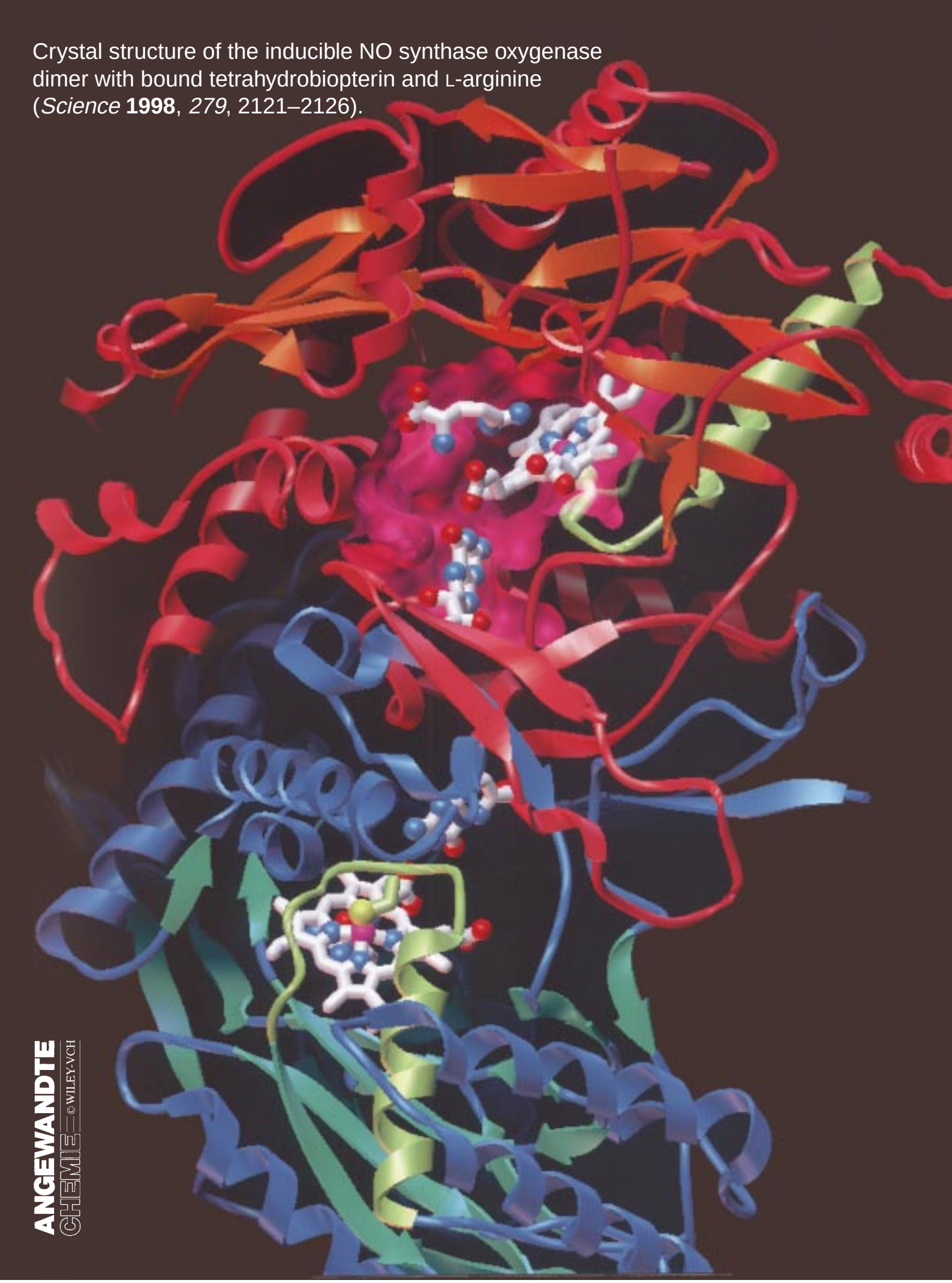
Crystal structure of the inducible NO synthase oxygenase dimer with bound tetrahydrobiopterin and L-arginine (*Science* **1998**, *279*, 2121–2126).



Nitric Oxide: Chemical Puzzles Posed by a Biological Messenger**

Silvia Pfeiffer, Bernd Mayer,* and Benjamin Hemmens

About ten years ago, the inorganic gas nitric oxide (NO) was discovered to have a biological role as a signaling molecule in the cardiovascular system. It is produced by the cells that line blood vessels, and promotes relaxation of the muscle cells in the wall of the vessel, thus lowering blood pressure. Since then, other signaling functions have been discovered. In some muscles that are directly controlled by nerves, NO is the transmitter released by the nerve terminal that causes relaxation. NO is also produced in the brain, where it functions as a regulator of synaptic activity and may be important for development of memory. Furthermore, macrophages—cells of the immune system—produce a variety of toxic compounds, including NO, at the scene of an infection. Research on NO has thrown up a variety of challenging chemical problems. How can it function as a specific and reversible signaling molecule? How can it survive long enough in biological fluids to exert its physiological effects? Which of its many reactions with biological molecules contribute to its useful or harmful effects? How is it produced, and how is its production regulated? In this review, we present some of the current discussions about these issues.

Keywords: heme proteins · nitric oxide · peroxynitrite · radicals · signal transduction

1. Introduction

Nitric oxide (NO) is currently one of the most studied molecules in the biomedical sciences. This interest is driven by the multiplicity of roles that NO plays and by the prospect of developing new drugs to tackle important disease states. The cardiovascular role of NO[1] is already exploited by drugs that release NO, thus improving blood supply to the heart muscle. Another signaling role of NO—control of blood flow into the corpus cavernosum of the penis—is exploited by the anti-impotence drug Viagra. In this case NO functions as a peripheral neurotransmitter.[2]

NO can also have damaging, even fatal effects. In the often fatal condition of septic shock, excessive NO synthesis is induced in blood vessels, leading to vascular leakage and a disastrous drop in blood pressure. In chronic diseases such as rheumatoid arthritis, NO contributes to the tissue damage caused by an inappropriate inflammatory response. In stroke, NO may contribute to brain damage. The race is on to find specific inhibitors of NO synthesis that may limit this harmful overproduction.[3, 4]

In the first part of this review, we show that the physiological effects of NO can only be understood by studying its basic chemistry—which has turned out to be surprisingly complex. For a radical like NO, biological fluids present a wide choice of substances with which to react. Some of the products of these reactions may be biologically inert; some may have similar activity to NO itself; some may be poisonous. The partitioning of NO between these different fates is made all the more complex because the decisive steps may involve highly reactive intermediates that are hard to detect.

In the second part, we present current discussions on the enzymes responsible for NO biosynthesis. The catalytic machinery of these enzymes offers many challenges for basic biochemical research. The essential catalytic puzzle, in our opinion, centers on the division of roles between the heme and the tetrahydrobiopterin cofactor. Much of the hope of new drugs centers on discovery of specific inhibitors of one of the three isozymes that are involved in different functions of NO. Some substrate analogues show some specificity; the basis of this is only partly understood.

Although many of the topics we discuss here are of direct physiological relevance, we have tried to focus on the progress on understanding underlying chemical events. Readers interested in pursuing the physiological aspects in more detail are referred to excellent reviews by others on the role of NO in the peripheral nervous system,[2] in the brain,[5] and in the cardiovascular system.[1] A visit to the NO homepage (http://www.apnet.com/no) is also to be recommended.

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[**] A list of the abbreviations used is presented in Section 5.

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2. Biological Chemistry of NO

2.1. Reactions of NO

2.1.1. Reaction with Oxygen

NO reacts with O_2 both in the gas phase and aqueous solution to form NO_2. The reaction is second order with respect to NO and first order with respect to O_2 [Eq. (1)].[6]

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

Consequently, lower concentrations of NO react much more slowly than higher concentrations: In air, for example, the time required for half of a given concentration of NO to react is 24 s for 10000 ppm of NO, but 7 h for 10 ppm. In aqueous solution, the stoichiometry of the reaction between NO and O_2 is different from the gas phase, although the form of the rate law is the same.[7, 8] The rate constant in aqueous solution has been calculated to be $6.3 \times 10^6\,M^{-2}s^{-1}$.[8, 9]

Whereas in the gas phase NO_2 is the stable oxidation product of NO, in aqueous solution NO_2 gives stoichiometric amounts of NO_2^- and NO_3^- [Eqs. (2) and (3)]. In oxygen-containing aqueous solution NO reacts to yield exclusively NO_2^-.[10] The difference could be explained if during NO autoxidation NO_2 does not accumulate in quantities sufficient to dimerize to N_2O_4, but reacts with another molecule of NO to give N_2O_3. Hydrolysis of N_2O_3 in aqueous solution would then give NO_2^- [Eqs. (4)–(6)]. The sum equation [Eq. (7)]

$$2NO_2 \rightarrow N_2O_4 \quad (2)$$
$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (3)$$
$$2NO + O_2 \rightarrow 2NO_2 \quad (4)$$
$$2NO + 2NO_2 \rightarrow 2N_2O_3 \quad (5)$$
$$2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (6)$$
$$4NO + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^- \quad (7)$$

agrees well with the overall stoichiometry of NO autoxidation in aqueous solution.[10, 11] The formation of N_2O_3 as an intermediate in this process is controversial, but would account not only for the lack of NO_3^- formation, but also for the nitrosative activity of NO (see Section 2.1.3).[12, 13]

2.1.2. Reaction with Superoxide

As a radical, NO is capable of reacting with other radicals. An important reaction of NO in biological media is a direct bimolecular reaction with $O_2^{\bullet -}$, yielding peroxynitrite [oxoperoxonitrate(1−)].[14] Peroxynitrite is not a free radical because the unpaired electrons on NO and $O_2^{\bullet -}$ have combined to form a new N–O bond. Formation of peroxynitrite from NO and $O_2^{\bullet -}$ occurs at nearly diffusion controlled rates ($4.3 \times 10^9 - 6.7 \times 10^9\,M^{-1}s^{-1}$).[15, 16] The rate constant is about three times higher than the rate constant for dismutation of $O_2^{\bullet -}$ by superoxide dismutase ($2.4 \times 10^9\,M^{-1}s^{-1}$).[17] Therefore, NO outcompetes the reaction of $O_2^{\bullet -}$ with superoxide dismutase at steady-state concentrations that are likely to exist in vivo.[18]

Peroxynitrite is a potent oxidant ($E^\circ(ONOO^-, 2H^+/NO_2, H_2O) = 1.6$ V at pH 7.0) and has been shown to react with virtually all classes of biomolecules in vitro.[19] Table 1 summarizes the rate constants of the most relevant reactions

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Bernd Mayer was born in Graz, Austria. He studied chemistry at the Karl-Franzens-Universität in Graz and did his doctoral work with Prof. Hermann Esterbauer on the physiology and pathophysiology of eicosanoids. Staying with a signal transduction theme, he worked from 1983–1989 with Prof. Walter Kukovetz at the Institut für Pharmakologie und Toxikologie in Graz and investigated various aspects of cGMP-mediated signal transduction. In 1989 he was awarded a Humboldt Fellowship to work with Prof. Eycke Böhme and Prof. Günther Schultz at the Free University of Berlin. Since his return to Graz in 1991 he has built up a research group working on a variety of biochemical and pharmacological projects. Since January 1998 he has been interim head of the institute.

Silvia Pfeiffer was born in Waidhofen an der Thaya, Austria. She obtained a degree in biotechnology at the Universität für Bodenkultur in Vienna. Her PhD thesis focussed on effects of NO on cGMP formation in plants. She joined Bernd Mayer's research group in 1995 and works mainly on the biological chemistry of NO and peroxynitrite.

Benjamin Hemmens was born in Dublin, Ireland. He studied Biochemistry at Trinity College, Dublin. He obtained his PhD from the University of Dundee, Scotland, for work on enzymes of pyrimidine biosynthesis. He spent two years in the research group of Dr. Irmgard Ziegler at the GSF-Forschungszentrum in Munich, working on pteridine biosynthesis. In 1995 he moved to Graz to join the research group of Bernd Mayer, and works on the enzymology of NO synthases.

Table 1. Rate constants of peroxynitrite reactions with enzymes, biomolecules, and some antioxidants.[a]

Reactant	k [$M^{-1}s^{-1}$]	Ref.
myeloperoxidase	6.2×10^6 [e]	[229]
horseradish peroxidase	3.2×10^6 [c]	[229]
lactoperoxidase	3.3×10^5 [e]	[229]
glutathione peroxidase	4.5×10^4 [b]	[230]
alcohol dehydrogenase	2.6×10^5 [d]	[231]
aconitase	1.4×10^5 [c]	[232]
oxyHb	2.0×10^4 [b]	[76]
cytochrome c	1.4×10^4 [b]	[76]
CO_2	3.0×10^4 [b]	[74]
ebselen	2.0×10^6 [c]	[86]
cysteine	5.0×10^3 [b]	[80]
glutathione	1.5×10^6 [b]	[233]
methionine	1.8×10^2 [c]	[234]
ascorbate	2.3×10^2 [c]	[235, 236]
NO	9.1×10^4 [b]	[72]

[a] Reported rate constants were obtained from the literature and represent the apparent values from pH 7.2–7.6. [b] $T = 37\,^\circ C$. [c] $T = 25\,^\circ C$. [d] $T = 23\,^\circ C$. [e] $T = 12\,^\circ C$.

of peroxynitrite with enzymes, antioxidants, and some other biologically relevant molecules.

2.1.3. Nitrosation Reactions

Under physiological conditions, the reaction of NO with O_2 is complicated by the presence of a variety of other possible reactants. In particular reduced thiols are more or less ubiquitous in vivo. At physiological pH, O_2, NO, and thiols react to form *S*-nitrosothiols.[12] Two possible mechanisms for this reaction are in discussion.[20] The first of these involves electrophilic nitrosation, that is, transfer of a nitrosonium (NO^+) equivalent. Nitrous acid, alkylnitrite, nitrosyl halide, N_2O_3, and N_2O_4 are all able to act as carriers of NO^+. Possibly, in the presence of oxygen, N_2O_3 is formed, which can react in this way [Eq. (8)].[21, 22] The other proposal is a radical mechanism based on formation of a thiyl radical [Eq. (9)].

$$N_2O_3 + 2\,RSH \rightarrow 2\,RS{-}NO + H_2O \quad (8)$$

$$RS^{\cdot} + NO \rightarrow RSNO \quad (9)$$

In vivo, thiols such as glutathione are used as antioxidants, and thus a low steady-state concentration of thiyl radicals is to be expected—sufficient, perhaps, to sustain this type of nitrosation reaction. *S*-Nitrosothiols have in fact been shown to occur endogenously in various tissues, including human plasma,[23] airways,[24] white blood cells,[25] and rat cerebellum.[26] These compounds act as NO donors and can thus mediate the usual spectrum of effects expected for NO.[27] Release of NO from *S*-nitrosothiols is rather slow, but can be greatly accelerated by Cu^I in vitro.[28] They survive longer than NO in blood because they are not subject to the same inactivation reactions, and in principle they could transmit NO signals through the circulation—particularly if the target cells contained a catalyst of their decomposition. Stamler and co-workers discovered one example of such signaling.[29] Cysteine 93 of the β subunit of hemoglobin (Hb) can be rapidly nitrosated when the protein is in the R (oxy) state, but not in the T (deoxy) state. *S*-nitroso-Hb was detected in arterial but not in venous blood in rats; moreover, it caused vasodilation in perfused blood vessels if glutathione was present in the perfusion medium. The sum of these reactions would be a net transport of NO from the lungs to the tissues.

In contrast to *S*-nitrosation, which seems to have fairly benign effects, *N*-nitrosation reactions of NO have deleterious consequences. An intermediate of NO autoxidation, presumably N_2O_3, can directly nitrate primary amines on DNA bases. In the presence of oxygen, NO may damage DNA also by nitrosation of various secondary amines to form *N*-nitrosamines [Eq. (10)], which can then be metabolized to form

$$N_2O_3 + R_2NH \rightarrow R_2N{-}NO + NO_2^- + H^+ \quad (10)$$

strongly alkylating electrophiles that react with DNA.[30, 31] Synthetic nitrosamine derivatives (NONOates), however, are useful tools in NO research because their rates of NO release can be engineered.[32, 33]

2.1.4. Reaction with Hemoglobin

NO reacts with oxyhemoglobin (oxyHb) with a second-order rate constant of about $3 \times 10^7\,M^{-1}s^{-1}$ to form metHb and NO_3^- [Eq. (11)].[34] The first-order dependence on NO

$$Hb{-}Fe^{II}(O_2) + NO \rightarrow Hb{-}Fe^{III} + NO_3^- \quad (11)$$

concentration and the lack of a limiting rate at high NO concentrations show that NO reacts directly with bound O_2 without first displacing it from the heme.[35] Stopped-flow spectroscopy studies recently showed that this reaction occurs by the intermediate formation of peroxynitrite.[36] NO also interacts with deoxyHb and metHb, binding to the heme iron center. In the case of metHb the binding is hindered by a water molecule coordinated to the heme Fe^{III} atom, and the association rate constant is about 100-fold smaller than for ferrous deoxyHb.[35, 37]

The reaction with oxyHb strictly limits the transport of NO in blood, so that free NO, whether produced endogenously or released from NO-donating drugs, does not have systemic effects. As an NO sink, it is probably important for deactivation of NO-stimulated sGC, at least in vascular smooth muscle (see Section 2.1.6). The impact of the reaction with oxyHb on outward diffusion of NO from vascular endothelium has been addressed by mathematical modeling.[38, 39] Neural NO signaling may be less subject to these effects because of greater distances to the nearest blood vessels.

2.1.5. Reaction with Other Metal Centers

Cells contain a variety of other metalloproteins that could bind or react with NO, such as cytochromes P450 and cytochrome c oxidase.[40] NO competes with oxygen for binding to cytochrome c oxidase: The resulting inhibition of mitochondrial respiration may be a significant toxic effect of NO, particularly under inflammatory conditions with increased NO production or in ischemic states with depressed

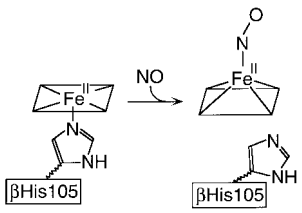
oxygen tension.[41, 42] The discovery of an NO synthase in mitochondria[43] raises the prospect that the balance of NO versus O_2 binding to cytochrome c oxidase may also be used to regulate respiration under normal physiological conditions. Some reactions of NO with zinc- and copper-containing proteins have been reported.[44–46]

NO has been shown to bind to iron–sulfur clusters, leading to the formation of iron–nitrosyl complexes.[47, 48] Recent studies have shown that NO inactivates aconitase due to a direct reaction of NO with the iron–sulfur cluster resulting in the reversible formation of an iron–nitrosyl complex. Cytosolic aconitase has a second function as the iron-regulatory protein (IRP). The NO-inhibited form appears to have increased IRP activity; this activity inhibits translation of genes responsible for iron storage and utilization, while stabilizing the mRNA for the transferrin receptor, which is responsible for iron uptake.[49] Thus NO is proposed to be a signal that counteracts iron deficiency.

2.1.6. Activation of Soluble Guanylyl Cyclase

The effects of NO as a physiological signaling molecule in smooth muscle cells and neurons are due to its activation of soluble guanylyl cyclase (sGC) by up to 200-fold above the basal activity.[50–52] sGC catalyzes, in the presence of Mg^{2+} or Mn^{2+} ions, the conversion of guanosine 5′-triphosphate (GTP) to guanosine 3′,5′-monophosphate (cGMP). cGMP is an intracellular messenger that connects the NO signal to the cellular response by activating specific protein kinases, phosphodiesterases and ion channels. sGC is a heterodimer of α and β subunits. The N-terminal half of each subunit is not needed for basal catalytic activity, but is essential for the response to NO. NO exerts its effect by binding to a heme group bound in the N-terminal portion of the β subunit.

Spectral studies of sGC in the absence of exogenous heme ligands suggest a pentacoordinate, high-spin Fe^{II} state, with a Soret maximum at 431 nm and a single broad $\alpha|\beta$ peak at 555 nm,[53] consistent with histidine as a proximal ligand. Site-directed mutagenesis showed that NO-dependent activation was completely eliminated by the mutation H105F in the β subunit.[54, 55] Binding of NO to the heme results in a pentacoordinate complex: The bond to the proximal histidine is lost (Scheme 1).[56, 57]



Scheme 1. Binding of NO to soluble guanylyl cyclase. βHis105 = His 105 of the β subunit.

This dissociation of the proximal ligand is believed to be the trigger that activates the enzyme. This is supported by a variety of results. sGC can be activated by protoporphyrin IX (PPIX) as well as by Fe^{II}PPIX(NO).[58] The enzyme is only marginally activated by CO, which binds to the heme without causing the proximal histidine to dissociate.[59] An Mn^{II}PPIX-substituted enzyme fails to be activated by NO, and also retains the proximal ligand.[60] Co^{II}PPIX-substituted sGC was fully activatable by NO, and the activation was accompanied by loss of the proximal ligand.[60]

Surprisingly, sGC binds oxygen very poorly. Also, the dissociation of NO from the heme is unusually fast. NO dissociates from sGC with a k_{obs} of about $6 \times 10^{-4} - 8 \times 10^{-4}\ s^{-1}$, several times faster than from myoglobin ($k_{obs} = 10^{-4}\ s^{-1}$; both at 20 °C).[61] The dissociation from sGC was found to be faster still ($k_{obs} = 0.04\ s^{-1}$) in the presence of substrate and Mg^{2+}: Extrapolated to 37 °C, this would give a half-life of about 5 s.[62] One element of the effect may be the negative *trans* effect of the proximal histidine moiety. In pentacoordinate nitrosyl heme, the iron atom is displaced by 0.14 Å out of the plane of the porphyrin, whereas in hexacoordinate complexes this displacement is reduced by half, which brings the NO nitrogen atom up against the four porphinato nitrogen ligands.[61] Imidazole speeds up NO dissociation by 1000-fold without a significant amount of hexacoordinate complex appearing in the absorbance spectrum: Presumably a small steady-state concentration of the hexacoordinate intermediate is sufficient.[61] Taken together, these results all point to a special protein environment around the heme in sGC, the nature of which will probably be resolved only by crystal structures of the enzyme.

Finally, it should be noted that the dissociation rates just discussed were measured in the presence of large concentrations of NO scavengers (oxyHb or dithionite) and thus represent the maximal rates that can be reached.[61, 62] Therefore the effectiveness of physiological NO sinks is highly relevant to the kinetics of NOS-sGC signaling. Oxidation of the heme[63] would also cause NO dissociation: This is the mechanism of action of the specific sGC inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ).[64] There is no evidence yet that controlled heme oxidation of sGC is exploited under normal physiological conditions. Deactivation mechanisms that override the effect of bound NO are also conceivable.

2.2. Peroxynitrite, Nitration, and Disease

In many disease states, tyrosine residues in tissues are found to be nitrated. From studies on tyrosine nitration of some proteins in vitro, it seems that this modification can have considerable effects on protein and enzyme function; thus it seems likely to be a contributing factor to pathological dysfunction. The prime suspect for the cause of nitration is peroxynitrite; however, as the following discussion shows, this small molecule has unexpectedly complex chemistry, and the link between it and pathological tissue damage is still a matter for lively debate. Table 1 lists rate constants for a variety of reactions of peroxynitrite.

2.2.1. Decomposition of Peroxynitrite in Aqueous Solution

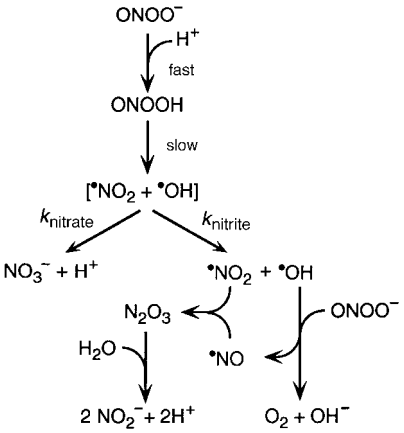
At alkaline pH, peroxynitrite is quite stable (it can be stored at pH 13 for weeks). This stability is ascribed to the adoption of the *cis* conformation under alkaline conditions.[65] Ab initio calculations suggest that a barrier of 21–24 kcal mol^{-1} limits isomerization between the *cis* and the *trans* anion.[66] When protonated (peroxynitrous acid), isomerization to the *trans* conformation is facilitated by a substantially lower energy barrier of 10–12 kcal mol^{-1}.[65]

Stopped-flow and pulse radiolysis studies revealed that the decay of peroxynitrite follows first- (or lower) order kinetics between pH 1–11, and that peroxynitrite exhibits a pK_a of 6.8 at 37 °C.[19] Independently, the decomposition of peroxynitrite was also studied in the presence of the ·OH scavenger 2,2′-azinobis-(3-ethyl-1,2-dihydroxybenzothiazoline-6-sulfonate (ABTS). From the pH-dependent formation of ABTS·+ radicals during the decomposition reaction, a pK_a of 8.0 was derived.[67, 68] The different pK_a values were ascribed to the *cis* and *trans* isomers of peroxynitrite.

The mechanism of the decomposition reaction is still not well understood.[68] Under acidic conditions peroxynitrous acid isomerizes to NO_3^-.[19, 69] The mechanism of this transformation is the subject of debate. Proposed intermediates include a vibrationally excited form of peroxynitrous acid (ONOOH*) on the reaction pathway and a complex of $^{\bullet}NO_2$ and ·OH radicals, although it is not certain whether a specially activated intermediate is necessary. Whether homolytic cleavage of peroxynitrous acid into $^{\bullet}NO_2$ and ·OH radicals is thermodynamically possible is under investigation.[70, 71]

In addition to NO_3^-, O_2 and NO_2^- are formed in a 1:2 stoichiometry during peroxynitrite decomposition in neutral to alkaline solutions.[72] The two reactions yielding NO_2^- and NO_3^- have different temperature dependences and a difference in free energy of activation of about 26 kJ mol^{-1}. The mechanism leading to NO_2^- and O_2 formation is still unclear, but a bimolecular reaction between peroxynitrite and peroxynitrous acid as the rate-determining step can be excluded[73] since decomposition of peroxynitrite does not follow second-order kinetics and the partitioning of the two pathways does not depend on the concentration of peroxynitrite.[72]

Scheme 2 shows a hypothetical mechanism of peroxynitrite decomposition to NO_2^- and NO_3^-, involving a conversion of ONOOH into an activated intermediate as the rate-limiting



Scheme 2. Decomposition of peroxynitrite.

step in both reactions. According to this mechanism, the caged $^{\bullet}NO_2$ and ·OH radicals can recombine to NO_3^-, or at alkaline pH, ·OH may react with peroxynitrite anion to form O_2, NO, and OH^-. NO could then react with $^{\bullet}NO_2$ radicals to yield N_2O_3 and finally NO_2^-. It may be that intermediates of this pathway of peroxynitrite decomposition have significant biological activities as well as peroxynitrite itself.

2.2.2. Reaction with CO_2

Peroxynitrite anion reacts rapidly with carbon dioxide ($k = 3 \times 10^4$ M^{-1} s^{-1} at pH 7.4 and 37 °C) to yield the adduct nitroso peroxycarbonate, which decomposes to NO_3^- and CO_2 in the absence of other reactive molecules [Eqs. (12) and (13)].[74, 75]

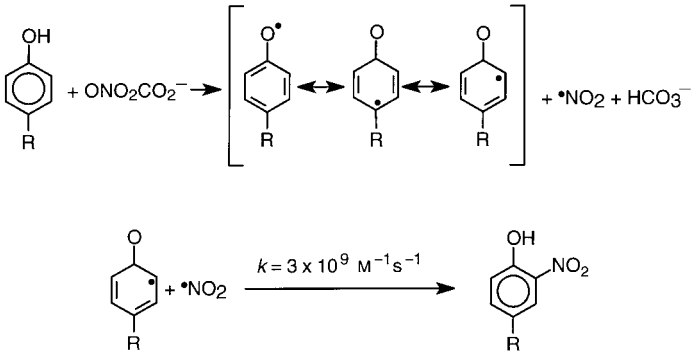
$$ONOO^- + CO_2 \rightarrow ONO_2CO_2^- \quad (12)$$
$$ONO_2CO_2^- \rightarrow NO_3^- + CO_2 \quad (13)$$

The rate constant of this reaction is large enough that it could be the predominant route of peroxynitrite disappearance in normal biological fluids, where the total carbonate concentration is between 1 and 25 mM. $ONO_2CO_2^-$ exhibits a different reactivity than peroxynitrite.[75] For instance, in the presence of CO_2 nitration yields are usually increased by two- to four-fold,[76–78] whereas thiol oxidation is dramatically reduced.[76]

The mechanism of $ONO_2CO_2^-$ decomposition is not well characterized, but homolysis of the weak oxygen–oxygen bond is favored, leading to the formation of $^{\bullet}CO_3^-$ and $^{\bullet}NO_2$. These radicals ($k = 1 \times 10^9$ M^{-1} s^{-1}) are postulated to recombine to form NO_3^- and CO_2 [Eqs. (14) and (15)].[79] The

$$ONO_2CO_2 \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}CO_3^- \quad (14)$$
$$^{\bullet}NO_2 + {}^{\bullet}CO_3^- \rightarrow NO_3^- + CO_2 \quad (15)$$

enhanced nitration promoted by peroxynitrite in the presence of CO_2 could be explained by one-electron oxidation of a phenolic ring by $^{\bullet}CO_3^-$ in concert with a termination reaction of the phenoxyl radical with $^{\bullet}NO_2$ (Scheme 3).[79]



Scheme 3. Tyrosine nitration by peroxynitrite/CO_2.

2.2.3. Reaction with Thiols

A particularly important reaction is the oxidation of protein-bound and low molecular mass thiols such as glutathione or cysteine.[80] The reactions occur with moderate second-order rate constants at physiological pH and 37 °C, but the high intracellular concentration of thiols (1–10 mM) makes this a relevant route for the cellular detoxification of peroxynitrite. One- and two-electron oxidations of thiols have been reported, mainly resulting in the formation of disulfides (>90%).[19, 80] Small amounts (≤1%) of *S*-nitrosothiols,[81, 82] which release NO in a Cu^+-catalyzed reaction,[28] are also formed by a direct nucleophilic nitrosation mechanism,[83] This accounts for the apparent ability of peroxynitrite to activate soluble guanylyl cyclase,[81] to induce vasodilation,[84] and to inhibit platelet aggregation and leukocyte adhesion.[82]

Another related reaction is the oxidation of selenium, both in naturally occurring proteins such as glutathione peroxidase and synthetic complexes such as ebselen.[85, 86] These have been found to reduce peroxynitrite to NO_2^- with formation of a selenoxide, which can be reduced back by thiols. It has been proposed that this reaction plays a major role in cellular detoxification of peroxynitrite.

2.2.4. Tyrosine Nitration and Disease

3-Nitrotyrosine has been detected in injured tissues for a wide variety of disease states. These include neurodegenerative diseases,[87, 88] acute lung injury,[89] atherosclerosis,[90, 91] bacterial and viral infections,[92, 93] and chronic inflammation.[94] Tyrosine nitration appears to represent a prominent pathway of pathophysiological protein modification under inflammatory conditions with increased expression and/or activity of NO synthases.[69, 95] These diseases often involve increased plasma levels of cytokines and endotoxin, which increase the expression of inducible NO synthase in many different cell types, causing a high output of NO over relatively long periods of time.[96]

Nitration of tyrosine residues has been shown to alter the functional properties of a variety of proteins, and it is proposed to be causally involved in the pathogenesis of certain diseases.[69, 97] Nitration of cardiac actin may impair contractile function of the heart in myocarditis.[98] Nitration of human neurofilament L has been shown to interfere with filament polymerization in amyotrophic lateral sclerosis.[69] Nitration of surfactant protein A impairs its ability to aggregate lipids and to bind mannose, two functions that are essential for building a functional surfactant monolayer at the air–water interface in the lung.[89, 99] In addition to these structural proteins, tyrosine nitration may affect the function of enzymes, including Mn-SOD[100, 101] and glutamine synthetase.[102, 103] Nitration of tyrosine residues can also disrupt signal transduction cascades that depend on reversible phosphorylation of tyrosine.[104–106] Potentially, many more proteins could be affected: 140 mammalian proteins are known whose activity is dependent upon tyrosine residues.[107] The main culprit in these phenomena has been believed to be peroxynitrite, the product of the reaction of NO with superoxide. As a basis for discussion of these ideas, we first need to consider the basic chemistry of peroxynitrite.

2.2.5. What Nitrates Tyrosine?

In vitro, 3-nitrotyrosine can be formed by the reaction of tyrosine with a variety of nitrogen oxide species (NO_x), such as peroxynitrite, nitrogen dioxide (NO_2), nitrous acid (HNO_2), nitronium ion (NO_2^+), and nitryl chloride (NO_2Cl).[97] NO by itself does not directly nitrate tyrosine,[69, 97] but can cause nitration in the presence of additional oxidants that generate tyrosyl radicals. The second-order reaction of NO with tyrosyl radicals ($k > 10^9\,M^{-1}s^{-1}$)[108] leads to formation of *C*-nitroso and/or *O*-nitrosotyrosine addition products, which can be converted into 3-nitrotyrosine in a two-electron oxidation reaction.[109, 110] NO_2 formed by the autoxidation of NO has been shown to nitrate tyrosine residues in proteins in vitro.[111] At micromolar concentrations of NO, the third-order autoxidation reaction[7] is certainly not fast enough to generate sufficient amounts of NO_2 to explain 3-nitrotyrosine formation in cells. Also, small amounts of ascorbate or thiols have been shown to strongly inhibit tyrosine nitration by NO_2.[111] However, recent data indicate that the reaction of NO with O_2 is about 300-fold faster in the hydrophobic phase of biological membranes, so that these may be important sites for formation of NO-derived reactive species.[112] Under acidic conditions ($pH < 2$) the stable end product of NO autoxidation, NO_2^-, forms nitrous acid (HNO_2), which is also capable of nitrating tyrosine residues. Such a reaction might occur at the low pH values present in the stomach,[113] but in most other tissues the pH probably never becomes sufficiently low to promote significant protonation of NO_2^-.

Of these nitrogen oxide species, peroxynitrite is postulated to make the largest contribution to the pathogenesis of inflammatory and infectous disorders.[97] Peroxynitrite-dependent nitration has been extensively studied in vitro by bolus addition of synthetic peroxynitrite from alkaline stock solutions.[97, 114, 115] It has been pointed out that this approach may be a poor approximation to in vivo situations, in which the peroxynitrite is formed by continuous reaction of NO and $O_2^{\bullet-}$ at physiological pH.[116, 117] Recently, methods have been developed that should simulate these conditions more closely, by combining NO-generating and superoxide-generating systems. Surprisingly, tyrosine nitration by NO and $O_2^{\bullet-}$ generated simultaneously at equal rates was about 100-fold less efficient than the reaction triggered by synthetic peroxynitrite.[117] These results may reopen the question whether peroxynitrite is really the main mediator of tyrosine nitration in vivo.

Some alternative mechanisms of tyrosine nitration involve myeloperoxidase, which is secreted by monocytes and polymorphonuclear neutrophils under inflammatory conditions. Myeloperoxidase generates tyrosyl radicals[118] which can be trapped by NO, leading to formation of 3-nitrotyrosine in the presence of additional oxidants, for example H_2O_2.[110] This mechanism could account for the extensive tyrosine nitration observed in atherosclerotic lesions of blood vessels.[91, 119] In the presence of H_2O_2, myeloperoxidase may also cause nitration by oxidation of NO_2^- to NO_2.[120] Finally, myeloperoxidase catalyzes formation of hypochlorous acid (HOCl), which reacts non-enzymatically with NO_2^- to form the potent nitrating agent nitryl chloride (NO_2Cl).[121, 122] Thus, tyrosine nitration may become significant at sites where NO synthase (NOS) is activated together with oxidative pathways generating tyrosyl radicals and H_2O_2 or other oxidants. The presence of myeloperoxidase appears to be important, but would not be obligatory if other enzymatic pathways acting in a similar manner were activated together with NO synthase in tissue inflammation. The origins of tyrosine nitration and its relevance to particular disease states seem likely to occupy researchers for some time to come.

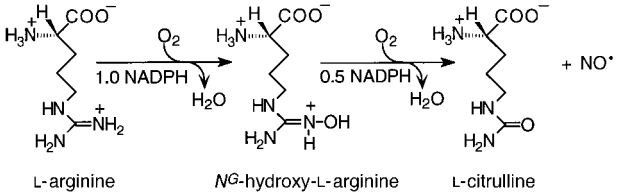
2.2.6. Other Deleterious Effects of Peroxynitrite

In addition to tyrosine nitration, some other deleterious reactions have been attributed to peroxynitrite. Being a strong

oxidant, it can cause DNA strand breaks. In response, cells activate the nuclear enzyme poly(ADP-ribose) synthetase (PARS; E.C. 2.4.2.30).[123] However, this can deplete the intracellular pool of reduced nicotinamide adenine dinucleotide, causing breakdown of energy metabolism and ultimately cell death. Peroxynitrite-mediated activation of PARS has been implicated to importantly contribute to the cellular injury in endotoxic shock, inflammatory pancreatic islet cell destruction, and central nervous system ischemia. Another damaging effect of peroxynitrite is its ability to oxidize hemoproteins.[48]

3. Enzymology of NO Synthases

Scheme 4 shows the biosynthesis of NO catalyzed by the three isozymes of NO synthase (NOS) listed in Table 2. The structure and mechanism of the three isozymes are basically similar, but they display adaptations that allow different modes of regulation.



Scheme 4. The NO synthase reaction.

Neuronal NOS (nNOS) is expressed in postsynaptic terminals of neurons in the brain, where it is activated by Ca^{2+} influxes caused by binding of the neurotransmitter glutamate to receptors in the cell membrane: Here, the NO produced contributes to long-term potentiation, a phenomenon that may underlie learning and memory formation. In peripheral nerves, nNOS is also activated in response to Ca^{2+} influx, this time through voltage-gated Ca^{2+} channels opened by a membrane depolarization. In these nitrergic neurons, NO is the neurotransmitter that triggers the response in the target organ (blood vessels, gastrointestinal tract, corpus cavernosum of the penis, etc.); these responses are mediated by NO activating sGC (see Section 2.1.6).

Endothelial NOS (eNOS) is expressed in endothelial cells lining blood vessels and produces the endothelium-derived relaxing factor (EDRF). NO diffuses into the smooth muscle cells of the blood vessel wall and elicits a cGMP-dependent relaxation, facilitating increased blood flow through the vessel. This was the first signaling role of NO to be discovered.[124–126]

Inducible NOS (iNOS) is expressed in macrophages in response to inflammatory cytokines and other stimuli associated with infection. In this case, NO is produced as one of a palette of toxic compounds used to inactivate pathogens.

This rough sketch of the main roles and locations of the NO synthases is helpful for basic orientation, but is somewhat oversimplified. Thus it should be remembered that eNOS is present in blood vessels in the brain and can therefore influence events in the adjacent neurons; it has also been discovered inside some neurons[127] and in cardiomyocytes.[128] iNOS is not only present in activated macrophages, but its synthesis can be induced in smooth muscle,[129, 130] cardiac muscle,[131, 132] liver,[133] and glial cells.[134]

3.1. Domain Architecture

The active form of each NO synthase isozyme is a homodimer. Each subunit consists of a C-terminal reductase domain and an N-terminal oxygenase domain (Figure 1);

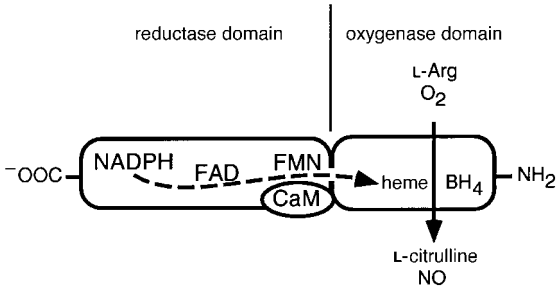


Figure 1. Domains and cofactors of NO synthase. The dashed arrow shows the path of electron transfer.

these domains remain folded independently of each other. The isolated oxygenase domain remains homodimeric whereas the isolated reductase domain is monomeric, showing that

Table 2. The isozymes of NO synthase.

NOS isozyme	Alternative descriptions	M_r [kDa]	Distinctive properties	Subcellular localization	Tissue expression
neuronal	type I, nNOS, ncNOS, bNOS	160	Ca^{2+}-dependent, constitutively expressed	binds to specific membrane proteins through an N-terminal PDZ domain[237]	neuronal cells, skeletal muscle
endothelial	type III, eNOS, ecNOS	134	Ca^{2+}-dependent, constitutively expressed	targets to the Golgi and to caveoli through N-terminal myristoylation and palmitoylation	endothelial cells, epithelial cells, cardiomyocytes, some neurons
inducible	type II, iNOS, macNOS	130	Ca^{2+}-independent, induced by inflammatory stimuli (cytokines, LPS[a])	soluble?	macrophages, hepatocytes, astrocytes, smooth muscle cells, and many more

[a] LPS = lipopolysaccharides.

the dimeric interface of the enzyme is formed by the oxygenase domain.[135, 136] The reductase contains one molecule each of bound FAD and FMN, and has a binding site for NADPH.[137] The oxygenase contains a heme group and one binding site each for the pteridine cofactor tetrahydrobiopterin and the substrate L-arginine. Between the reductase and oxygenase domains is a binding site for calmodulin.[137, 138] On the N-terminal side of the oxygenase domain, each isozyme has an extension that is not essential for the enzyme activity, but may have a role in positioning the enzyme correctly within the cell.

3.1.1. The Reductase Domain

Like the homologous protein cytochrome P450 reductase, the NOS reductase domain is made up of nucleotide-binding modules that bind NADPH, FAD, and FMN.[137, 139] The only break in the homology to P450 reductase is an insert of about 45 amino acid residues within the FMN-binding region; this insert is present in eNOS and nNOS, but absent in iNOS.[140] The function of the reductase domain is to shuttle electrons from NADPH to the oxygenase domain. This electron transfer activity requires the binding of calmodulin to its site between the oxygenase and reductase domains.

3.1.2. The Reductase – Oxygenase Interface

NOS is the only P450 heme containing enzyme in animals that has a dedicated reductase as an integral part of the protein (another, cytochrome P450 BM-3, occurs in bacteria[141, 142]), suggesting that a special oxygenase – reductase interface is crucial to its function. Indeed, attempts to reconstitute NOS using isolated oxygenase and reductase domains resulted in low activity.[143] Both EPR[144] and time-resolved fluorescence experiments[145] indicated a structural linkage of the heme to the flavins. No crystal structure of a full-length NOS has been published yet, so that the three-dimensional orientation of the domains is still a matter of conjecture. However, a pair of recent papers[146, 147] indicated that the dimeric structure of the enzyme is important for reductase – oxygenase communication. Both depended on the construction of heterodimers of iNOS in which one subunit was full-length, whereas the other consisted only of the oxygenase domain. In the first study it was shown that maximally one heme per heterodimer could be reduced, suggesting that the single reductase domain could only reduce one of the two heme groups.[146] In the second,[147] a mutation[148] in the oxygenase domain was used to disrupt L-arginine binding to one of the subunits (Figure 2). If the mutation was in the full-length subunit (Figure 2 A), the activity of the heterodimer was unaffected. If it was in the oxygenase-only subunit (Figure 2 B), the activity was abolished. Thus it was concluded that the reductase domain can only reduce the heme of the opposite subunit.

3.1.3. The Oxygenase Domain

The CO complex of reduced NOS has a strong absorbance band at 446 nm, like the cytochromes P450, indicative of a proximal thiolate ligand.[149–151] The ligation is somewhat modified (as in chloroperoxidase) by extra hydrogen bonds to the proximal ligand, as revealed by resonance Raman spectroscopy[152] and the recent crystal structures.[153, 154] A view of the crystal structure of the iNOS oxygenase dimer is shown in the frontispiece on page 1714 (more images can be seen at http://www.scripps.edu/~jat/nos.html). The heme is cupped within an assembly that is comparable to a left-handed baseball catcher's mitt. The "thumb" (bright green) is formed by an α helix and loop carrying the heme proximal ligand cysteine. The distal side of the heme is shielded by a winged β sheet corresponding to the palm and fingers (cyan in the lower subunit, orange in the upper one).

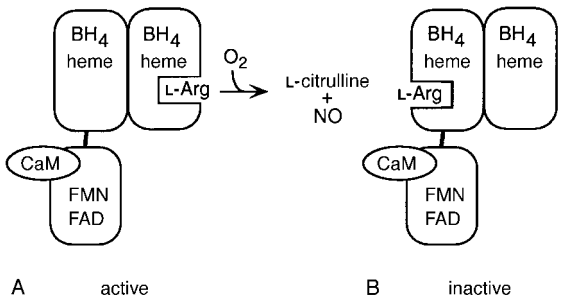


Figure 2. Domain swapping in NO synthase. A) When the L-arginine binding site and the reductase domain are on opposite subunits, NOS activity is observed. B) When L-arginine can only bind to the subunit that contains a reductase domain, no NOS activity is observed.

The large content of β sheets is very unusual among hemoproteins. This restricts the scope for movements based on sliding of secondary structural elements, often exploited in α-helical proteins, for example in substrate binding by P450s.[155] While this article was in preparation, a crystal structure of the eNOS oxygenase was published, confirming that the NOS isoenzymes share the same fold.[156]

3.1.4. Heme and Dimerization

The native form of NOS is a homodimer. However, under conditions of heme deficiency, monomeric forms of the enzyme have been observed.[157, 158] They contained no heme and could bind neither H_4biopterin (see Section 3.2.3) nor L-arginine,[158] but their flavin content and cytochrome c reductase activity (see Section 3.1.1) were normal. In the case of iNOS, dimerization was observed on incubating the subunits simultaneously with haem, H_4biopterin, and L-arginine;[157] for nNOS, heme alone seemed to be sufficient.[158] Both isozymes could be reactivated.[157, 159] Heme binding did not drastically affect the UV CD spectrum (a measure of secondary structure content),[158] but did cause strong quenching of tryptophan fluorescence.[159] Similar quenching was observed with PPIX, Mn-PPIX, or Co-PPIX. Of these, only Mn-PPIX stimulated dimerization. It was also the only one for which absorbance spectra (by comparison with metal-substituted $P450_{cam}$) suggested the acquisition of a thiolate ligand from the enzyme. It was proposed that the picking-up of the thiolate ligand by the heme metal could be the trigger for dimerization.[159]

3.2. Ligand Binding and Allosteric Effects

3.2.1. Ca^{2+}/CaM

In nNOS and eNOS, only the Ca^{2+}-bound form of calmodulin can bind and activate the enzyme.[160, 161] In contrast, iNOS already binds CaM, and is fully active at such low calcium concentrations that its activity in vivo can never be limited by Ca^{2+}.[162] This difference between iNOS and the other isozymes has been ascribed both to the presence of the 40-residue insert in the FMN domains of nNOS and eNOS and to different characteristics of the CaM-binding sequences. Synthetic peptides corresponding to the insert in the FMN domain inhibited the enzyme and displaced CaM from its binding site.[140] Several other CaM-dependent enzymes contain a flexible loop that occupies the CaM-binding site in the absence of Ca^{2+}/CaM, and must be displaced in order for the enzyme to be activated. It was suggested that the insert may play a similar role in NOS.

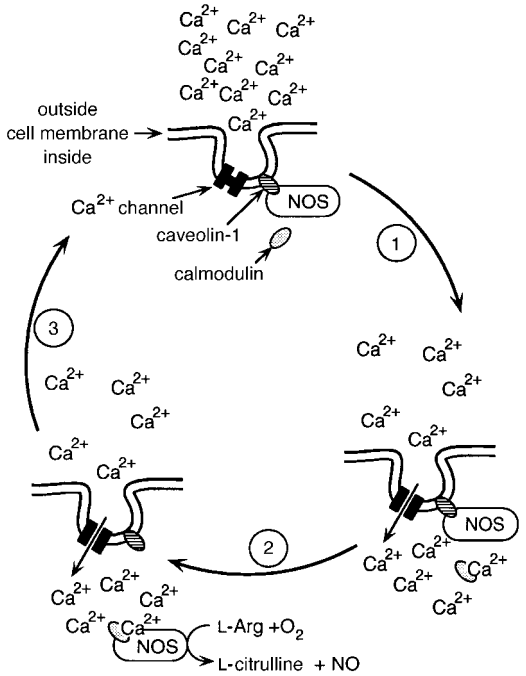
Another study showed, however, that the Ca^{2+}-independent binding of CaM to iNOS cannot be explained merely by the absence of this inhibitory loop.[163] A series of chimeric enzymes were made in which the CaM-binding sequences were exchanged between isozymes. The modified eNOS containing the CaM-binding site from iNOS bound CaM Ca^{2+}-independently, but still required Ca^{2+} to become active. This suggests that the sequence of the CaM-binding site determines the nature of CaM binding, whereas the inhibitory insert confers Ca^{2+}-dependent activation.

Binding of calmodulin improves electron flow from NADPH to the flavins.[164] It also facilitates electron transfer from FMN to the heme,[165] though in the native enzyme this does not seem to be the rate-limiting step.[166] The reductase can also catalyze reduction of external electron acceptors such as oxidized cytochrome c;[167] this reaction is also largely dependent on calmodulin binding. The nature of the change induced by CaM that allows electron transfer is still poorly understood.

3.2.2. Caveolin

Another mode of regulation of NOS activity has come to prominence in the last couple of years. Caveolae are specialized invaginations of the plasma membrane found in many cell types.[168] They are organized by the protein caveolin, of which there are three subtypes, caveolin-1, caveolin-2, and caveolin-3.[169] Caveolin is proposed to be a generalized negative regulator of signal transduction that sequesters signaling proteins in an inactive form at the cell membrane, ready to respond to an incoming signal. In endothelial cells, a fraction of the eNOS is found in the caveolae.[170] Treatment of the cells with agents that cause a calcium influx into the cells leads to dissociation of the eNOS from the caveolae and its activation (Scheme 5). The association with the caveolae is also largely dependent on fatty acylation of the eNOS at sites near its N terminus.[171]

Studies on purified eNOS have found effective inhibition by a few micromolar caveolin that was reversed by addition of Ca^{2+}/CaM.[172] A peptide corresponding to residues 82–101

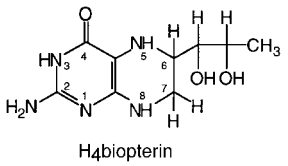


Scheme 5. Regulation of NO synthase by caveolin and calmodulin. 1) A Ca^{2+} channel in the membrane opens. 2) The rising initial concentration of Ca^{2+} allows calmodulin to compete with caveolin for NOS; NOS dissociates from caveolin and becomes active. 3) When the Ca^{2+} signal subsides, the equilibrium shifts back in favor of NOS binding to caveolin.

of caveolin-1 inhibited eNOS as effectively as the full-length protein. The peptide inhibited the cytochrome c reductase activity (though not ferricyanide reductase activity) with an IC_{50} of 3 μM, identical to that for the complete NOS reaction.[173] Hydrogen peroxide dependent conversion of NOHLA into L-citrulline, a reaction that can be catalyzed by the oxygenase alone,[174] was not inhibited. Immunoblotting showed that both the isolated reductase and oxygenase domains bound to immobilized caveolin-1, although the binding of the oxygenase domain was weaker. Thus caveolin-1 appears to inhibit NOS by interfering with electron transfer within the reductase domain. It is not yet clear whether the additional, apparently noninhibitory, binding to the oxygenase is functionally significant.

3.2.3. Negative Cooperativity of H_4biopterin Binding

Purified NOS enzymes usually contain one molecule of tightly bound H_4biopterin per heme, yet in the presence of exogenously added H_4biopterin, they can be activated a further twofold, suggesting the presence of a second H_4biopterin binding site.[175] This was clarified using a H_4biopterin-free version of the enzyme, prepared by expressing nNOS under conditions of low pteridine availability.[176] Binding studies with radiolabeled H_4biopterin and L-NNA (an L-arginine analogue) provided clear indications that H_4biopterin binding occurs in two steps with about a 1000-fold difference in



affinity. The first molecule of H_4biopterin binds with an affinity in the subnanomolar range, whereas the second molecule binds with an affinity of around 1 μM.

This negative cooperativity of H_4biopterin binding suggested a strong structural communication between the subunits. This was probed further in a study using 1,4-dithiothreitol (DTT).[177] Inhibition of nNOS containing one equivalent of H_4biopterin per dimer was monophasic and competitive with respect to L-arginine, with a K_i of about 10 mM. In contrast, binding of DTT (as measured by the absorbance of the bisthiolate complex, absorbing at 456 nm) had an additional, L-arginine-independent component at higher affinity. H_4biopterin-free NOS bound DTT monophasically at the higher affinity. Thus, in NOS containing one molecule of bound H_4biopterin per dimer, NO synthesis at the H_4biopterin-containing subunit was not affected by DTT binding to the opposite subunit. In this sense, the hemes of the two subunits function independently of one another.

3.2.4. H_4biopterin, L-Arginine, and the Heme Spin State

Like the P450s, the heme of NOS can exist in either high-spin, pentacoordinate or low-spin hexacoordinate states.[151, 176, 178] In contrast to most P450s, in which the high-spin state only appears upon substrate binding,[155] the haem of NOS as isolated is usually already mainly in the high-spin form. The reason for this was revealed by studies on H_4biopterin-free nNOS, which was found to have a substantially higher low-spin content but converted into high spin after addition of H_4biopterin.[176] The conversion of nNOS from low to high spin was rather slow (about 0.1 min^{-1} at 25 °C), allowing its comparison with enzyme activity. The gain of activity after addition of H_4biopterin to pteridine-free nNOS correlated exactly with the shift to high spin. Interestingly, the rate of conversion was independent of the concentration of added L-arginine or H_4biopterin, suggesting a slow spin-state equilibrium that determines substrate and cofactor binding. In other words, dissociation of the distal ligand (probably water, by analogy to P450s[179]) is a precondition of L-arginine or H_4biopterin binding. This also seems to hold for eNOS[180] and iNOS (though pteridine-free iNOS has a higher high spin content than nNOS).[181]

Surprisingly, imidazole can bind to the heme of nNOS to form a low-spin complex in the presence of H_4biopterin, although its binding is competitive with respect to L-arginine.[182] The crystal structures have provided an explanation, in that H_4biopterin binds on the proximal side of the porphyrin plane, whereas L-arginine as expected binds on the distal side (see the frontispiece on page 1714).[154] Thus, the loss of the distal ligand is more directly coupled to L-arginine than to H_4biopterin binding. Indeed, the recent crystal structure of the eNOS oxygenase domain revealed no significant conformational differences between the H_4biopterin-free and H_4biopterin-bound forms.[156] Thus the linkage of H_4biopterin binding to spin state may be electrostatic rather than steric in nature.

In both isoenzymes hydrogen bonds were identified from N3 (directly) and the carbonyl O atom at C4 (via a water molecule) of H_4biopterin to a heme propionate, which in turn is hydrogen bonded to the α-amino group of the substrate L-arginine.[154, 156] Also, the carbonyl O atom at C4 and N5 of the pteridine have hydrogen bonds to the helix that defines the substrate-binding channel.[154] This network of interactions may account for effects of H_4biopterin on both the coordination behavior of the heme and on L-arginine binding without a large movement of the protein.

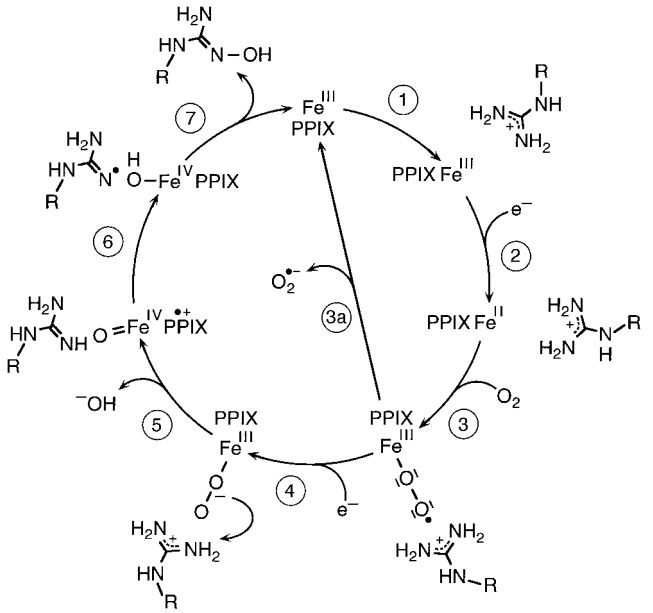
3.2.5. Dimer Stabilization

Another structural correlate of H_4biopterin and L-arginine binding is the stabilization of the NOS homodimer. In sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis, NOS migrates according to the relative molecular mass (M_r) of the monomeric subunits—as would be expected in this method, which normally involves heating the samples briefly to 95 °C to ensure full denaturation in the detergent. However, if the enzyme is preincubated with L-arginine and/or H_4biopterin, and the heating step before electrophoresis is omitted, a substantial fraction of the enzyme resists dissociation by the SDS.[180, 181, 183] This unusual stabilization may be accounted for by the large buried surface area in the subunit interface (2800 $Å^2$),[154] and by the fact that each H_4biopterin molecule makes some contacts with the opposite subunit. A new factor to be considered in NOS dimer stability is suggested by the discovery in the eNOS oxygenase structure of a zinc ion coordinated by two cysteine residues from each subunit.[156]

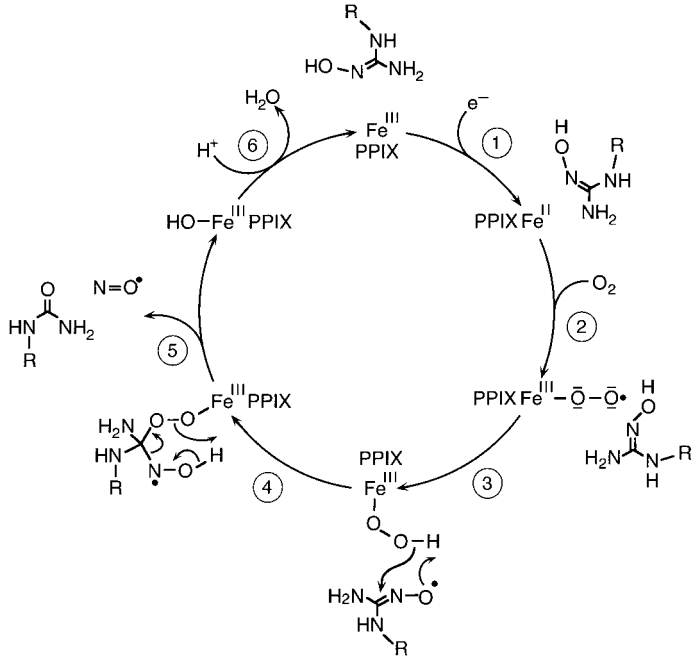
3.3. NOS Catalysis

NOS is the only known heme iron dependent H_4biopterin enzyme. However, apart from NOS, four enzymes are known that use H_4biopterin as a cofactor: phenylalanine monooxygenase, tyrosine 3-monooxygenase, tryptophan 5-monooxygenase, and glycerylether monooxygenase. In these enzymes H_4biopterin is directly involved in the hydroxylation of the substrate, being oxidized in the process to H_2biopterin, which is recycled to H_4biopterin by dihydropteridine reductase.[184, 185] In contrast the NOS reaction does not result in net oxidation of H_4biopterin.[186] Also, H_4biopterin binds too far away, and on the wrong side of the porphyrin, to participate as a hydroxylating cofactor at the distal side of the heme iron center.[154]

Current discussion of the NOS mechanism is often based on concepts derived from P450 research.[187–189] This is justified partly by the involvement of the thiolate-ligated heme of NOS in both stages of the reaction,[149, 190] and partly by results on analogous reactions of guanidines and *N*-hydroxyguanidines catalyzed by classical P450s. However, mechanisms such as we present in Schemes 6 and 7 should be handled with caution. Even for P450s, after many years of sophisticated investigations, crucial steps of the reaction are still the subject of much debate.[155] For NOS, though we can report rapid progress, the direct evidence on details of the reaction is still fragmentary. Nonetheless, recent work has begun to provide intriguing hints, particularly about the unconventional role of the pteridine cofactor.



Scheme 6. Hydroxylation of L-arginine. See text for details.



Scheme 7. Oxidation of NOHLA to L-citrulline and NO. See text for details.

3.3.1. What Does H_4Biopterin Do?

H_4biopterin is certainly an allosteric activator of NOS, with conformational effects reported by L-arginine binding, the heme spin conversion, and dimer stabilization (see Sections 3.2.3 and 3.2.4). Functionally, it is needed for coupling of NADPH oxidation to NO synthesis. The H_4biopterin-free rat nNOS catalyzes efficiently oxidation of NADPH and production of H_2O_2 (step 3a in Scheme 6). This reaction continues in the presence of L-arginine. When the enzyme in the presence of a constant L-arginine concentration (100 μM) is titrated with H_4biopterin, there is a progressive and quantitative shift from H_2O_2 production to citrulline production by the enzyme (Figure 3);[176, 191] Thus, H_4biopterin is truly indispensible for the correct outcome of the reaction: In terms of Scheme 6,

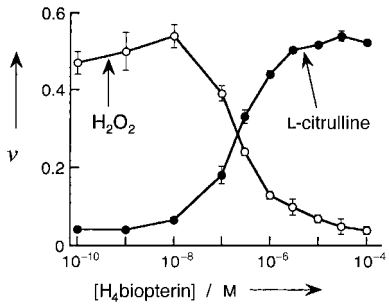
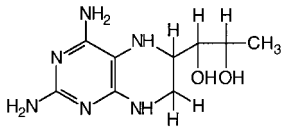


Figure 3. Effect of H_4biopterin concentration on coupled and uncoupled reactions of NO synthase (reproduced from reference [176]). Pteridine-free rat nNOS was titrated with H_4biopterin in the presence of 100 μM L-arginine, Ca^{2+}, CaM, and NADPH. Formation of H_2O_2 was monitored by absorbance at 492 nm and was corrected for CaM-independent rates. The coupled NOS reaction was assayed by measuring the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline. The rate of formation of H_2O_2 or L-citrulline (v) is given in μmol min^{-1} mg^{-1}.

H_4biopterin is needed for step 4 to outcompete step 3a. Probably the hottest issue in NOS catalysis currently is whether this shift is the result of a chemical involvement in the reaction—perhaps as a transient electron donor—or just another aspect of the allosteric role of H_4biopterin, perhaps adding a crucial final adjustment to the spatial juxtaposition of the substrate, the heme, and the protein.

One approach to solving this question is the study of analogues of H_4biopterin and their effects on the NOS reaction. For example, H_2biopterin binds with considerably lower affinity, and even at saturating concentrations does not activate the enzyme.[192, 193] Thus the tetrahydro form seems to be necessary. One analogue, 4-amino-H_4biopterin (for the numbering of atoms in H_4biopterin, see Section 3.2.3), has been found that binds with even higher affinity than H_4biopterin, and brings about, as far as current assays can measure, the same allosteric changes.[181, 194, 195] Although it exists in the tetrahydro state (and can be oxidized) 4-amino-H_4biopterin does not support NOS activity; in fact, it inhibits both L-arginine hydroxylation and the conversion of NOHLA into L-citrulline and NO. Among the possible reasons why it does not support the NOS reaction are 1) subtle structural changes; 2) that although it can undergo reversible oxidation, some aspect of its redox mechanism is incompatible with the NOS reaction (for example, it might be less amenable to reversible one-electron oxidation than H_4biopterin); and 3) that the charge distribution on the pteridine is important in fine-tuning the heme redox potential, which is coupled to the protonation state of the propionates and is likely to be sensitive to the hydrogen bonds from N3 and carbonyl O atom at C4 of the pteridine.[154]



4-amino-H_4biopterin

3.3.2. Regulation of Heme Reduction

Reduction of the heme (step 2 in Scheme 6) is controlled by the supply of electrons from the flavins (see Section 3.2.1), but also by adjustment of the heme redox potential. P450s

generally use the binding of substrate to raise the heme midpoint potential into the range where reduction becomes possible.[196] This avoids running down the cellular stores of reducing equivalents only to produce harmful reduced oxygen species. iNOS similarly links heme reduction to binding of L-arginine and H_4biopterin.[197] In contrast, the heme midpoint potential of nNOS is higher in the absence of L-arginine, and is not significantly altered by L-arginine and H_4biopterin binding.[197] These findings would explain the previous observation that of all three isozymes, nNOS has by far the highest NADPH oxidase activity in the absence of substrate and cofactor.

3.3.3. Oxygen Activation and L-Arginine Hydroxylation

Two groups have observed spectral intermediates formed after addition of oxygen to ferrous nNOS (steps 3–5 of Scheme 6); Abu-Soud et al.[198] observed a complex absorbing at 427 nm by the stopped-flow technique at 10 °C, whereas Bec et al.,[199] in a study done at −30 °C, found an intermediate with the Soret maximum at 416 nm. The reason for the discrepancy is not known; most ferrous-oxy P450s absorb maximally at around 418–420 nm. In the stopped-flow study, L-arginine inhibited the decay of the 427-nm complex, whereas H_4biopterin accelerated it by 70-fold.

In the low-temperature study, a further complex absorbing at 405 nm was observed, but only in the presence of both L-arginine and H_4biopterin as well as dithionite-reduced heme. Hydroxylation of L-arginine was also observed only under these conditions, suggesting that this complex might be an intermediate on the reaction pathway. It was suggested to be an oxo-ferryl complex (product of step 5 in Scheme 6), but further spectral analysis is necessary for a firm assignment.

The supply of protons to the active site may also be crucial to fission of the dioxygen bond (step 5 in Scheme 6): In the structure with bound L-arginine, the guanidino group is well shielded from solvent. It was suggested that one of the protons may be donated by the substrate itself.[154]

Though the hydroxylation of L-arginine is formally similar to that of P450—in that it requires one equivalent of NADPH and results in the incorporation of one oxygen atom from O_2 into the product—it has never been found to be catalyzed by a classical P450.[200, 201] Interestingly, P450-dependent hydroxylation of at least one non-physiological guanidine has been observed, showing that this type of reaction is chemically feasible without H_4biopterin, though apparently at very low rates.[202] Again, one could suppose that H_4biopterin helps poise a heme–oxygen intermediate at a more favorable redox potential for attack on the L-arginine, but a more radical deviation from a P450 paradigm cannot be ruled out.

3.3.4. From N^G-Hydroxy-L-arginine to L-Citrulline and NO

We must emphasize at the beginning of this section that the second stage of the NOS reaction, from NOHLA to L-citrulline and NO (see Scheme 7) is also completely dependent on H_4biopterin.[195] That similar reactions can occur non-enzymatically[203, 204] or be (very inefficiently) catalyzed by H_4biopterin-independent enzymes[200, 201] should not obscure this.

This stage requires the oxidation of only 0.5 equivalents of NADPH.[189, 205] This contrasts with the hydroxylation of L-arginine by NOS and with P450 reactions, in which a further reduction of the heme is needed to achieve oxygen fission (steps 4 and 5 of Scheme 6). However, $O_2^{\bullet-}$ has been shown to oxidize some *N*-hydroxyguanidine model compounds to urea products with release of NO, and the Fe^{III}-O-O$^\bullet$ complex that would be formed after one-electron reduction of the heme represents a nearly equivalent redox state of oxygen to $O_2^{\bullet-}$.

NOHLA itself does not reduce the ferric iron of NOS;[206] therefore the reaction is likely to be initiated by NADPH-dependent reduction of the iron center (step 1 of Scheme 7) followed by O_2 binding (step 2 of Scheme 7). It was suggested that an electron transfer from NOHLA to the Fe^{III}-O-O$^\bullet$ complex might then occur, leading to the formation of Fe^{III}-O-O$^-$ and the radical cation NOHLA$^{\bullet+}$.[187, 207] Countering this proposal, it was pointed out, with the aid of an electrochemical experiment on NOHLA, that the formation of the radical cation would be very unlikely, and that a hydrogen-atom abstraction would be more feasible (step 3 in Scheme 7).[188]

3.3.5. Recognition of L-Arginine Analogues

In the iNOS crystal structure,[154] specific binding of the guanidino group of L-arginine, involving hydrogen bonds with the carboxylate group of E371, can be seen. This is in agreement with the earlier result that the mutation E371A disrupts L-arginine binding. However, several modifications to the guanidino group are tolerated by the enzyme and even increase the binding affinity, giving rise to a series of effective inhibitors that are competitive with L-arginine (Figure 4).

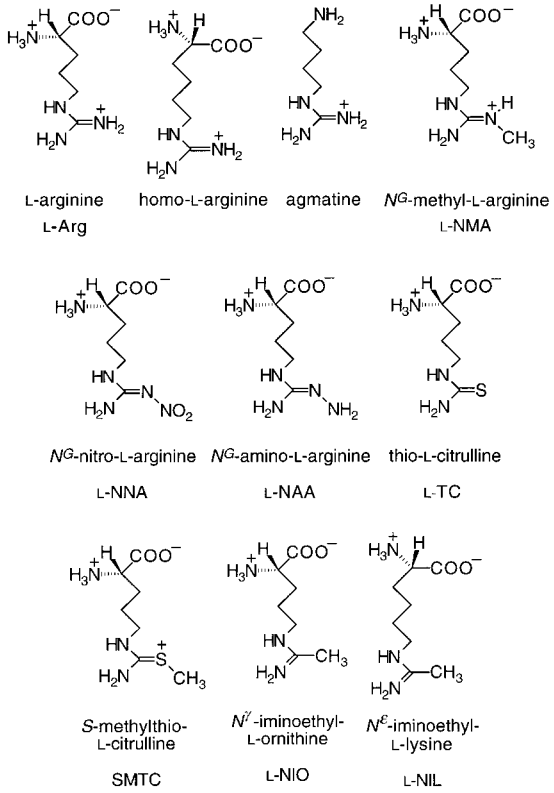


Figure 4. Schematic representations of L-arginine and analogues that are competitive with L-arginine.

Recognition of the α-aminopentanoic acid part of the substrate is also important: Besides L-arginine, only homo-L-arginine and agmatine are detectably active as NOS substrates, whereas neither guanidine nor hydroxyguanidine is a substrate.[208–210] Removal of the α-aminopentanoic acid part of L-NMA, L-NAA, and L-NNA to give the corresponding guanidine derivative also resulted in a loss of binding affinity.[208, 209] Another series of inhibitors is based on L-thiocitrulline. In this case, the removal of the α-aminopentanoic acid portion to give the corresponding isothiourea derivative increases the affinity.[211, 212] An iNOS crystal structure with bound L-TC has been determined,[154] but structures with isothiourea derivatives will be needed before this change in affinity can be understood.

Apart from the competition with L-arginine, these inhibitors have diverse additional effects. Some analogues (L-NMA,[213, 214] L-NIL,[215] L-NIO[216]) exhibit mechanism-based inhibition, causing irreversible inactivation when both oxygen and NADPH are present.[210] L-NMA[213, 214, 217] and L-NIL allow NADPH oxidation (albeit with progressive inactivation), whereas the others inhibit it. The same pattern was observed with the corresponding guanidine analogues, without the α-aminopentanoic acid part of the substrate.[209, 211] Inhibition of NADPH oxidation has been suggested to be due to an electrostatic effect of an electron-rich substituent on the guanidine/isothiourea group on the heme redox potential. Another factor that may be relevant is the access of oxygen to the heme: Binding of CO, for example, is slowed by the presence of L-arginine in its binding site.[218] Analogues that inhibit H_2O_2 formation do not necessarily prevent reduction of the heme: Aminoguanidine allows NADPH-dependent heme reduction, whereas L-NNA blocks heme reduction even by dithionite.[197]

3.3.6. Isozyme-Specific Inhibitors

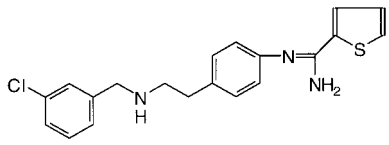
A major difficulty of all NO-related drugs is to avoid crossover between different physiological functions of NO. It is hoped that isozyme-specific NOS inhibitors will offer a way to avoid analogous problems with drugs aimed at suppressing NO biosynthesis. Selective nNOS inhibitors might help to alleviate ischemic brain damage: Mice lacking nNOS were more resistant to experimentally induced stroke.[219] Selective iNOS inhibitors would have a wider range of possible indications, including, for example, the severe hypotension in septic shock, chronic inflammatory diseases such as rheumatoid arthritis, and adverse immune reactions associated with transplantation. As a rule of thumb, lead compounds should have 100-fold isozyme selectivity and a submicromolar IC_{50}.

Most of the L-arginine analogues mentioned in Section 3.3.5 exhibit only marginal selectivity between isozymes. One exception is L-NIL, which is about 30-fold more selective for iNOS[215] and was effective in both adjuvant-dependent arthritis and leishmaniasis models in mice.[220, 221] The substituted isothioureas are also mostly nonselective,[211] though *S*-methylisothiourea is about 20-fold selective for iNOS,[222] whereas 1-amino-*S*-methylisothiourea is selective for nNOS (8-fold versus iNOS and 34-fold versus eNOS) in vitro.[223]

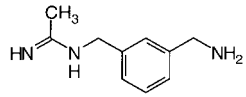
7-Nitroindazole is selective for nNOS in vivo, and is protective in experimental stroke[224] as well as having antinociceptive activity. However, it inhibits the purified NOS isozymes with about equal potency. Its selectivity may be due to differences in its uptake or metabolism in different tissues, or to its unusual mode of inhibition: It competes with both H_4biopterin and L-arginine binding.[225] Thus the nNOS selectivity could be due to relatively low concentrations of L-arginine and H_4biopterin in certain neurons.

4-Amino-H_4biopterin (see Section 3.3.1) exhibits little isozyme selectivity with the purified enzymes,[181, 194, 195] but appears to be selective for iNOS in cultured cells.[226] It was speculated that this may be because 4-amino-H_4biopterin does not readily replace tightly bound endogenous H_4biopterin from the enzyme,[194] so that NOS would be most sensitive to the inhibitor during de novo protein synthesis. This effect would cause more efficient inhibition of the iNOS, because this isozyme is synthesized rapidly on induction, whereas the other isozymes are subject to much slower protein turnover.[3, 195]

The most selective nNOS inhibitor available at present(100-fold) is the Astra Arcus compound ARL 17477. It substantially reduced the volume of infarcts caused by



ARL 17477



1400W

temporary occlusion of the middle cerebral artery in rats. The doses used caused nearly complete inhibition of cortical NOS activity, but did not affect mean arterial blood pressure.[227]

The most selective iNOS inhibitor reported to date is the Glaxo Wellcome compound 1400W (*N*-(3-aminomethyl)benzyl)acetamidine).[228] It exhibits rapidly reversible, low-potency ($K_i \approx 2-50$ μM) inhibition of all NOS isozymes, but an additional slow phase of binding to iNOS has an estimated binding constant of 7 nM. This slow phase required the presence of NADPH, but did not appear to involve chemical conversion of the inhibitor. In endothelium-intact rat aortic rings, it caused only a slight contraction at 300 μM, reflecting a lack of inhibition of eNOS. In contrast, in endothelium-removed aorta from LPS-treated animals, 1400W caused contraction with an EC_{50} of 0.8 μM, reflecting inhibition of iNOS. In LPS-treated rats it was shown to protect against miocrovascular damage as reflected by vascular leakage into the ileum. It could be administered simultaneously with the LPS without adverse effects, unlike nonspecific NOS inhibitors that actually cause increased vascular leakage in this model. While continuous infusion at 120 mg kg^{-1} per day was tolerated, a bolus of 50 mg kg^{-1} given intravenously was immediately lethal. This toxicity is lower than that of

isothioureas. It remains to be seen whether the toxicity of such compounds can be lowered sufficiently to make them clinically useful. In the meantime, 1400W should allow much better definition of experimental disease models in which iNOS inhibition can produce a therapeutic benefit.

4. Summary and Outlook

The most important signaling functions of NO are certainly mediated by activation of sGC and the consequent increases in intracellular cGMP. The reversible binding of NO to the heme of sGC is in striking contrast to its irreversible reaction with oxyHb, leading to formation of metHb and nitrate, which can be considered a major inactivation mechanism for NO. The causes of these differences are to be found in the special heme environment of sGC and can probably only be assessed exactly when a crystal structure is solved.

Reactions with superoxide and thiols can also determine the biological effects of NO. The reaction with oxygen leads to formation of reactive intermediates that can (unlike free NO) nitrosate amines and thiols. Some of the resulting nitroso compounds—particularly *S*-nitrosothiols—probably have important functions in extending the range of NO signals or as stores of NO. Reaction of NO with $O_2^{\bullet-}$ yields peroxynitrite, whose oxidative activity contributes substantially to tissue damage in inflammatory and infective diseases. Resolving the question of which reactions of peroxynitrite are causing the most damage is likely to lead to new therapeutic strategies.

The biosynthesis of NO is catalyzed by NO synthases. These are complex homodimeric enzymes in which each subunit contains a thiolate-bound heme group that binds molecular O_2 and catalyzes, in the presence of the cofactor H_4biopterin, the oxidation of L-arginine to L-citrulline and NO. The reducing equivalents needed for the reaction are supplied by NADPH through the FMN- and FAD-containing reductase domain of the enzyme. This electron transport is gated by the binding of calmodulin. The constitutively expressed neuronal and endothelial isozymes bind calmodulin only in the presence of 0.1–1 μM Ca^{2+}, whereas the inducible isozyme is practically independent of Ca^{2+} concentration. To understand the reaction mechanism it will be decisive to establish the role of H_4biopterin. In addition to its known allosteric effects it seems likely to have an as yet undefined redox function. These questions seem likely to be answered in the near future by a combination of biophysical and functional experiments, and by crystal structure analysis of specific NOS mutants and NOS–pteridine complexes. Finally, the development of isozyme-selective NOS inhibitors is expected to open new perspectives in the treatment of infective and inflammatory diseases and ischemic conditions.

5. Abbreviations

4-amino-H_4biopterin	2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine
H_4biopterin	2-amino-4-oxo-(6*R*)-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine
H_2biopterin	2-amino-4-oxo-(6*R*)-7,8-dihydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine
CaM	calmodulin
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
Hb	hemoglobin
IC_{50}	concentration causing 50% inhibition
IRP	iron regulatory protein
L-NAA	N^G-amino-L-arginine
L-NNA	N^G-nitro-L-arginine
L-NMA	N^G-methyl-L-arginine
L-NIL	N^ε-iminoethyl-L-lysine
L-NIO	N^δ-iminoethyl-L-ornithine
L-TC	thio-L-citrulline
SMTC	*S*-methylthio-L-citrulline
Mn-SOD	manganese superoxide dismutase
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NMDA	*N*-methyl-D-aspartate
NOHLA	N^G-hydroxy-L-arginine
NOS	nitric oxide synthase (EC 1.14.13.39)
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
PARS	poly(ADP-ribose) synthetase
PPIX	protoporphyrin IX
sGC	soluble guanylyl cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2.)

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