and complexity of its regulatory power.

Because of NO’s prolific and universal signalling properties, there has been intense interest in its synthesis and mechanism of action. Early work on the biochemical action of NO showed that NO activates guanylate cyclase binding to the haem domain of guanylate cyclase (Arnold et al., 1977; Denninger and Marletta, 1999; Zhao et al., 1999) leading to vasodilation, neurotransmission, and other effects (Bredt and Snyder, 1989; Warner et al., 1994; Denninger and Marletta, 1999). However, further work has failed to identify another receptor or sensor for NO.

* To whom correspondence should be addressed. E-mail: ncrawford@ucsd.edu

© The Author [2005]. Published by Oxford University Press [on behalf of the Society for Experimental Biology]. All rights reserved. For Permissions, please e-mail: journals.permissions@oxfordjournals.org
Instead, nitrosylation of proteins at specific cysteines has emerged as a primary mechanism for NO action (Stamler et al., 2001; Hess et al., 2005). Such reactions are not enzymatically catalysed and depend on the local concentrations of NO, which is controlled by NO synthesis and scavenging rates. In animals, NO synthesis is arginine-dependent and requires one of several enzymes. The discovery of these enzymes and their mechanism provided not only a new biochemical mechanism in cellular metabolism but also major targets for pharmaceutical research.

**Discovery of animal NO synthases**

In animals, synthesis of NO is primarily accomplished by three different isoforms of nitric oxide synthases (NOS) (Stuehr, 1999; Alderton et al., 2001). The three isoforms of NOS are iNOS (for inducible NOS), eNOS (for endothelial NOS), and nNOS (for neuronal NOS). The overall reaction for these enzymes is the same: NADPH-dependent oxidation of L-arginine to N-hydroxy arginine and then to NO and citrulline (Fig. 1). The location, regulation, properties, and discovery of these enzymes differ, however, and correspond to their respective functions.

The discovery of iNOS activity arose from the observation that humans and germ-free rats secrete nitrate at levels that exceed intake, and this secretion increases markedly upon infection (Green et al., 1981a, b; Wagner et al., 1983). Treatment of isolated macrophages with an immunogenic elicitor (bacterial lipopolysaccharides or LPS) induced nitrate and nitrite synthesis (Stuehr and Marletta, 1985). This experiment was key as it provided a tissue culture system to study nitrate and nitrite synthesis and was used to show that L-arginine is needed by macrophages to produce inorganic nitrogen (Hibbs et al., 1987; Iyengar et al., 1987) and that nitrate and nitrite arise from oxidation of NO (Marletta et al., 1988). These experiments established arginine as the substrate for NO synthesis.

nNOS activity was uncovered by studying the induction of cGMP synthesis in the central nervous system by the excitatory neurotransmitter glutamate (Bredt and Snyder, 1989). Glutamate functions by activating ionotropic and kainite receptors to induce a rise in intracellular Ca\(^{2+}\), which leads to the production of NO from arginine, which, in turn, activates guanylate cyclase (Ignarro, 2000). eNOS activity was first identified by showing that vasodilator-induced production of EDRF (NO) was dependent on L-arginine but not D-arginine in perfused endothelial cells (Sakuma et al., 1988). It was then shown that NO was made by extracts of endothelial cells in a Ca\(^{2+}\)-dependent manner using cGMP synthesis as an assay (Forstermann et al., 1991).

The subsequent purification of NOS and, ultimately, cloning of all three isoforms provided a detailed picture of the structure and activity of these enzymes (Bredt and Snyder, 1990; Bredt et al., 1991; Pollock et al., 1991; Lamas et al., 1992; Xie et al., 1992). All three forms are bi-domain enzymes related to cytochrome P450 enzymes (Fig. 2) (Stuehr, 1999; Alderton et al., 2001; Li and Poulos, 2005). The C-terminal reductase domain contains the NADPH binding site as well as FAD and FMN cofactors. The C-terminal oxygenase domain contains a protoporphyrin IX haem iron and tetrahydrobiopterin (H\(_4\)B) and the binding sites for arginine and oxygen. Electrons are shuttled from NADPH through the flavins to the haem and then to oxygen, which then reacts with a guanidino nitrogen of arginine producing N-hydroxyarginine (NOHA). NOHA is oxidized further to produce NO and citrulline. Between the two domains is a site that binds calmodulin, which activates the enzyme. These enzymes vary from 130–160 kDa in size, form dimers, and are about 50–60% identical in mammals.

The primary differences among these enzymes are in their regulation and in their output rates of NO (Stuehr, 1999; Alderton et al., 2001; Li and Poulos, 2005). iNOS constitutively binds calmodulin and needs such low levels of Ca\(^{2+}\) that it is not regulated post-translationally. Instead, its synthesis is inducible by a variety of immunological signals at the mRNA level. It produces large quantities of NO, which, with superoxide anion, serves as a cytostatic or cytotoxic agent against pathogens and tumour cells. nNOS and eNOS, however, are typically expressed constitutively
and are activated by increases in Ca\(^{2+}\) levels in response to neuronal or endothelial signals. Calmodulin binds reversibly so regulation is primarily post-translational. These two enzymes are often referred to as constitutive NOS (cNOS). They produce much lower levels of NO than iNOS and are involved in signalling.

NOS enzymes are quite conserved and are found in mammals, fish, amphibians, and invertebrates (Torrellas, 2001). For example, a NOS enzyme has been cloned from Drosophila and its function has been investigated (Regulski and Tully, 1995; Regulski et al., 2004). No NOS gene has been found in Saccharomyces, but the oxygenase domain of NOS has been found in multiple species of bacteria (Adak et al., 2002, 2003; Kers et al., 2004), leading to the idea that eukaryotic NOS arose by fusion of a prokaryotic-like oxygenase domain to a reductase domain (Zemojtel et al., 2003).

**Plant nitric oxide synthesis**

The history of NO research is different in plants. This history has influenced our current models for NO synthesis and signalling in plants, yet the advances made in animal systems have also helped to shape our ideas. Heavy emphasis has been placed on production of NO by nitrate reductase (NR) as this enzyme provided the first known mechanism to make NO in plants. NR normally reduces nitrate to nitrite, but it can also further reduce nitrite to NO. Subsequently, other nitrite-dependent mechanisms for NO synthesis were found. Interestingly, evidence for arginine-dependent NO synthesis has accumulated in plants; however, the search for the responsible enzyme was fraught with several false leads. Recently, a protein was identified that has no similarity to animal NOS enzymes, yet has the biochemical properties of cNOS. This discovery has made genetic tools available to elucidate NO synthesis and action and suggests that there is a novel mechanism for making NO in plants.

It has long been known that plants can emit NO from their leaves and that this release is enhanced by herbicide or NO\(_2\) treatment (Klepper, 1979; Nishimura et al., 1986). The amount of NO release correlates with tissue nitrite levels, and NO was thought to arise from reactions between nitrite and plant metabolites (Klepper, 1979). Subsequently, it was shown that *in vivo* NR assays release NO (Harper, 1981; Klepper, 1987). These assays are done under anaerobic conditions and result in nitrite accumulation, and NR activity is needed for NO formation (Dean and Harper, 1988; Klepper, 1990). This line of experiments culminated in the demonstration that purified NR reduces nitrite to NO (Yamasaki et al., 1999; Yamasaki, 2000; Rockel et al., 2002). For maize NR, the *K_m* for nitrite is 100 \(\mu\)M, and nitrate is a competitive inhibitor with a *K_i* of 50 \(\mu\)M (Rockel et al., 2002). Thus, under normal circumstances where nitrate levels are high (1–5 mM) and nitrite is low (10 \(\mu\)M in illuminated leaves), one would not expect much NO production from NR. However, under anaerobic condition, nitrite levels increase, and NO release can increase 100-fold (Rockel et al., 2002). High NO levels can also be generated by applying nitrite under anaerobic conditions (Rockel et al., 2002), by using nitrite reductase-deficient lines (Morot-Gaudry-Talarmain et al., 2002; Planchet et al., 2005), which accumulate nitrite, or by deregulating NR with mutations in its phosphorylation site (Lea et al., 2004). With NR being the only verified enzyme for making NO in plants at that time, NR became the primary candidate for generating NO *in vivo* for signalling and defence responses (Desikan et al., 2004; Garcia-Mata and Lamattina, 2003; Yamasaki et al., 1999). However, it is not easy to understand how an enzyme that has a strong preference for nitrate, which is normally much more abundant than nitrite, could produce a universal signal from nitrite, which accumulates to significant levels only under special conditions (e.g. anoxia). The idea that a ‘housekeeping’ process such as stomatal movements can depend on a mechanism that is nitrate-dependent has also been questioned (Raven, 2003). A recent review provides an excellent discussion of these issues (Meyer et al., 2005). Nevertheless, a key paper linking NR and NO signalling showed that a NR-defective mutant is impaired in NO-mediated stomatal closure induced by ABA (Desikan et al., 2002). Other papers have also supported the involvement of NR (Xu and Zhao, 2003; Simontacchi et al., 2004; Vanin et al., 2004) while others have questioned it (Garcés et al., 2001; Zhang et al., 2003). Several words of caution are needed about using NR mutants for these experiments. First, the nitrogen nutrition of a plant is dramatically altered in the NR mutant. If WT and mutant plants are fed ammonium nitrate, the mutant will be at a disadvantage as it can use very little to no nitrate and thus will have the metabolism and growth of an ammonium-grown plant, which is quite different from a plant that has access to nitrate (Wilkinson and Crawford, 1993; Bloom et al., 2002; Wang et al., 2003, 2004). In addition, the NR mutant will accumulate nitrate to higher levels (Unkles et al., 2004).

Several recent discoveries have introduced an interesting twist to our understanding of nitrite-dependent NO synthesis. Plant mitochondria also make NO from nitrite (Tischner et al., 2004; Planchet et al., 2005), as can animal mitochondria (Kozlov et al., 1999). Anaerobic conditions are still needed *in vivo* and *in vitro* for appreciable NO synthesis. Inhibitors of mitochondria electron transport inhibit NO synthesis, suggesting that electrons from the mitochondria electron transport chain drive nitrite reduction. This raises the possibility that NO release under hypoxic or anoxic conditions depends on NR for the production of nitrite, but it is the mitochondria that produce the bulk of NO from nitrite. This would, of course, depend on the ratio of nitrite to nitrate in the cytosol. This idea is supported by the fact that NR mutants of *Chlorella* release
copious amounts of NO when provided nitrite under anoxic conditions (Tischner et al., 2004).

Several other nitrite-dependent mechanisms have been reported for NO synthesis. Nitrite is converted to NO at acidic pH in the presence of a reductant such as ascorbic acid. Such conditions exist in the apoplast and have been invoked to explain NO effects on germinating seeds (Beligni and Lamattina, 2000; Bethke et al., 2004a, b). A nitrite-dependent NO reductase activity has been observed for tobacco root plasma membranes and has been hypothesized to play a role in nitrate signalling (Stohr et al., 2001; Stohr and Ullrich, 2002).

**Plant nitric oxide synthase**

In addition to the nitrite-dependent mechanisms described above, an arginine-dependent mechanism, analogous to that used by animal NOS, has emerged from plants (Cueto et al., 1996; Ninnemann and Maier, 1996; Barroso et al., 1999; Ribeiro et al., 1999; Wendehenne et al., 2001). This is based on biochemical assays showing that plant extracts have arginine-dependent citrulline and NO synthesis activity that can be inhibited by classic NOS inhibitors (arginine analogues). In addition, these inhibitors block NO production and some NO-mediated responses such as ABA-induced stomatal closure. These results suggested that plants have orthologues to animal NOS enzymes. This idea was supported by immunological experiments showing that anti-mammalian NOS antibodies cross-react with plant proteins (Ribeiro et al., 1999). However, a proteomic analysis showed that such proteins are not related to NOS and include such proteins as heat shock proteins and glycolytic enzymes (Butt et al., 2003). In addition, no protein or gene was identified that had any sequence similarity to the complete animal NOS proteins. Thus, the NOS activity was a puzzle. Was it an artefact or do plants have a unique NOS?

A clue to this question came from an unexpected source. In 1997, an enzyme in the neurons of *Helix pomatia* (snail) was found that had something to do with NO synthesis (Huang et al., 1997). The authors were trying to identify the location of nNOS activity in an invertebrate nervous system. They found activity in specific regions of snail central ganglia based on NADPH-diaphorase histochemical staining. Since dehydrogenase activity is not specific to NOS, the authors needed further evidence to support their localization of NOS. They purified the NOS activity (using ADP-affinity and ion exchange chromatography) and obtained a preparation that had several major proteins from 30–150 kDa. Using anti-human nNOS antibody, they performed a western blot on this preparation, expecting to see the 150 kDa protein cross-react with the antibody. They found that the 150 kDa protein did not bind the antibody, but the 60 kDa protein did. They cloned a cDNA for the 60 kDa protein, sequenced it, and expressed it in *E. coli*. This protein increased NO synthesis ~15-fold in crude bacterial lysates, and its activity was stimulated by NADPH, Ca²⁺, and CaM. However, the protein had no sequence similarity to known mammalian NOS enzymes and had no consensus binding sites for NADPH, FAD or arginine. It was similar to a class of GTP-binding and GTPase proteins from bacteria. They concluded that this protein could be an accessory or regulatory protein for NOS. The inference is that it was interacting with an *E. coli* protein to increase its NOS activity. The other possibility was this protein was a novel NOS.

The snail protein is not unique to molluscs as similar proteins can be found in bacteria, insects, mammals, and plants (Zemojtel et al., 2004). A gene in *Arabidopsis* (At3g47450) encodes a protein (AtNOS1) that is 16% identical to the snail protein (Guo et al., 2003). To see if the *Arabidopsis* protein had anything to do with NO synthesis in plants, a T-DNA insertion mutant (Alonso et al., 2003) was obtained from the *Arabidopsis* Biological Resource Center. The characterization of this mutant led to some startling findings that showed this protein is a central player in NO synthesis in *Arabidopsis* (Guo et al., 2003).

The first phenotype observed in the mutant was that its first true leaves were yellow. If plants were treated with a NO donor (SNP or sodium nitroprusside), the phenotype was partially reversed, indicating that it was due to a lack of NO. NO has been shown to increase Fe accumulation and availability and can increase chlorophyll levels in corn plants grown under limiting Fe (Graziano et al., 2002; Murgia et al., 2002; Graziano and Lamattina, 2005). These findings encouraged further investigation of AtNOS1.

The key experiments were direct measurements of NO synthesis in intact roots and in leaf extracts of mutant plants. Using the NO-specific fluorescent dye (DAF-2 DA), it was found that NO levels were much lower in the roots of *Atnos1* mutant plants compared with wild type (WT). This was shown in roots of plants grown on plates, where the majority of fluorescence was at the root tip, and in roots of ABA-treated plants, where enhanced NO accumulation was observed throughout the root. As for the leaves, protein extracts from the mutant had only 20% of the NOS activity of WT extracts (as measured by the citrulline assay), the latter being inhibited by the NOS inhibitor l-NAME (1 mM). These findings have been confirmed in several other laboratories, which have reported that *Atnos1* has 20% the WT level of NO measured using NO-specific electrodes (He et al., 2004) and using DAF-FM fluorescence in *Arabidopsis* leaves (Zeidler et al., 2004). These results show that AtNOS1 is needed for most but not all of the NO accumulation in *Arabidopsis* tissues under these conditions.

Having a mutant with greatly reduced levels of NO allows the role of NO in a developmental or metabolic pathway of choice to be examined. Given the ubiquitous and universal nature of NO signalling, it might be expected...
that an NO synthesis mutant would have some noticeable phenotypes. It was encouraging to find that some of the processes known to involve NO were in fact impaired. Seed germination was inhibited as was shoot growth and leaf greening. Root growth and fertility were also impaired. A key experiment tested ABA-induced stomatal closure. In isolated epidermal peels, Atnos1 mutant stomates produced less NO and had reduced closure when treated with ABA. This demonstrated that AtNOS1 was needed for normal growth and development and was involved in hormonal signalling.

Subsequently two key papers expanded the role of AtNOS1 in unexpected ways. The first demonstrated that NO is a signal that regulates flower timing by controlling the expression of flower timing genes (He et al., 2004). A NO-overproducer mutant (nox1) was identified and shown to be an allele of CUE, which encodes a chloroplast phosphoenolpyruvate/phosphate translocator (Streatfield et al., 1999). This mutant has higher levels of arginine and NO. This mutant flowers later than WT plants. In addition, the application of NO donors delays flowering. The Atnos1 mutant, however, shows earlier flowering in a closed system.

A second paper expanded the role of AtNOS1 to defence responses. This was most surprising as it deviates from the animal NOS system, where signalling NOS enzymes (eNOS and nNOS) differ from macrophage NOS (iNOS). Since AtNOS1 was shown to play a role in ABA signalling and because its biochemical properties were more similar to eNOS than iNOS (see below), it had been assumed that another NOS would mediate defence and hypersensitive responses in plants. However, the Atnos1 mutant produces only 20% the WT level of NO in response to LPS and is more sensitive to bacterial infection (using low levels of a virulent pathogen) (Zeidler et al., 2004). In addition, a genomic response to an LPS elicitor is almost abolished in the Atnos1 mutant. These results show that AtNOS1 is a major source of NO needed not only for signalling but also for some defence responses.

With these findings demonstrating the importance of AtNOS1 for NO synthesis in vivo, it became imperative to determine the biochemical function of AtNOS1. Is it an accessory or regulatory protein as suggested by the work in snail or is it a novel NOS enzyme? To answer this question, AtNOS1 was fused to GST and expressed in E. coli (Guo et al., 2003). NO synthesis increased dramatically in crude E. coli extracts compared with vector-transformed controls. The fusion protein was enriched by glutathione affinity chromatography, and the specific activity (citrulline assay) increased 80×. The activity was dependent on NADPH, Ca2+ and CaM, but not on added FAD, FMN or BH4. NO synthesis was verified by the Griess (nitrite) assay and shown to have a \( V_{\text{max}} \) of 5.0 nmol min\(^{-1}\) mg\(^{-1}\). These results indicate that AtNOS1 has NOS activity that is dependent on Ca\(^{2+}\) and CaM.

A comparison of AtNOS1 activity with that of mammalian NOS shows that it behaves more like eNOS than iNOS (Table 1). eNOS and AtNOS1 show Ca\(^{2+}\)-dependent activity and have low outputs of NO [\( V_{\text{max}} \) for eNOS is about 15 nmol min\(^{-1}\) mg\(^{-1}\) (Pollock et al., 1991)]. Another key distinguishing feature of eNOS (and nNOS) is that it is not induced at the mRNA level while iNOS is. AtNOS1 regulation was examined by quantitative PCR and shown not to be responsive to ABA at the mRNA level (Guo et al., 2003), indicating that its regulation is like eNOS and nNOS in that it is not regulated at the transcriptional level; instead, it is regulated by Ca\(^{2+}\) at the post-translational level.

### Conclusions

At present, AtNOS1 is the only reported arginine-dependent NOS in plants. This protein provides the strongest evidence for plant arginine-dependent NO synthesis. Mutations in this gene reduce NO accumulation in vivo but not completely, indicating that other mechanisms or genes are involved in NO synthesis. Consistent with this result is that null mutants still grow but have many defects in growth, development, flowering, hormonal signalling, and defence responses. It will be interesting to see what other processes are defective in this mutant.

The evidence to date indicates that AtNOS1 encodes a novel NOS enzyme that behaves most like the constitutive class of mammalian NOS enzymes (eNOS and nNOS), showing Ca\(^{2+}\)-dependent regulation but little transcriptional control. However, the protein’s sequence and its biochemical analysis give few clues about how it can mediate the NADPH-dependent oxidation of arginine to produce NO. Because it is most similar to GTP binding proteins, it is possible that a redox cofactor is required by the enzyme to function. It is also possible that AtNOS1 interacts with other proteins to form a complex that synthesizes NO. The fact that AtNOS1 is part of a family of genes that are conserved among bacterial, insects, mammals, and plants suggests that the mechanism for NO synthesis employed by AtNOS1 may also apply to animals and bacteria.

### Acknowledgements

The research from my laboratory described in this review was supported by a grant from the National Institutes of Health (GM40672).
References


Klepper LA. 1990. Comparison between NO evolution mechanisms of wild-type and nr1 mutant soybean leaves. Plant Physiology 93, 79–82.


Wagner DA, Young VR, Tannenbaum SR. 1983. Mammalian nitrate biosynthesis: incorporation of $^{15}$NH$_3$ into nitrate is enhanced by endotoxin treatment. Proceedings of the National Academy of Sciences, USA 80, 4518–4521.


