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Biochemistry of Nitric Oxide and Its Redox-Activated Forms

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Nitric oxide ($NO^{\bullet}$), a potentially toxic molecule, has been implicated in a wide range of biological functions. Details of its biochemistry, however, remain poorly understood. The broader chemistry of nitrogen monoxide (NO) involves a redox array of species with distinctive properties and reactivities: NO^+ (nitrosonium), $NO^{\bullet}$, and NO^- (nitroxyl anion). The integration of this chemistry with current perspectives of NO biology illuminates many aspects of NO biochemistry, including the enzymatic mechanism of synthesis, the mode of transport and targeting in biological systems, the means by which its toxicity is mitigated, and the function-regulating interaction with target proteins.

Nitrogen monoxide (NO) has been implicated in a number of diverse physiological processes, including smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, and penile erection (*1, 2*). The biochemical pathways in these processes share two common features: the enzymatic synthesis of NO from L-arginine and the formation of an iron-nitrosyl complex in a target (heme) protein to evoke the functional response (*1, 2*).

Implicit in current perspectives of NO biochemistry is the identification of NO with the free radical nitric oxide. The broader chemistry of NO, however, involves an array of interrelated redox forms: nitrosonium cation (NO^+), nitric oxide ($NO^{\bullet}$), and nitroxyl anion (NO^-). This array is reminiscent of the redox forms of dioxygen—O_2, $O_2^{\bullet-}$, and O_2^{2-}—the implications of which for O_2 biochemistry are well understood. For example, mammalian systems express superoxide dismutase and catalase activities to prevent the toxicities, which are distinct from O_2 itself, of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), respectively. Fundamental to the understanding of NO biochemistry is the integration of established concepts in NO chemistry—the properties and reactivities of NO^+, $NO^{\bullet}$, and NO^-—with current perspectives on the biological functions of NO.

Physical Properties of NO

Some of the physical properties that illustrate the distinctions among the nitrogen monoxides are presented in Table 1. Neutral $NO^{\bullet}$ has a single electron in its $2p$-π antibonding orbital (*1*). The removal of this electron forms NO^+; conversely, the addition of an electron to this orbital forms NO^- (*3–7*). Each electron added in going from NO^+ to NO^- decreases the bond order, as reflected in the serially increasing bond lengths and decreasing vibrational energies. The measurement of these properties (for example, by infrared spectroscopy) provides a means to assess the character of the nitrosyl groups in compounds containing NO (*4–6*). Iron-nitrosyl compounds of biomedical interest have also been studied by electron paramagnetic resonance (EPR) (*8*); the paramagnetism of these complexes is a composite property of both the metal and ligand electrons and not simply a consequence of the paramagnetism of the $NO^{\bullet}$ radical. In addition to paramagnetic differences, an obvious distinction among the nitrogen monoxides is their charge (the pK for NO^- is 4.5) (*5–7*). Although the charge neutrality of $NO^{\bullet}$ has been assumed to facilitate its free diffusibility in aqueous medium and across cell membranes (*9*), neither the diffusion coefficient of $NO^{\bullet}$ in water nor its phospholipid membrane permeability has been reported.

NO Reactivity

Nitric oxide (*$NO^{\bullet}$*). From a biological standpoint, the important reactions of $NO^{\bullet}$ are those with (di)oxygen in its various redox forms and with transition metal ions (Fig. 1). Nitric oxide may also participate in reactions with other free radicals; for example, $NO^{\bullet}$ quenches the tyrosyl radical of ribonucleotide reductase (*10*). Such reactions, however, do not appear to dictate its general aqueous phase chemistry (*11*).

The reaction of $NO^{\bullet}$ with O_2 in both the gas phase (*1*) and aqueous solution (*12*) is a complicated process that is second order in $[NO^{\bullet}]$ ($k[NO^{\bullet}]^2[O_2]$); the biological half-life of $NO^{\bullet}$, generally assumed to be on the order of seconds, thus depends critically on its initial concentration. Nitric oxide also reacts rapidly with $O_2^{\bullet-}$ in aqueous solution ($k \sim 3.7 \times 10^7\ M^{-1}\ s^{-1}$), yielding peroxynitrite ($OONO^-$) (*13*).

Nitric oxide readily forms complexes with the transition metal ions, including those regularly found in metalloproteins. The reactions with heme-containing proteins have been widely studied, particularly in the case of hemoglobin (*14*). The ratio of rates of NO uptake and release for Fe(II)-hemoglobin is five to six orders of magnitude greater than that of O_2 (*14*). The association rate constants with deoxyhemoglobin are comparable ($\sim 5 \times 10^7\ M^{-1}\ s^{-1}$), but the dissociation rate constants are substantially different ($\sim 10^{-5}\ s^{-1}$ for NO and $\sim 20\ s^{-1}$ for O_2) (*14*). Nitric oxide, unlike CO and O_2, binds to Fe(III)-porphyrins (*15*). The association rate constants are markedly influenced by the protein structure at the heme pocket and range from 10^3 to $10^7\ M^{-1}\ s^{-1}$ (*15*). The Fe(III)$NO^{\bullet}$-heme complex appears to undergo a charge transfer reaction to form Fe(II)NO^+ (*16, 17*). Subsequent loss of NO^+ occurs readily by attack of ambient nucleophiles (*16, 17*). The net effect is that the Fe(III)-heme adduct exhibits rapid loss of NO, with rate constants in the range of 0.65 to 40 s^{-1} (*15*).

Nitric oxide also forms nonheme transition metal complexes. In the biochemical

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Table 1. Properties of nitrogen monoxides (3–6).

Molecule	Nitrogen oxidation number	Bond length (Å)	N–O stretch (cm^{-1})
Nitrosonium (NO^+)	3+	0.95	2300
Nitric oxide ($NO^{\bullet}$)	2+	1.15	1840
Nitroxyl anion (NO^-)	1+	1.26	1290

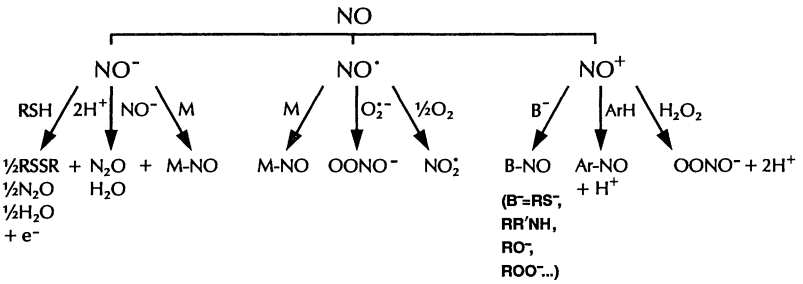

Fig. 1. Summary of the chemistry of the redox-interrelated forms of NO of potential biological significance. The primary reactions of NO• involve oxygen, superoxide, and redox metals (M). NO^- is reactive with metals and with sulfhydryls. Spontaneous dimerization of NO^- results in the formation of N_2O. Nitrosation reactions involve electrophilic aromatic substitutions (Ar) and addition to bases (B^-), including peroxide.

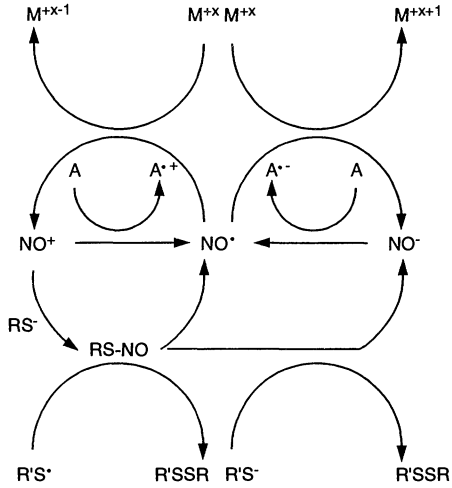

Fig. 2. Interconversion of the redox-related forms of NO. Three pathways of potential biological significance are shown, involving metal (M) nitrosyl complexes, charge transfer to electron acceptors (A), and coupling to thiol/disulfide redox reactions.

literature, interest has focused on its reactivity toward iron-sulfur centers in proteins, including several proteins involved in mitochondrial electron transport and enzymes such as aconitase (*8*). The cytotoxic effects of cytokine-activated macrophages are accompanied by the formation of iron-dinitrosyl-dithiolate complexes, with EPR spectra (*18*) similar to those of model compounds studied by Vanin and by Commoner and colleagues (*19*). Many of the transition metal nitrosyls are complexes where NO donates formally as NO^+ (these are best regarded as NO^+-metal adducts) (*6*, *20*). Such complexes exhibit NO stretching frequencies that range from ~1700 to 2000 cm^{-1}, reflective of the extent of charge transfer (*6*, *20*). Bottomly has suggested that molecules with NO stretching frequencies greater than 1886 cm^{-1}, or where the force constant for N-O is greater than 13.8 $mdyn Å^{-1}$, may be thought of as NO^+ carriers (*21*). In the context of NO^+ chemistry, such metal-nitrosyl complexes act as electrophilic nitrosating agents—that is, they transfer NO^+ to electron-rich substrates, as elaborated in the next section. NO can also behave formally as NO^- (*6*, *20*). The N-O stretching frequencies for metal nitrosyls of this nature are generally in the region of 1500 to 1700 cm^{-1} (*6*, *20*). In this case, the complexes are susceptible to attack by electrophiles (for example, $M\text{-}NO^- + H^+ \rightarrow M\text{-}NOH$, where M is a metal) (*20*). In addition, the formal conversion of NO• to NO^- is supported by aqueous Fe(II) ions (*22*) and by Fe(II)-containing metalloenzymes (*23*). Thus, metal nitrosylation provides a direct link among the redox states of NO (Fig. 2).

Nitrosonium (NO^+). The chemistry of NO^+ is characterized by addition and substitution reactions with nucleophiles such as electron-rich bases and aromatic compounds, as indicated in Fig. 1. Nitrosation in aqueous phase can occur at -S, -N, -O, and -C centers in organic molecules and appears to involve NO^+ or related NO^+ equivalents (*24*) (Fig. 1). There is abundant chemical evidence for the existence of NO^+ in aqueous media under stringent acidic conditions. Acidified NO_2^-, a medium used frequently in biomedical studies to produce NO•, is equivalent, through dehydration, to NO^+. The biological relevance of NO^+ under weakly acidic or physiologic conditions, however, has been disputed (*24*). Nevertheless, a variety of nitroso-compounds that form effectively under neutral physiological conditions (*8*, *25*, *26*) can be conveniently viewed as NO^+ carriers. Important examples of such compounds are the metal-nitrosyl complexes described above, thionitrites (RS-NO), nitrosamines (for example, RNH-NO), alkyl and aryl nitrites (RO-NO), and dinitrogen tri- and tetra-oxides (N_2O_3 and N_2O_4) (*6*, *8*, *24*–*26*). Similar to the metal-nitrosyl species, the nonmetal X-NO compounds show a variation in NO stretching frequency that reflects their nitrosonium character and correlates with their nitrosative reactivity (*4*, *27*). Forward reaction rates for the nitrosation reactions involving these species are thus not encounter-limited, in contrast to NO^+ (H_2ONO^+)-mediated nitrosation, but vary according to the electronegativity of X and the nucleophilicity of the substrate.

In biological systems, there are numerous nucleophilic centers whose potential susceptibility to nitrosative attack is precedented in in vitro studies. An extensive literature exists on amine deamination (primary) and *N*-nitrosation (secondary and tertiary) in the context of carcinogenesis (*25*, *28*). The propensity for thiol nitrosation (thionitrite formation) under physiological conditions (*26*, *29*) and in biological systems (*30*) has been recognized more recently. Nitrosation reactions sustained by various other nucleophilic centers of biological importance—including amides, carboxyls, and hydroxyls—have also been studied (*24*); however, their biological relevance is more speculative. Aromatic ring nitrosation has been suggested to involve the formation of charge-transfer complexes between NO^+ and aromatic electron donors [$Ar\text{—}NO^+ \rightleftharpoons Ar^{\bullet+}\text{—}NO^\bullet$] (*4*). Such one-electron transfer processes may provide a means, analogous to metal nitrosylation, to interconvert redox-related forms of NO (Fig. 2).

The enumeration of nitrosative chemistries of biological importance must be viewed within the context of relative reactivities and product stabilities. Although both amines and thiols are highly reactive toward nitrosation, physiologic conditions favor the formation of thiol adducts owing to the propensity of basic amines to exist in their unreactive protonated forms. This overall relative reactivity has led to the use of thiols as nitrous acid traps in reactions involving amines (*24*). Moreover, thiols are immune to the diazotization and deamination processes that deplete the nitrosamine product. The relative basicity of the nitrosated products governs the relative rate at which nitrosation is reversed by hydrolysis (*31*). The characteristic basicity of amines, including nitrosamines, together with the greater basicity of oxygen as compared to sulfur render nitrosated thiols least prone to hydrolytic degradation. These facts rationalize the presence of *S*-nitrosothiol pools in biological systems and their apparent predominance over other nitrosated forms (*30*). In further support of this notion, in the presence of thiol *S*-nitrosation occurs preferentially, perhaps to the exclusion of *N*- (or O-) nitrosation (*32*). Additional complexity, with regulatory potential for a chemically controlled process, derives from the differential reactivities of various thiolate anions and the stabilities of the derivative thionitrites (*24*, *26*, *27*, *29*, *33*). These properties are functions of thiol

pK, redox milieu, and molecular structure.

Nitroxyl anion (NO^-). The chemistry of NO^- has received significantly less attention, particularly in aqueous solution. Established reactions under physiological conditions are shown in Fig. 1. Nitroxyl anion converts rapidly to N_2O through dimerization and dehydration ($k \sim 2 \times 10^9\ M^{-1}\ s^{-1}$) (*34*) and is known to react with Fe(III)heme ($k \sim 6.4 \times 10^5\ M^{-1}\ s^{-1}$) (*9, 23*), analogous to the reaction of Fe(III)heme with $NO^\bullet$ (*15, 23*). Nitroxyl anion also undergoes reversible addition to low molecular weight (*35*) and protein (*23*) thiols leading to sulfhydryl oxidation (*23, 35*), with the intermediate formation of RSNOH. The chemistry of NO^- in the gas phase is rich and varied. Electron transfer and collisional detachment reactions are common and generally yield $NO^\bullet$ as a major product (*3*). *S*-nitrosothiols are believed to be a (minor) product of the reaction of NO^- with disulfides (*3*).

Enzymatic NO Generation

Nitrogen monoxide is generated enzymatically in vertebrates by the family of enzymes termed NO synthases, which are homologous to cytochrome P450 reductase (*36*). Moreover, like P450 itself, the enzyme contains iron-protopophyrin IX, probably with cysteine as the axial ferric (III) iron ligand (*37*). A five-electron oxidation of a terminal guanidino nitrogen of arginine is supported by reduced nicotinamide adenine dinucleotide phosphate (NADPH) and flavins; tetrahydrobiopterin and thiol are additional enzyme cofactors (*38*). Assuming a mechanistic analogy with the cytochrome P450 (*39*), reduction of Fe(III) would be required before the binding of molecular oxygen. Current views hold that there is successive activation of two O_2 molecules as a means to insert a pair of oxygen atoms into the arginine substrate, thus yielding $NO^\bullet$ and citrulline (*40*). *N*-hydroxy-arginine is an early intermediate in the biosynthesis of $NO^\bullet$ (*38*); however, its oxidative fate is not known in detail.

N-hydroxy-guanidines can also generate NO^-(HNO) (*41*). This process presumably involves a four-electron oxidation of the guanidino nitrogen. The preferential generation of $NO^\bullet$ or NO^- from the *N*-OH-arginine intermediate is determined by the oxidative conditions used (*41*); NO^- may be subsequently oxidized to $NO^\bullet$ by coupled redox pathways (*3, 7*). Precedent for the conversion of $NO^\bullet$ to NO^- by enzymatic pathways has been established for the bacterial $NO^\bullet$ reductase (*42*).

Nitrosonium equivalents are also produced as a result of NO synthase activity (*26, 40*); accordingly, NO synthase readily effects nitrosation of thiols (*26*) and amines (*40*). Nitrosation by way of N_2O_3 and N_2O_4 formed in the enzyme milieu is one possible mechanism by which to explain this observation (*43*). An alternative mechanism involves the oxidative activation of $NO^\bullet$ through binding to the Fe(III)-heme site [$Fe(III)NO^\bullet \rightarrow Fe(II)NO^+$], with subsequent attack by biological nucleophiles to form nitroso adducts (*17, 44*). This chemistry is exhibited by other heme proteins, including cytochrome P450 (*17*), and nonheme metalloproteins, such as bacterial NO_x reductases (*42, 44*). For NO synthase, redox activation of $NO^\bullet$ might (i) establish a means for binding molecular oxygen to heme through reduction of iron to the ferrous (II) state; (ii) establish a cofactor function for reduced thiol (*38*) that can react with the redox-activated NO complex to form an *S*-nitrosothiol (*17*); (iii) facilitate liberation of NO, by way of formed adduct, from an active site environment necessarily accessible to O_2 and containing heme; and (iv) at the same time provide an autoprotective function by limiting the toxicity of $NO^\bullet$ in the cell of the synthesis that is attributed to oxidative reactions with O_2 and $O_2^{\bullet-}$, which result in the formation of $NO_2^\bullet$ and $OONO^-$, respectively. These species are highly cytotoxic as a result of initiating free radical–mediated lipid peroxidation and sulfhydryl oxidation (*45*). Moreover, under circumstances of arginine depletion, NO synthase reduces O_2 with preferential generation of H_2O_2 (*46*). The oxidative conditions produced by H_2O_2 would directly support the oxidation of $NO^\bullet$ to NO^+ (redox potential $\sim$ +0.5 V), which in turn could react nitrosatively with H_2O_2, giving $OONO^-$ (*24*). The presence of thiol (cofactor) would effectively limit this undesired reaction (*47*). Thus, one might conceive of situations in which substrate availability influences the redox state of the enzymatic NO product and where available reducing equivalents modify the toxic potential of the species.

Intermediary NO Equivalents in Biological Systems

The form in which NO is released from cells has been much debated. The identity of endothelium-derived relaxing factor (EDRF) in particular remains in dispute, the major contenders being $NO^\bullet$ (*48*) and a short-lived nitroso-compound, perhaps *S*-nitroso-cysteine (*33*). However, more recently a case has been made for NO^- (*7, 41*) and iron-nitrosyl complexes (*49*) [the latter possibly liberated from the heme complex by displacement of a weakly bound *trans*-imidazole ligand (*50*)]. Notwithstanding the fact that EDRF shows close physiochemical and pharmacological resemblance to $NO^\bullet$ (*48*), this controversy highlights the potential impact that redox conditions may have on EDRF identity and activity.

The predominant redox forms of NO in (human) plasma are *S*-nitrosothiols (RS-NO, where RS is a thiyl group), mostly as *S*-nitrosoproteins (*30*). These *S*-nitrosylated proteins, the most abundant of which is serum albumin, are present in micromolar concentrations in normal subjects (*30*). This bioactive pool is thought to serve as a source and sink of NO, buffering the concentration of free NO (*30*). The S-NO bond can participate in both homolytic (*27, 31, 51*) and heterolytic (*27, 30, 31, 51*) cleavage mechanisms and thereby regulate the free plasma concentrations of $NO^\bullet$ and NO^+ adducts (*30*); under strong reducing conditions, RS-NO may also liberate NO^- (*52*) (Fig. 2). Products of *N*-nitrosation are found in the urine of normal subjects but are not readily detected in blood or plasma (*28, 30*). The NO-generating species N_2O_3 and N_2O_4 likely form as intermediates in plasma, urine, saliva, and cells (*25, 28, 30, 43*). Although both are potent nitrosating species, reflecting their NO^+ character (*24*), they are also in equilibrium with $NO^\bullet$ [$N_2O_3 \rightleftharpoons NO^\bullet + NO_2^\bullet$; $NO_2^\bullet \rightleftharpoons 1/2N_2O_4$].

The nitrosyl Fe(II)-hemoglobin adduct is measurable in blood during sepsis (*53*) and allograft rejection (*54*), but concentrations of this compound are otherwise below the limits of detection (*55, 56*). Iron-nitrosyl complexes form intracellularly in macrophages (*8, 18*) and in cocultured target cells (*55*) as a consequence of cytokine-induced NO synthesis; they have also been detected in tissues undergoing rejection (*54*). Nitrosylation of Fe-S centers is generally associated with loss of protein activity and is believed to participate in modulation of the immune response (*1, 8*). More recently, these complexes were identified in activated endothelial cells (*56*) and were proposed as candidates for the NO storage pool, loosely defined as a source of NO not immediately dependent on NO synthase activity. The iron-nitrosyl complexes share the potential stabilizing function of the protein *S*-nitrosothiol reservoir (*26, 30*), both of which are generated intracellularly in response to cell activation (*51*).

Implicit in the stability of *S*-nitrosothiols is the relative lack of reactivity of NO in this form toward O_2 and reactive oxygen species, therein limiting the generation of toxic NO_x. Thus, the formation of RS-NO may provide a means to control the toxicity of $NO^\bullet$. Supporting this notion, RS-NO have recently been identified in human airway-lining fluid, under the high ambient concentrations of O_2 and $O_2^{\bullet-}$ in the lung (*51*). In addition, modulation of thiol groups on the *N*-methyl-D-asparate (NMDA)–type glutamate receptor by RS-NO (NO^+) can afford

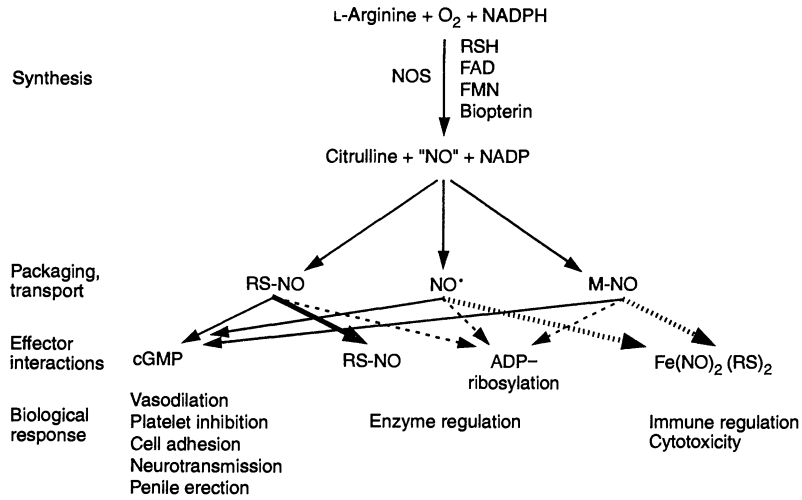

Fig. 3. Extended paradigm of NO biochemistry. NO synthase (NOS) converts L-arginine in the presence of oxygen, requisite cofactors, and NADPH to citrulline and a redox form of NO. Production of distinct NO-containing compounds, either accompanying or occurring after the action of NOS, may provide a means of packaging NO to tailor its lifetime and transport properties and to elicit specific biological responses.

protection from receptor-mediated neurotoxicity that is dependent on NO• generation (*57*). Accordingly, the form in which NO is delivered and transported may determine its toxic potential and resolve the apparent paradoxical observations of both cytotoxic and cytoprotective actions of NO.

The generation of NO-containing compounds may be viewed as a means to "package" NO in forms that are better suited to its intermediary roles. Specifically, the appropriate packaging of NO might serve to facilitate its transport, prolong its life in the blood and tissues, target its delivery to specific effectors, and mitigate its adverse-cytotoxic potential. Moreover, the existence of various types of NO pools, with relative abundance determined, for example, by pH and redox potential of the microenvironment, provides a means through which the transport, lifetime, and targeting properties of the various redox forms of NO can be tailored to evoke specific biological responses. An overview of these concepts is presented in Fig. 3.

Target Interactions

Nitrogen monoxide has the capacity to modulate the activity of proteins through reversible reaction with available functional groups, notably cofactor Fe and thiols (*1, 8, 26, 29, 50, 58*). The activation of guanylyl cyclase by the binding of NO to its heme iron is a particularly important example of this principle: many target cell responses to NO, including vasorelaxation and platelet inhibition, are the result of guanylyl cyclase–mediated increases in guanosine 3′,5′-monophosphate (cyclic GMP) (*1, 29, 50*). It has been conjectured that the binding of NO• induces a structural change, reminiscent of the effect of O_2 binding in hemoglobin, that activates the enzyme (*29*). This mechanism requires some means of deriving NO• from NO^+-containing vasodilators (for example, RS-NO and -Fe-NO). The spontaneous liberation of NO• from the latter compounds to account for enzyme activation has been invalidated in certain experiments (*59*). The activation of guanylyl cyclase by NO^- (*41*) is similarly unexplained. Moreover, redox conditions—specifically the redox state of protein thiol groups—are an important determinant of guanylyl cyclase activation (*60*). Thus, thiol alone can activate the enzyme (*60*), and activation of guanylyl cyclase by peroxides and OH• may result from interaction with regulatory thiol groups as opposed to the heme-iron (*61*). Taken together, these observations suggest that the oxidation state of both NO and the enzyme itself critically affect the NO-dependent activation of guanylyl cyclase.

Nitrosylation of free thiols has been shown to modulate the activity of certain enzymes (*26, 58*). In view of the presence of critical thiols in proteins of diverse function, this chemistry may have broad regulatory implications. The reaction of NO with cell-surface thiols, for example, has been associated with antimicrobial effects (*62*), modulation of ligand-gated receptor (NMDA) activity (*57*), and alterations of smooth muscle cell function (*51, 59*). In addition, signal transduction mediated by adenosine diphosphate (ADP)–ribosylation of protein sulfhydryls may be regulated by *S*-nitrosylation and thus link NO biohemistry to cyclic GMP–independent processes (*63*). In related observations, *S*-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase promotes ADP-ribosylation of the enzyme and is associated with inhibition of its activity (*63*). Nitroxyl has also been implicated in the inhibition of sulfhydryl-dependent enzyme activity (*64*).

The redox state of NO influences its preference for various target functional groups. This is exemplified in the important case of hemoglobin, which reacts with NO• at the heme site (to inactivate NO) (*1, 14, 29, 48*) and by way of alternative redox forms of NO at intramolecular amine (NO^+) and sulfhydryl (NO^+ and NO^-) centers (*23, 65*). These observations raise the question as to whether NO^+ or NO^- equivalents (or both) activate guanylyl cyclase and, if so, whether through reaction with thiol or redox metal.

Conclusion

Under physiological conditions, NO can be interconverted among different redox forms with distinctive chemistries. We have discussed the impact of this redox versatility on fundamental aspects of NO biochemistry: the enzymatic mechanism of NO production; the packaging of NO in forms suited to its intermediary roles; and the function-regulating interaction of NO with proteins (Fig. 3). Given the ultimate goal of therapeutic intervention, the distinction among the different NO species assumes added importance. The current teaching is that nitroso-vasodilators carry out their physiological functions by releasing NO•. It is notable that by far the most widely studied of the nitroso-compounds in clinical use, nitroprusside, is an iron-nitrosyl with strong NO^+ character (*20*); nitrosation reactions, in fact, dominate its chemistry (*20*). Thus, nitroprusside provides a cogent reminder that the term NO neither adequately identifies its redox form nor delineates the chemical reactivity of nitrogen monoxide in biological systems.

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