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Nitric oxide (NO) is a gaseous diatomic radical that is involved in a wide range of physiological and pathological functions in biology. Conceptually, the biochemistry of NO can be separated into three stages: generation (stage 1), translocation (stage 2), and action (stage 3). In stage 1 the oxygenase domain of NO synthase converts L-arginine to L-citrulline and NO (g). Owing to its short-lived nature, this molecule is converted into a different nitrogen oxide such as NO₂, an organonitrosyl such as a nitrosothiol, or a metal nitrosyl such as a heme-nitrosyl, for transportation in stage 2. Each of these derivatives features unique physical characteristics, chemical reactivity, and biological activity. Upon delivery in stage 3, NO exerts its physiological or pathological function by reaction with biomolecules containing redox-active metals or other residues.

Introduction

Historically, nitric oxide (NO) was known as an industrial and automotive pollutant that was highly toxic and environmentally damaging (Culotta and Koshland, 1992). The photochemical reaction of NO with ozone causes O₃ to break down into dioxygen and leads to depletion of the ozone layer. Additionally, atmospheric NO can be converted to nitric acid and has been implicated in acid rain. For these reasons one of the primary functions of catalytic converters in automobiles is to reduce the amount of NO released into the atmosphere. Although this diatomic molecule is formed as an intermediate in the Ostwald process, whereby ammonia is oxidized to nitric acid, it has almost no practical value in industry (Sadykov et al., 2000). Recently, NO has been utilized in the fabrication of semiconductors, and the number of patents concerning these processes has increased dramatically (Yun et al., 2007).

By the end of the 1980s, the uniformly negative perception of NO had begun to change as research in various fields, including immunology, oncology, and cardiology, began to divulge diverse physiological roles for the molecule. Interest in NO grew rapidly, and in 1992 it was named “Molecule of the Year” (Culotta and Koshland, 1992). Subsequently, the Nobel Prize in Physiology or Medicine was awarded for the identification of NO as a signaling molecule in the cardiovascular system (Furchgott, 1999; Ignarro, 1999; Murad, 1999). The known functions of NO in biology continue to grow and now range from neuroprotection and the immune response to protein regulation and chemotherapeutic resistance (Bonavida and Baritaki, 2011; Bronte and Zanovello, 2005; Calabrese et al., 2007; Carreau et al., 2011; Heo, 2011; Keswani et al., 2011; Turchi, 2006).

The diverse identities and activities of NO in vivo can be understood by considering three distinct stages in its lifetime, namely, generation, translocation, and action (Figure 1), most of which involve metal ions. During the first stage, NO is generated by nitric oxide synthase (NOS), an enzyme that converts L-arginine into NO (g) at an iron-porphyrin, or heme, center. Next, NO (g) either diffuses directly to its target, or it is converted to: (1) a different oxide of nitrogen, such as NO₂ or N₂O₃; (2) an organonitrosyl (E-NO) compound, where E is a sulfur-, nitrogen-, or carbon-containing moiety; or (3) a metal-nitrosyl (M-NO) complex. Some of these species are better suited for delivery of NO and others for longer-term storage. Finally, NO or a derivative thereof can exert its physiological or pathophysiological functions by interaction with: (1) redox-active metals; (2) redox-active metals bound to or in the vicinity of redox-active ligands; (3) redox-inactive metals supported by redox-active ligands, such as zinc finger proteins; or (4) metal-free organic species including peptides containing cysteine, tryptophan, and/or tyrosine.

Stage 1—Generation by NOS

Investigation of the mechanism by which NO is generated in living systems requires detailed kinetic studies of the various isoforms of NOS or, as a minimum alternative, their oxygenase domains. Small-molecule biomimetic complexes of metalloenzyme active sites typically do not reproduce the second- and third-coordination spheres provided by the protein architecture, which can supply hydrogen-bonding or redox-active amino acid side-chain interactions necessary for proper enzyme function. In this section we describe the structure of NOS and address possible mechanisms by which the enzyme could generate NO from L-arginine. Ultimately, elucidation of the mechanism will require more extensive use of genetic, biochemical, and bioanalytical techniques.

Structure

NOSs are enzymes that effect the synthesis of mammalian NO from L-arginine in a dioxygen- and NADPH-dependent manner (Valance and Leiper, 2002). During one complete catalytic cycle, a molecule of L-arginine and two molecules of dioxygen are converted to one molecule of NO, one molecule of L-citrulline, and two molecules of H₂O in a process that requires three exogenous electrons and protons. Crystallographic studies have revealed...
the active site in NOS to contain a cysteine-ligated heme unit, analogous to that found in cytochrome P450, with tetrahydrobiopterin in close proximity to the heme active site as well as a reductase domain farther away (see Figure 2 for all NOS components and Figure 3 for the oxygenase domain) (Li et al., 2006).

**Mechanism**

The conversion of L-arginine into NO and L-citrulline occurs in two discrete mechanistic steps (Figure 4). First, L-arginine is hydroxylated to form N-hydroxy-L-arginine (NHA), which is then converted to L-citrulline with concomitant release of NO. Current understanding of this cycle invokes an [FeIV = O(porr/C15+)] intermediate, similar to that observed in cytochrome P450, as the species that hydroxylates L-arginine (Li et al., 2007). However, recent experiments cast some doubt on this assignment because iodosylbenzene-generated ferryl NOS does not result in conversion of L-arginine to NHA (Zhu and Silverman, 2008). Therefore, an alternative mechanism for this step has been proposed whereby an iron(III) peroxy species forms that deprotonates a guanidinium nitrogen atom in L-arginine (Li et al., 2007). However, recent experiments cast some doubt on this assignment because iodosylbenzene-generated ferryl NOS does not result in conversion of L-arginine to NHA (Zhu and Silverman, 2008). Therefore, an alternative mechanism for this step has been proposed whereby an iron(III) peroxy species forms that deprotonates a guanidinium nitrogen atom in L-arginine. The lone pair on the neutral imine can then effect nucleophilic attack at the terminal, or distal, oxygen atom in the iron(III) hydroperoxo unit, leading to formation of NHA. During the second step the same iron(III) peroxy unit attacks the imine carbon atom of NHA, and subsequent collapse of this intermediate releases NO and L-citrulline (Li et al., 2007; Woodward et al., 2010). Recently, detailed studies of this NOS-catalyzed process by mutagenesis, substrate/product analysis, and magnetic resonance methods (EPR and ENDOR), suggest that both ferric peroxy and ferryl intermediates can be generated during a typical catalytic cycle (Woodward et al., 2009). Each intermediate might effect different steps, with L-arginine conversion to NHA catalysis promoted by the ferric peroxy species and NHA decomposition to L-citrulline by a ferryl (Davydov et al., 2009).

**Model Complexes**

Although no complexes that mimic both the coordination environment and reactivity of NOS yet exist, several notable approximations have been prepared over the past 10 years. One of the simplest examples is the ruthenium complex I, which can catalyze the transformation of arginine to citrulline in the presence of H2O2, with concomitant generation of NO (Figure 5A) (Marmion et al., 2001). However, given the structure of the NOS active site, there is greater interest in heme-type mimics. The fluorinated heme complex [Fe(TPPF20)] (IIa, TPPF20 = tetra-kis(pentafluorophenyl)porphyrin) catalyzes the H2O2-driven oxidation of NHA to citrulline and the nitrile, forming NO in the process (Figure 5B) (Keserü et al., 2000). Similarly, reaction of [Fe(TMP)] (IIb, TMP = tetra(mesityl)porphyrin) with fluorenone oxime in the presence of O2 affords NO (trapped by the heme) and fluorenone, analogous in this context to citrulline (Figure 5C) (Wang et al., 1999).
Biochemistry

There are three isoforms of the homodimeric NOS enzyme in mammals—endothelial, neuronal, and inducible—that share roughly 50% sequence homology (Vallance and Leiper, 2002). The first two, endothelial NOS (eNOS) and neuronal NOS (nNOS), are constitutive, whereas the last, inducible NOS (iNOS), is inducible, typically by an immune response. The individual monomers do not display any NOS activity but form the active homodimeric enzyme when linked by a [Zn(Cys)₄] unit in which two cysteine ligands are supplied by each monomer unit. Calmodulin binding to eNOS and nNOS is required for enzymatic activity, and its affinity for the enzyme may or may not depend on Ca²⁺ concentration (Roman et al., 2002). In contrast, the binding of calmodulin to iNOS, also necessary for enzyme function, is calcium independent, and thus, the NOS activity of this isoform is independent of [Ca²⁺].

eNOS is an important part of the cardiovascular system, and is essential for vasodilation and the maintenance of a healthy cardiovascular state (Vallance and Leiper, 2002). Chemical inhibition or knockdown of eNOS causes vasoconstriction, hypertension, and severe aneurisms in some instances. This isoform is also responsible for the regulation of vascular-endothelial growth factor and, thus, plays a role in angiogenesis.

Despite the name, nNOS can be found in nerve endings as well as muscles (Vallance and Leiper, 2002). Outside of the brain, nNOS modulates processes ranging from bladder relaxation to respiration. Mice in which this enzyme has been genetically deleted exhibit impaired balance and night vision. Although nNOS has been implicated in long-term potentiation, these knockout mice are still capable of normal learning and memory tasks. A multitude of physiological functions are performed by the neuronal isoform under normal conditions, such as neurogenesis, whereas pathologies such as ischemic brain damage and Parkinson’s disease can also arise when the enzyme malfunctions (Zhou and Zhu, 2009).

ιNOS functions in an immunoprotective capacity, serving to fight off infection from sources ranging from bacteria to viruses (Vallance and Leiper, 2002). This isoform is not expressed in healthy somatic cells but can be rapidly transcribed in response to disease. Unlike the other isoforms, iNOS can produce large concentrations of NO in a short period of time.

Bacterial NOS (bNOS) is analogous to the mammalian enzyme and has a similar heme oxygenase domain (Gusarov et al., 2008). This enzyme is required for normal Gram-positive bacterial growth, successful infection of a target, and defense against oxidant-based immune response. One notable difference between bNOS and the mammalian isoforms is that the former lacks an integrated reductase domain as an integral part of the enzyme. Bacteria expressing this enzyme can produce NO without a dedicated reductase by recruiting one from the host. Presumably, such behavior could serve as a foundation for the development of a new class of antibiotics against bNOS enzymes in pathogenic bacteria, such as those associated with anthrax, sepsis, and other infectious diseases (Kim et al., 2007, 2008; Shatalin et al., 2008).

Recently, substantial evidence has accumulated that suggests the existence of an entirely separate isoform of NOS localized within mitochondria (mtNOS) (Finocchietto et al., 2009). However, this hypothesis has been the subject of controversy, the prevailing contrary argument stipulating that mtNOS is merely a localized variant of one of the three major isoforms (Lacza et al., 2006).

Stage 2—Translocation and Storage

After synthesis, NO must diffuse or be transported away from NO to exert its effects. Recent studies have suggested that the lifetime of NO in aerobic, biological milieu could be too short to account for its observed functions (Liu et al., 1998; Thomas et al., 2001). Other oxides of nitrogen (Figure 6), S-nitrosothiols, and metal-nitrosyl complexes might serve as storage vessels or delivery vehicles that retain NO until needed or transport it to sites where it is required. S-Nitrosothiols and metal-nitrosyl complexes can release NO by thermal decomposition, metal-catalyzed processes, or illumination. Because translocation of NO in vivo involves the movement of NO (g) or one of its derivatives, investigations into this behavior under physiological and pathophysiological conditions require tools for the detection of NO (g) or related species. To address this need, we have devised sensors for the turn-on fluorescent detection of NO (g) selectively over other potentially interfering species (Lim and Lippard, 2007; McQuade et al., 2010; Pluth et al., 2011). Enormous progress has been achieved with “indirect” NO probes, which detect nitrogen oxides derived from NO (g) (Nagano and Yoshimura, 2002; Yang et al., October 28, 2011 ©2011 Elsevier Ltd All rights reserved 1213
et al., 2010), yet studies of the spatiotemporal distribution of NO (g) under various healthy or disease states will nonetheless require a sensor that is selective for NO (g). A sensor that is not selective for NO (g) may afford a false-positive response, incorrectly reporting HNO, ONOO\(^{-}/\)CO\(^{2-}\), or other reactive nitrogen species (RNS) derived from NO, thus obfuscating the relationship between NO (g) generation and the physiology or pathophysiology being studied. Similarly, investigations into the biological effects of other RNS, such as HNO, require sensors that are selective for HNO over NO (g) and other derivatives. Such constructs have recently been reported (Rosenthal and Lippard, 2010; Tennyson et al., 2007; Zhou et al., 2011).

**Nitrogen Oxides**

As a consequence of its generation from NOS in vivo, NO is formed in the presence of O\(_2\) and water. From this environment and the redox-active nature of the molecule, NO can be converted to a variety of nitrogen oxide (NO\(_x\)) species, with formal N-atom oxidation states ranging from +1 to +5.

**Nitroxyl, N(1+)**. Nitroxyl (HNO) may play an important role in biology, with a signaling pathway possibly orthogonal to that for NO (Fukuto et al., 2005; Miranda, 2005). Studies to elucidate the separate roles of these molecules have been hampered by a lack of spectroscopic tools that can distinguish the two. To address this limitation, our lab has pursued bioimaging agents selective for HNO over NO and other RNS, and we have recently succeeded in preparing fluorescent sensors for the selective detection of HNO in aqueous solution (Rosenthal and Lippard, 2010; Tennyson et al., 2007; Zhou et al., 2011). Although HNO is toxic at high concentrations, causing severe depletion of cellular glutathione, HNO can play a beneficial role in the cardiovascular system, where it interacts with targets that do not react directly with NO (Fukuto et al., 2005; Miranda, 2005). One possible source of HNO in vivo is from reaction of an S-nitrosothiol with another thiol to afford a disulfide and HNO. Although one-electron reduction of NO followed by protonation would afford HNO, this process is spin forbidden (Shafirovich and Lymar, 2002). The lowest-energy configuration for NO\(^{-}\) is the singlet state, but for HNO it is the triplet state. Realization of this spin change led to revision of the pK\(_a\) value for HNO from 3.4 to 11.4. However, coordination to a transition metal could favor the singlet state for NO\(^{-}\), and metal-bound HNOs have been previously reported to exhibit much lower pK\(_a\) values (∼7) (Southern et al., 2001). The free triatomic HNO molecule can react with thiols to form disulfide and hydroxylamine, whereby HNO may function as a one-electron reductant or a hydrogen-atom source (Fukuto et al., 2005).

**Nitrite (NO\(_2\)), N(2+)**. Although NO is a product of the NOS catalytic cycle, this diatomic radical is unstable in aerobic, aqueous solutions, especially in the presence of redox-active metal ions and organic compounds (Nagano and Yoshimura, 2002). Under physiological conditions, the half-life for NO can range from minutes to milliseconds. Early studies suggested that the intravascular concentrations of NO might be too low to account for its observed vasodilative behavior because of its limited diffusion range and stability in a hemoglobin-rich environment (Liu et al., 2007). More recent findings have revealed a more complicated picture, where factors such as the local environment and oxygen concentration can significantly affect NO diffusion (Liu et al., 2010). As a result, consideration must be given to various nitrogen oxides and other NO-derived species that can be formed from NO.

**Nitrate (NO\(_3\)^{-}), N(3+)**. Simple autooxidation of NO in aerobic, aqueous solution will give rise to NO\(_2\)^{-}, but not nitrate (NO\(_3\)^{-}) (Lewis and Deen, 1994). Reduction of NO\(_2\)^{-} to NO\(_2\)^{-} is effected by bacteria inhabiting humans, where studies of the NO\(_2\)^{-} anion have revealed it to have important biological effects and even serve as an indicator of infection (Lundberg et al., 2004, 2008).
Recent studies provide evidence that chemical reduction of NO₂⁻ could serve as an alternative to NOS for NO production in some cardiovascular and gastroesophageal tissues (Asanuma et al., 2007; Zweier et al., 2010). Although the majority of NO₃⁻ in humans is excreted in the urine, the absence of bacteria therein precludes any formation of NO₂⁻ (Carlsson et al., 2001). However, during bacterial infection of the urinary system, NO₃⁻ production occurs, and its presence can be used as a diagnostic indicator of such infection. Acidification of nitrite-enriched urine below pH 5.5 is lethal to all known urinary infective bacteria, and both modern treatments and folk remedies for bacterial urinary infections employ deliberate acidification via dietary habits, such as cranberries and citrus. Because the physiological and bactericidal functions of NO₂⁻ are dependent on acidic conditions, the relevant intermediate is believed to be HNO₂, which disproportionate to the presumed active species N₂O₂ and water. NO₂⁻ can also be reduced by deoxyhemoglobin or xanthine oxidoreductase to afford NO and hydroxide ion. Conversely, reaction of NO₂⁻ with oxyhemoglobin results in the formation of NO₃⁻.

Peroxynitrite (ONOO⁻, N(3+)). ONOO⁻ is formed in vivo by the diffusion-limited reaction between NO and superoxide (Koppennol et al., 1992). This anion is highly oxidizing and can even effect tyrosine nitration, resulting in a variety of pathophysiological effects ranging from inflammation to cancer (Salvemini et al., 2006). Additionally, ONOO⁻ can cause oxidation of cysteine to cystine or sulfone formation from methionine (Ulrich and Kissner, 2006). When ONOO⁻ is combined with two molecules of NO, N₂O₂ and NO₂⁻ are formed.

Nitrogen Dioxide (NO₂/N₂O₄) N(4+) and N(5+). NO₂/N₂O₄ is an unstable species in aqueous solution and will readily behave as a one-electron oxidant to generate NO₂⁻ (Hughes, 2008). Dimerization of NO₂ to N₂O₄ affords a species best viewed as [NO⁺][NO₃⁻], and subsequent hydration affords NO₂⁻ and NO₃⁻ (Addison, 1980). Because N₂O₃ formally contains an NO⁺ unit, it can act as a nitrosating agent in the generation of organonitrosyl compounds.

Dinitrogen Trioxide (N₂O₃). N(2+) and N(3+). N₂O₃ can be formed by the disproportionation of nitrous acid or by radical coupling of NO with NO₂ (Lundberg et al., 2004, 2008). This species is too unstable in aqueous solution to exert any direct influence but can deliver both NO⁺ and NO. In the former capacity, N₂O₃ functions as a nitrosating agent and enables the formation of species such as S-nitrosothiols and N-nitrosamines with concomitant formation of NO₂⁻. In the latter capacity, N₂O₃ serves as a source of NO and NO₂⁻. A commonly proposed mechanism for the response of "indirect" NO (g) probes involves reaction with N₂O₃ derived from the aerobic oxidation of NO (Nagano and Yoshimura, 2002; Yang et al., 2010).

NO₃⁻, N(5+). NO₃⁻ contains a nitrogen atom in its highest possible oxidation state (5+). It has long been considered to be an inert, thermodynamic sink for RNS (Lundberg et al., 2004, 2008). The bulk of NO₃⁻ in humans (60%–80%) is supplied by vegetable consumption. The remainder is produced by isomerization of ONOO⁻ or in the reaction of NO with oxygenated heme proteins. Because humans lack enzymes for which NO₃⁻ is a substrate, the majority of NO₃⁻ (60%) is excreted in the urine unaltered. Interestingly, nearly 25% of plasma NO₃⁻ is directed to the salivary glands and incorporated into saliva at concentrations 10-fold greater than those found in plasma. This observation is significant given the fact that there are a number of bacteria inhabiting the human mouth and gastrointestinal system. Whereas NO₃⁻ is an inert compound for humans, bacteria employ NO₃⁻ reductases in the conversion of NO₂⁻ to NO₂⁻. These bacteria can process the NO₂⁻ in saliva or elsewhere into NO₂⁻. Although NO₂⁻ has been associated with cancer and other diseases, as well as with beneficial NO-derived gastric bactericidal functions, its "effects" are due to the action of NO₂⁻ or other downstream RNS. For example ingestion of NO₃⁻ caused a large increase in plasma NO₂⁻ in test subjects, but if they did not swallow, no such increase was noted (Lundberg and Govoni, 2004). Other studies have shown that NO₃⁻ has a protective effect in the gastrointestinal system, such as the prevention of stress-induced injury in rats, accompanied by an increased generation of NO (Miyoshi et al., 2003). However, treatment of the rats’ oral cavities with topical antibiotics caused death of the resident bacteria and abolished the protective effect of the dietary NO₃⁻. Presumably, bacteria in the mouth reduce NO₃⁻ to NO₂⁻, which then migrates to the stomach, where the acidic environment can cause formation of a number of RNS through nitrous acid as an intermediate.

Organonitrosyls

Because N₂O₃ can function as a nitrosating agent, it is believed to be the intermediate by which nitrosamines and nitrosothiols are formed. Reaction of morpholine or glutathione with NO under aerobic conditions resulted in the formation of N-nitrosomorpholine and S-nitrosothiols, respectively (Keshive et al., 1996). By comparison, the same reaction performed under anaerobic conditions afforded no change. Kinetic analysis of this reaction at neutral pH indicated that the responsible intermediate was N₂O₃.

S-Nitrosothiols are commonplace in vivo, and there is evidence that these molecules are the predominant carriers of NO, but the mechanism of "NO" delivery by these species is disputed (Al-Sa’doni and Ferro, 2005; Tannenbaum and White, 2006; Tsikas and Fröhlich, 2004). Some evidence suggests that heterolysis of RS-NO directly transfers a nitrosonium (NO⁺) fragment to the target in a process termed nitrosation. Other experiments indicate that homolysis of the S-N bond liberates 0.5 equivalents of disulfide (RSSR) and 1 equivalent of NO, which
then reacts with the target in a process termed nitrosylation. The latter pathway is too slow for practical signaling purposes unless accelerated by a transition metal or other redox-active agent to facilitate homolytic decomposition of the RSN (Bazyliński and Hollocher, 1985). Most S-nitrosothiols decay with first-order kinetics under thermal conditions (Field et al., 1978) and with second-order kinetics when catalyzed by metals (Stanier and Toone, 2002).

\textit{N}-Nitrosamines can function as alkylating agents and are therefore potent carcinogens (Georgiadis et al., 1991; Hebels et al., 2010). However, these RNS can also exhibit antibacterial effects by a similar mechanism. Although the details for the biological activity of this class of compounds are unclear, loss of dinitrogen occurs, thus excluding possible involvement by derived RNS. Alternatively, nitrosamines can lead to the formation of reactive oxygen species (García et al., 2009), which are themselves potent biological effectors of physiology (Forman et al., 2010) and pathophysiology (Wang, 2008).

### Metal-Nitrosyl Complexes

Iron complexes bearing nitrosyl and thiolate ligands have been encountered in biology. They exhibit a range of functions from NO donation to antitumor activity (Butler and Megson, 2002). One intriguing feature of these \{Fe-S-NO\} complexes is their ability to release NO under photolytic conditions (Ford, 2008). Roussin’s black salt \([\text{Fe}_4\text{S}_3(\text{NO})_7]\) (RBS) has been used in vasodilation studies (Butler and Megson, 2002). Roussin’s red salt \([\text{Fe}_2\text{S}_2(\text{NO})_4]^{2-}\) (RRS) has been studied as a photolabile source of NO for the sensitization of cancer cells to radiotherapy (Bourassa et al., 1997). Roussin’s red esters \([\text{Fe}_2(\text{NO})_4(\mu-\text{SR})_2]\) (RREs) are attractive substrates for NO photodelivery studies, given the precedence for alkylations of the bridging sulfides in RRS to enhance solubility. However, the reverse reaction of NO photodelivery is NO alklycation, and this process occurs rapidly \((\sim 10^8 \text{ M}^{-1} \text{s}^{-1})\). Adaptation of this scaffold to incorporate longer wavelength or multiple photon chromophores within the R groups affords RREs with better photophysical properties and quantum yields, improvements beneficial for clinical applications (Ford, 2008).

Mononitrosyl iron complexes (MNICs) supported by thiolate ligands have only been detailed recently (Harrop et al., 2006, 2007). Measurements of NO transfer from MNICs revealed a quan-
titative process that was complete within 30 min of photoradiation (Ford, 2008). Although MNICs have yet to be observed unambiguously in biology, EPR spectra consistent with MNICs have been obtained from the nitrosylation of mammalian ferritin (Lee et al., 1994). Intriguingly, the properties of small-molecule MNICs suggest that these complexes could exert possibly orthogonal influences to those of dinitrosyl iron compounds (DNICs) or other (Fe-S-NO) complexes. For example MNICs could function as rapidly releasing donors of NO in vivo, whereas DNICs would behave as stores of NO. Thus, chemical modification of the (Fe-S-NO) storage complexes to afford MNICs would facilitate release of NO. Previous studies of other (Fe-S-NO) complexes have shown that photoinduced NO release occurs, but the quantum yields are poor, and back reactions are very rapid (Ford, 2008).

### Stage 3—Targets and Action

Whatever the means of NO transport and storage, it exerts its effects through reactions with biomolecules. Given the radical character of NO, it can react with a wide variety of redox-active species, including targets composed of: (1) redox-active metals, (2) redox-active metals bound to or in the vicinity of redox-active ligands, (3) redox-inactive metals supported by redox-active ligands, or (4) metal-free organic species. For example NO can inactivate mitochondrial aconitase by disrupting its [4Fe-4S] active site cluster through formation of a DNIC (vide infra). Similarly, NO binds tightly to cobalamin and inhibits the activity of methionine synthase. These results suggest regulatory roles for NO in energy production and C1 metabolism, processes that are essential for life. To gain a better understanding of the mechanisms by which NO can exert its influence in reactions with biomolecules, we have initiated a program to investigate the fundamental NO chemistry of transition metal thiolate complexes. The results for NO reactions with iron-, cobalt-, and nickel-thiolate coordination complexes have been recently re-
ported (Harrop et al., 2006, 2007, 2008; Tennyson et al., 2008; Tonzetich et al., 2009, 2010). In this section we present a few illustrative examples of the reactivity of NO with biomolecules and the functions that this chemistry affects. No attempt has been made to be comprehensive or to cover all classes of reactions.

### Complexes with Redox-Active Metals

#### Soluble Guanylyl Cyclase

One of the first targets identified for NO in biology was soluble guanylyl cyclase (sGC), an enzyme that features an NO-sensing and a catalytic cyclase domain. In the reduced state the active site of the NO-sensing domain contains a ferrous heme coordinated by two axial histidine ligands (Spiro, 2008). Crystallographic analysis of the inactive, ferric enzyme revealed that the pyrrole moieties within the heme unit were significantly distorted from the expected coplanar geometry. Reaction with NO by a reductive nitrosylation mechanism afforded an Fe(II) center with attendant flattening of the heme ring. Because a number of protein residues are within close proximity to this heme unit and potentially cause the steric distortion, the geometric change therein induces a significant displacement of the N-terminal helices and loops. Accompanying the heme flattening is the displacement of one of the coordinated histidine residues, a process that correlates with activation of the catalytic cyclase domain. Upon activation, sGC catalyzes the transformation of guanosine triphosphate to cyclic guanosine monophosphate (Gilles-Gonzalez and Gonzalez, 2005; Krumenacker et al., 2004), an effector for a wide variety of signal transduction events. Included are phototransduction, vascular smooth muscle modulation, electrolyte homeostasis, as well as the activation of protein kinases, ion channels, and phosphodiesterases.

To gain greater insight into the underlying chemical process by which the NO-heme interaction affords enzymatic activity, a number of fundamental studies have been performed. Reaction of CO with ferrous sGC caused displacement of one of the histidine ligands but did not induce activity (Burstyn et al., 1995). Reconstitution of this enzyme with nonnative metals allowed further investigation of the dependence of enzymatic activity on the coordination environment of the heme unit, i.e., five-versus six-coordinate (Dirks et al., 1997). Upon reaction of the manganese analog of sGC with NO, a six-coordinate metal nitrosyl was formed, and the enzyme lacked activity. However, treatment of the cobalt-reconstituted sGC with NO yielded a
five-coordinate cobalt-nitrosyl porphyrinoid complex, and the enzyme was active. Activation of the ferrous \([\text{Fe}(\text{por})(\text{His})_2]\) enzyme by NO proceeds by formation of the five-coordinate ferrous mononitrosyl \([\text{Fe}(\text{por})(\text{NO})]\) (Zhao et al., 1999). Other nitrogen oxides, such as \(\text{NO}_2^-\), also interact with these heme-based systems in a physiologically meaningful manner (Ford, 2010).

**Cytochrome P450.** This enzyme is responsible for the oxidative metabolism and degradation of a variety of physiological substrates and potential toxins. In the resting state the active site comprises ferric heme bound by a cysteine ligand at the proximal axial site. Treatment of cytochrome P450 with an NO donor caused a decrease in testosterone hydroxylation activity, which could be reversed upon addition of dithiotothreitol (Minamiyama et al., 1997). Analysis of the NO-inhibited enzyme by EPR spectroscopy revealed a 1:1:1 hyperfine-structured line at \(g = 2.0\), consistent with formation of an \([\text{Fe}-\text{NO}]^7\) complex. However, irreversible suppression of activity was also observed and attributed to conversion of the axial cysteine to S-nitroso-cysteine. Recent studies of NO coordination to different cytochrome P450 enzymes from *Mycobacterium tuberculosis* revealed multiple, distinct kinetic processes, suggesting that NO may regulate such events within the same organism (Ouellet et al., 2009).

The reactivity of cytochrome P450 with NO has been modeled with the biomimetic iron complex \([\text{Fe}(\text{TPPM})]\) (TPPM = 5,10,15-tris(o-pivalamidophenyl)-20-(o-(2-mercaptomethyl/phenoxo)-acetalidophenyl)porphyrin) (Franke et al., 2005). Addition of \(^1^5\text{NO}\) (g) to the ferric-heme complex followed by EPR spectral analysis revealed a signal at \(g = 2.02\) with three-line hyperfine coupling. Using \(^{19}\text{NO}\) (g) afforded a similar spectrum, albeit with a two-line coupling pattern associated with the signal at \(g = 2.02\). This heme-nitrosyl-thiolate can undergo subsequent reaction with excess NO to afford a complex featuring heme-nitrosyl and nitrosothiolate components.

**Iron-Sulfur Clusters.** Mitochondrial aconitase contains a [4Fe-4S] cluster in the active state that is reduced to a [3Fe-4S] configuration with nearly identical structural parameters upon inactivation (Robbins and Stout, 1989). Treatment of the EPR-silent enzyme with NO affords an inactive protein with an EPR spectrum consistent with formation of a [3Fe-4S] cluster and a DNIC (Asanuma et al., 2007; Duan et al., 2009; Kennedy et al., 1997). Similar reactivity is observed with the [4Fe-4S] cluster in the active site of dihydroxyacid dehydratase (Duan et al., 2009). In a similar manner, addition of NO to the redox-active transcription factor SoxR, composed of a [2Fe-2S] cluster in its active site, induces activation via DNIC formation (Ding and Demple, 2000).

**Ribonucleotide Reductase.** This enzyme is responsible for conversion of ribonucleotides to deoxyribonucleotides. Binding of NO to the enzyme was studied as a surrogate for dioxygen binding, and two \([\text{Fe}-\text{NO}]^7\) units were observed (Haskin et al., 1995). Reaction of this enzyme with NO inhibits activity by quenching a stable tyrosine radical at the active site. However, adventitious reduction of two molecules of NO yields a diiron(III) state without generating the tyrosyl radical.

This type of binding and reduction also occurs with NO reductases (Kurtz, 2007). Although the mechanism is unresolved, two steps that must be involved are iron-nitrosyl bond formation and nitrogen-nitrogen bond formation. For example the latter could occur on one iron bearing two nitrosyl ligands or between two separate \([\text{Fe}-\text{NO}]^7\) units. Related work on the binding of CO to this enzyme suggested that two separate \([\text{Fe}-\text{CO}]^7\) units form that interact with one another, implying that a similar configuration could be involved in the nitrogen-nitrogen bond-forming step with NO (Lu et al., 2004).

**Cobalamin.** The cobalt(III) unit in this cofactor is essential for methionine synthesis and C1 metabolism; treatment of cells with NO donors disrupts these functions (Danishpajooh et al., 2001). Although lacking a cobalt(III)-thiolate bond, cobalamin binds NO very rapidly and tightly (Wolak et al., 2001).

**Complexes with Redox-Inactive Metals**

**Zinc Finger Proteins.** Zinc finger domains are encoded by 3%–10% of the human genome (Andreini et al., 2006; Blasie and Berg, 2002) and are frequently employed in the recognition of specific sequences within DNA, RNA, and proteins (Dhanasekaran et al., 2006; Gamsjaeger et al., 2007; Lunde et al., 2007). NO can disrupt DNA binding of nuclear zinc finger proteins, and this loss of function may result from cysteine nitrosation followed by zinc release (Berendji et al., 1999; Garbán et al., 2005; Kröncke and Carlberg, 2000).

The action of NO on zinc thiolate complexes can regulate NOS activity. At the dimer interface of the enzyme is a \([\text{Zn}(\text{Cys})_2]^2^-\) unit, with two residues supplied by each monomer (Raman et al., 1998). Introduction of an NO donor under aerobic conditions produces S-nitrosation at these key residues, displacement of zinc, and subsequent loss of enzymatic activity (Mitchell et al., 2005). Replacement of the exogenous NO donor with an activator of iNOS activity also afforded an S-nitrosated enzyme. Variants of the enzyme in which a monomer is missing one or two of the key cysteine residues have much lower basal activity and are more sensitive to NO donors. Under aerobic, aqueous conditions, NO is converted to \(\text{N}_2\text{O}_3\), which can then nitrosate the cysteine residues in the bridging \([\text{Zn}(\text{Cys})_2]^2^-\) unit.

**Metallothioneins (MTs).** These enzymes contain multiple zinc cysite units and are involved in maintaining cellular zinc homeostasis. Addition of S-nitrosothiols to MTs caused labilization of zinc, presumably by cysteine nitrosation (Chen et al., 2002). This observation suggests a role for NO in the regulation of zinc homeostasis.

**Conclusions**

From its early recognition as an industrial pollutant and toxic gas, NO has become one of the most actively researched signaling and regulatory molecules in living systems. The three isoforms of NOS have been implicated in processes ranging from vasodilatation to the immune response, but the precise details of the mechanism of NO synthesis remain unknown. Furthermore, the belief that NO can freely diffuse over long distances has come under scrutiny, and there is growing evidence that other nitrogen oxides, organonitrosyls, and metal-nitrosyl complexes are important in the transportation and storage of NO. Delivery of NO at the target can induce effects ranging from activation of gene transcription to suppression of enzyme function. The fundamental chemistry of NO in vivo and the biochemical processes that it mediates depend on whether the target contains a complex of a redox-active metal and redox-active ligands, a complex composed of a redox-active metal only, a complex of a
redox-inactive metal supported by redox-active ligands, or organic-only substrates.

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REFERENCES


