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Nitric oxide signaling: no longer simply on or off

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Nitric oxide (NO) triggers various physiological responses in numerous tissues by binding and activating soluble guanylate cyclase (sGC) to produce the second messenger cGMP. *In vivo*, basal NO/cGMP signaling maintains a resting state in target cells (for example, resting tone in smooth muscle), but an acute burst of NO/cGMP signaling triggers rapid responses (such as smooth muscle relaxation). Recent studies have shown that the sGC heterodimer comprises at least four modular domains per subunit. The N-terminal heme domain is a member of the H-NOX family of domains that bind O_2 and/or NO and are conserved in prokaryotes and higher eukaryotes. Studies of these domains have uncovered the molecular basis for ligand discrimination by sGC. Other work has identified two temporally distinct states of sGC activation by NO: formation of a stable NO–heme complex results in a low-activity species, and additional NO produces a transient fully active enzyme. Nucleotides also allosterically modulate the duration and intensity of enzyme activity. Together, these studies suggest a biochemical basis for the two distinct types of NO/cGMP signal observed *in vivo*.

Introduction

The diatomic gas nitric oxide (NO) is a potent toxin and yet nature has harnessed it to transduce signals that control vital physiological processes. The same could be said for both oxygen (O_2), especially with regard to obligate anaerobes, and carbon monoxide (CO). Remarkably, the signal transduction receptors for these gases have evolved to discriminate among them and to translate their binding into specific physiological responses.

As a paracrine signaling molecule, NO is synthesized in a generator cell by NO synthase. NO, an uncharged diatomic gas with a half-life of several seconds at low concentrations in tissues [1], diffuses through lipid bilayers to adjacent cells, where it forms a complex with and activates soluble guanylate cyclase (sGC). The best-characterized isoform of sGC, α1/β1 (see Glossary), responds to nanomolar concentrations of NO *in vivo* by catalyzing the conversion of GTP to 3′,5′-cyclic GMP (cGMP) and pyrophosphate. cGMP binds to target proteins such as cGMP-dependent protein kinases, cGMP-regulated ion channels and several families of phosphodiesterases, resulting in cell-specific downstream outputs. Because NO signaling has a prominent role throughout the vasculature in regulating blood flow, and in the perfusion and function of many organs and tissues, dysregulation of NO signaling contributes to various diseases ranging from heart disease, hypertension and stroke, to gastrointestinal distress, erectile dysfunction and neurodegeneration among others (reviewed in Refs [2–4]).

Two molecular conundrums exist in NO/cGMP signaling. First, how does the heme domain of sGC, which has the same histidine-ligated Fe^{2+} protoporphyrin IX heme cofactor as O_2-binding globins, sense low (nanomolar) concentrations of NO in the presence of much higher (micromolar) concentrations of O_2? Second, numerous *in vivo* studies of the NO/cGMP pathway have revealed two distinct signaling modes – tonic and acute – both of which are mediated by sGC [5,6]; yet the accepted *in vitro* model of sGC activity, which is supported by many studies, is a binary mechanism in which NO binding to the heme switches the enzyme between basal and activated states. So how does sGC transmit two types of cGMP signal: one that responds to basal levels of NO, seems to be long-lived and produces low levels of cGMP; and another that responds to acute production of NO with a brief burst of cGMP synthesis? As we discuss here, recent results have shed light on these two puzzles, with profound implications for NO signaling in mammals.

Architecture of sGC

The β1 subunit of rat sGC comprises 620 amino acids and is part of a large family of sGC subunits that are conserved in higher eukaryotes. The β1 subunit forms a heterodimer

Glossary

α1/β1: the best-characterized isoform of soluble guanylate cyclase.
β2 subunit: a homolog of the β1 subunit of soluble guanylate cyclase.
Sf9 cells: pupal ovarian tissue cells from the fall army worm *Spodoptera frugiperda*.
BLAST: basic local alignment search tool (see http://www.ncbi.gov).
H-NOX: a family of conserved domains that binding heme, NO and/or O_2.
3D-PSSM: three-dimensional position-specific scoring matrix for structure prediction.

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with the homologous 690-residue α1 subunit to form the well-characterized NO-responsive enzyme. Evidence suggests that the β2 subunit of sGC can form homodimers and is weakly responsive to NO, although purification and spectral characterization of a β2 homodimer has not been accomplished [7].

Several experimental approaches have been used to determine which region of the α1/β1 isoform binds heme. In one study, histidine residues were systematically mutated to phenylalanine and His105 was found to be required for binding heme and responsiveness to NO [8]. In another approach, truncations of the β1 subunit revealed that the N-terminal 385 residues purified with heme bound and showed the same spectral traits as full-length sGC [9]. Mutation of His105 to glycine led to loss of heme from the truncated protein, but heme binding was rescued when the protein was expressed and purified in the presence of imidazole [10]. These studies indicated that the heme-binding region of sGC is localized to the N terminus of the β1 subunit and strongly suggested that His105 is the proximal heme ligand in sGC.

Further support for the role of the β1 N-terminal domain has been provided by a study in which deletion of the N-terminal 64 residues of the β1 subunit abolished NO responsiveness in lysates of Sf9 cells. Of note, deletion of the N-terminal 131 residues of the α1 subunit (which does not bind heme) severely reduced the activation of sGC by NO [11]. In this same study, the C-terminal halves of the α1 and β1 subunits were also expressed in Sf9 cells and showed basal catalytic activity. Taken together, these experiments show that the sGC enzyme comprises several domains that are required for activation by NO.

With the explosion of genome sequences in the past 5 years, alignment of sGC subunits now permits an evolutionary exploration of the conserved elements of the enzyme. Homology searches using BLAST on specified regions of the β1 subunit have revealed that most subunits of sGC comprise four distinct modular domains: a N-terminal heme binding region, a predicted Per/Arnt/Sim (PAS)-like domain, a putative amphipathic helix, and the C-terminal cyclase catalytic domain [12,13] (Figure 1).

Heme-binding domain

Although the heme-binding region of β1 sGC was initially localized to residues 1–385, it is now clear that ~190 amino acids at the N terminus of β1 comprise a heme-binding domain that is part of a conserved family of proteins in prokaryotes and non-plant eukaryotes [14]. The bacterial members of this family encode the domain as a stand-alone protein in facultative aerobes within operons that also encode putative histidine kinases, suggesting that the domain has a role in two-component signaling in bacteria. In obligate anaerobes, this domain of ~190 amino acids is predicted to be part of a methyl-accepting chemotaxis protein, again suggestive of a chemotactic or signaling function.

All of the bacterial domains characterized so far are indeed hemoproteins as predicted. Those from facultative aerobes have ligand-binding properties identical to those of sGC – namely, they bind NO and CO, but not O_2. The domain from the obligate anaerobe *Thermoanaerobacter tengcongensis* binds O_2 and NO with high affinity and has been proposed to function as an O_2 sensor [13]. Speculation on the function of the domain from the anaerobe *Clostridium botulinum* has centered on it acting as a NO sensor [15]. The heme-binding domain from a sGC subunit of *Caenorhabditis elegans* has been recently shown to form a complex with O_2; worms require this subunit for responses to hyperoxia, suggesting that this predicted sGC subunit in worms can sense and respond to O_2 [16].

We have termed these proteins the 'heme–nitric oxide and oxygen binding family' or H-NOX family to encompass

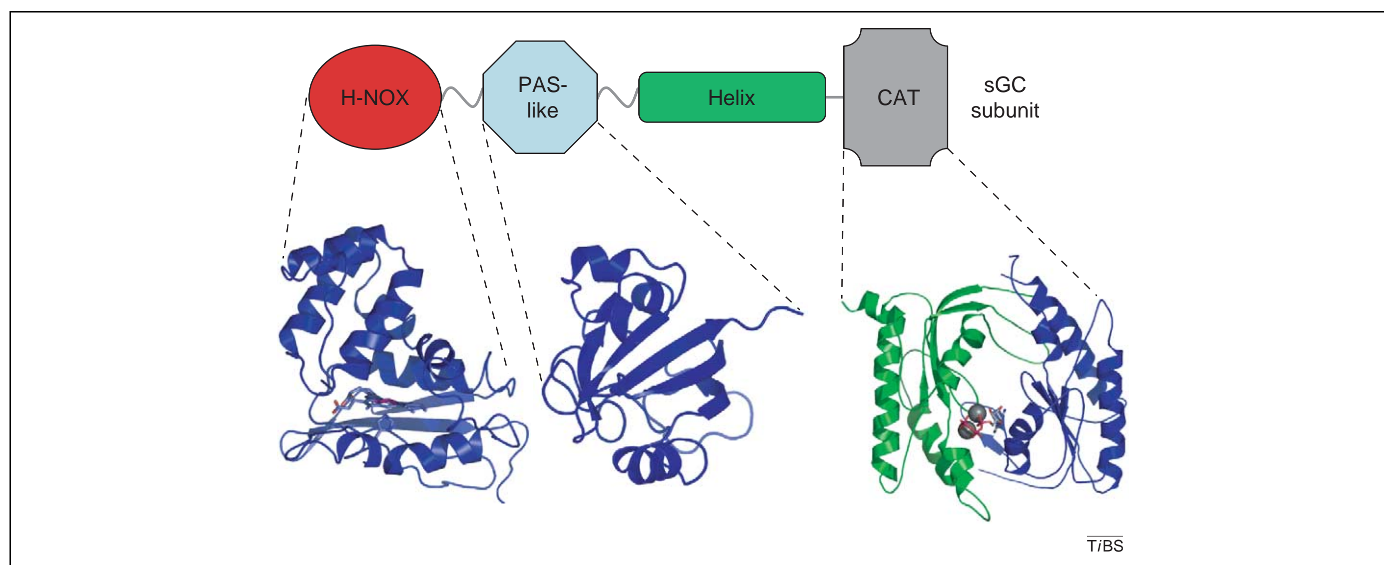

Figure 1. Architecture of soluble guanylate cyclase (sGC). The canonical sGC subunit has four modular domains, as determined by BLAST searches, sequence alignments and structure prediction. The H-NOX domain binds heme (stick structure) and is conserved from bacteria to mammals. H-NOX domains from facultative aerobic bacteria are most probably NO sensors, whereas those from obligate anaerobes can sense O_2 or NO. In mammalian sGCs the H-NOX domain binds NO, but in *Caenorhabditis elegans* it also binds O_2, suggesting the existence of O_2-regulated sGCs. The PAS-like domain predicted by 3D-PSSM is also conserved from bacteria to mammals, but so far its function is unknown. The predicted helix region and/or the PAS-like region might mediate the homo- and hetero-dimerization of sGC subunits. The catalytic domains of the sGC α1/β1 isoform have a catalytic site, where the substrate GTP (stick structure) and Mg^{2+} ions (spheres) are bound, and a symmetry-related pseudosubstrate site.

more broadly their ligand-binding properties. We consider that this appellation is a more accurate description of this family of proteins than other suggestions that refer only to NO binding.

PAS-like and helix domains

Several bacteria also encode another domain of sGC subunits. This domain corresponds to residues 210–355 of the β1 subunit, and structure prediction programs such as 3D-PSSM [17] suggest that it adopts a PAS-like fold. PAS domains, which are present in proteins in all classes of organisms, mediate protein–protein interactions and sometimes bind small molecules such as heme, flavins and nucleotides. Their function as a predicted domain in sGC remains to be determined, but for clarity here we refer them as ‘PAS-like domains’.

As mentioned earlier, conclusive identification of the heme-binding region of sGC used a construct encoding residues 1–385 of β1 sGC, which encompasses the H-NOX and PAS-like domains of the subunit and part of the helix region (β1 H-NOX–PAS–Helix) [9]. Whereas the β1 H-NOX domain of sGC (residues 1–194) is isolated as a monomer, the β1 H-NOX–PAS–Helix protein (residues 1–385) is a homodimer, indicating that the predicted PAS-like domain and/or the helix domain might be responsible for the homo- and heterodimerization of sGC subunits.

As also mentioned earlier, the third modular domain of sGC subunits is a putative helix region. The sequence of this helix is unique to sGC subunits and shares no homology with any other protein in the database. Whether it forms an amphipathic helix that mediates dimerization of the subunits or performs some other structural function remains to be determined.

Cyclase catalytic domain

The fourth and most C-terminal domain of sGC subunits forms one-half of the cyclase catalytic domain, which means that sGC subunits must homo- or heterodimerize to function catalytically. These domains are highly homologous to the catalytic domains of the particulate GC and adenylate cyclase.

The expression and purification of the predicted catalytic domains of the sGC α1 and β1 subunits has been recently reported [18]. Separately these domains form inactive homodimers, but when mixed together they heterodimerize and show cyclase activity. When the catalytic α1/β1 heterodimer of sGC is assayed in the presence of the β1 H-NOX domain (residues 1–194), cyclase activity is inhibited. Inclusion of the β1 H-NOX–-PAS–Helix protein (residues 1–385) has an even greater inhibitory effect on activity. NO does not affect this repression of activity. This inhibition *in trans* suggests that the H-NOX and PAS-like domains of sGC make contacts with the catalytic domains of the enzyme to regulate activity. Activation by NO in the context of the full-length enzyme might alter this interaction and derepress inhibition. This mechanism of activation of sGC could be similar to that of particulate GCs, which seem to undergo derepression on ligand binding (reviewed in Refs [19,20]), but evidence for the mechanism of activation of sGC requires further exploration.

Molecular basis for NO discrimination by the H-NOX domain

The mechanism of sGC regulation by NO or O_2 would be greatly enhanced by a crystal structure of the complete sGC α1/β1 heterodimer, but this task has proved to be a formidable challenge. A crystal structure of the *T. tengcongensis* H-NOX domain with O_2 bound, however, has been recently reported [15,21]. A key feature in the distal pocket of the heme is a tyrosine residue (Tyr140) that clearly interacts through a hydrogen bond with the bound O_2 (Figure 2). Mutagenesis studies of the distal pocket of several H-NOX proteins, including the *T. tengcongensis* H-NOX domain, the H-NOX domain from *Legionella pneumophilia* and the H-NOX domain from sGC, have been recently reported and revealed the crucial importance of Tyr140 in the H-NOX domains that are predicted to bind O_2 [22]. If Tyr140 in the *T. tengcongensis* H-NOX domain is mutated to leucine (Y140L), the affinity of the domain for O_2 is diminished.

A tryptophan in the distal pocket (Trp9) seems to position Tyr140 for hydrogen-bonding to the bound O_2. Mutation of Trp9 to a phenylalanine (W9F) also weakens O_2 binding, and the double mutant (Y140L/W9F) is completely unable to bind O_2. Surprisingly, O_2 binding can be restored to the Y140L mutant by converting Phe78 to tyrosine (F78Y). Phe78 is in the distal pocket but across from the normal position of Tyr140. It seems that a hydrogen-bond donor is required for O_2 binding and that, as long as the distance and angular requirements are met, the H-NOX domain will form a stable Fe^{2+}–O_2 bond.

In H-NOX proteins that do not bind O_2, an apolar residue is present instead of Tyr140 in the distal pocket, as

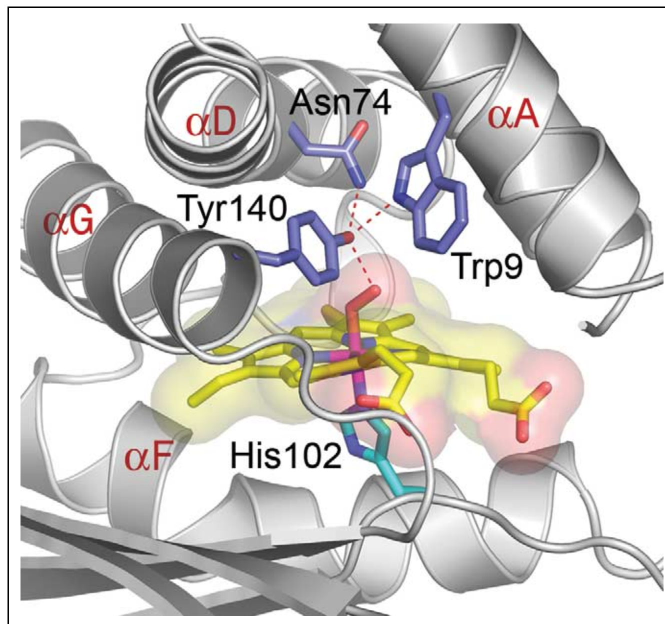

Figure 2. Ligand selectivity in H-NOX domains. This close-up view of the heme environment in the structure of the *Thermoanaerobacter tengcongensis* H-NOX domain shows O_2 bound to the heme iron in the distal pocket and His102 ligated to the heme iron in the proximal pocket. Tyr140 makes a hydrogen bond to the O_2 molecule. Trp9 and Asn74 position Tyr140 to make the hydrogen bond. The mutant Y140L has weakened affinity for O_2, and the double mutant Y140L/W9F has no detectable O_2 affinity. Mutating the apolar residues of NO-selective H-NOX domains to tyrosine converts them into H-NOX domains that bind O_2. Figure reproduced, with permission, from Ref. [21].

determined by sequence homology and structural modeling. When the apolar residue is mutated to tyrosine in both the *L. pneumophilia* H-NOX domain (F142Y) and the sGC β1 H-NOX–PAS–Helix construct (I145Y), the proteins are able to bind O_2. Taken together, these studies point to a crucial molecular determinant that prevents O_2 binding in NO-binding members of the H-NOX family—namely, the lack of a hydrogen-bond donor in the distal pocket of the heme (reviewed in Refs [23,24]).

Three *Drosophila melanogaster* homologues of sGC also have a tyrosine residue in the putative distal pocket of the H-NOX domain at a position analogous to Tyr140. Whether these H-NOX domains from fruitfly bind O_2 is not known, but preliminary experiments in mammalian tissue culture suggest that they are responsive to O_2 and synthesize cGMP [25]. Insects show irregular breathing as a way to avoid high concentrations of O_2 [26], and it would be worth exploring whether these putative O_2-regulated sGCs participate in this process.

Confoundingly, the H-NOX domain from gcy-35, a *C. elegans* sGC, binds O_2 but does not have a tyrosine residue at position 140 [16]. Several tyrosine residues are present in the H-NOX domain, but their position in the distal pocket is not known. Deletion of *gcy-35* in worms disrupts cGMP-dependent behavioral responses to hyperoxia, however, strongly implicating gcy-35 as a mediator of O_2 sensing in worms.

Thus, although significant progress has been made in understanding the basis for ligand discrimination in the H-NOX family, and especially in sGC, there remain unknown variables that might contribute to the specificity of the domains.

Activation and deactivation of sGC

The ligand-binding properties of the H-NOX domain are central to understanding sGC activation, especially because early observations established a causal link between vasodilation in blood vessels and NO binding to heme and activation of sGC *in vitro* [27–31]. The model established for activation and deactivation of sGC proposes that rapid binding of NO to the heme and severing of the proximal histidine bond leads to a conformational change in the protein that accelerates catalytic activity several hundred-fold [8,32–35]. In this model, then, dissociation of NO from the heme is the mechanism for deactivation of the enzyme. Slow dissociation of NO from the heme *in vitro* conflicts, however, with *in vivo* data showing that sGC rapidly deactivates as soon as NO levels drop [36,37].

Two studies [38,39] have recently uncovered a more complex activation process that relies on non-heme NO in addition to NO bound at the heme, and on the nucleotides ATP and GTP. These studies show that, in the physiological context of ATP and GTP, deactivated sGC still has NO bound to the heme and that full activation requires non-heme NO (Figure 3). The new model derived from these recent observations fundamentally alters the way in which NO signaling should be viewed *in vivo*.

For more than two decades, elucidating the details of the binary NO/sGC model has relied on two fundamental modes of observation: first, the spectral properties of the

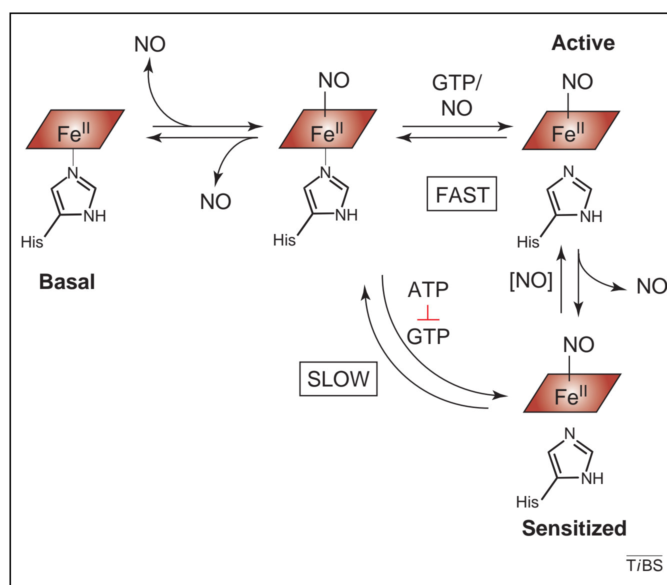

Figure 3. Allosteric modulation of NO–heme dynamics. The basal guanylate cyclase (sGC) heme contains five-coordinate Fe^{2+}. NO binds rapidly, at a rate approaching diffusion, forming a six-coordinate Fe^{2+}–nitrosyl intermediate. At a rate that depends on additional NO, histidine dissociates from the heme iron, resulting in a five-coordinate Fe^{2+}–nitrosyl complex. In the absence of excess NO, GTP accelerates conversion of the six-coordinate intermediate to the five-coordinate final species, which is fully active. ATP blocks GTP from accelerating conversion of the intermediate to the final species. When ATP is present with GTP, both conversion to the final species and dissociation of NO from the heme is slow. This species has low activity. Full activity requires additional NO. Fully active sGC rapidly deactivates in all cases.

heme ('heme dynamics', which entail the association and dissociation of ligands); and second, the correlation of these spectral properties with enzyme catalysis. In typical experiments, an excess of NO was added to sGC, the heme spectrum shifted to reflect binding of NO to the heme, and the enzyme became fully activated.

More detailed spectral studies have shown that formation of the final NO–heme complex occurs in two steps [34,35,40]. First, NO binds to the heme of sGC at the rate of diffusion, forming a six-coordinate Fe^{2+}–nitrosyl intermediate in which NO is bound on the distal side of the heme and His105 is ligated to Fe^{2+} from the proximal side [35]. Second, conversion of this intermediate to the five-coordinate Fe^{2+}–nitrosyl species requires dissociation of His105 from Fe^{2+}. This dissociation occurs more slowly than the initial NO-binding event, but unexpectedly its rate has been found to depend on the concentration of NO. At substoichiometric concentrations of NO, the conversion to the final species is even slower. Essentially, once NO is bound to the distal side of the heme Fe^{2+}, a second molecule of NO accelerates bond breakage between His105 and the Fe^{2+} ion. These data suggest that a second NO-binding site exists on sGC and mediates the kinetic properties of the NO–heme complex. Furthermore, full activation of sGC by excess NO occurs on the same timescale as cleavage of the Fe^{2+}–histidine bond, confirming that spectral and activation kinetics are correlated [34,35].

Values reported for the rate of dissociation of NO from the sGC heme vary widely, even though an accurate description of the off-rate of NO from the sGC heme is essential to understanding the rate of deactivation of sGC,

both *in vitro* and *in vivo*. Studies measuring the rates of dissociation of NO from the sGC heme have found that this process is slow [41,42]. In the study by Kharitonov *et al.* [41], for example, the rate of NO–heme dissociation was reported to be 6×10^{-4} s^{-1}, which translates to a half-life of 2 min at 37 °C. This rate is too slow to account for the rapid deactivation of sGC observed *in vivo*. Subsequently, the same authors reported that the inclusion of substrate with sGC accelerates dissociation of NO from the heme ~70-fold, in line with *in vivo* deactivation rates [43].

Consistent with these data, a study using sGC activity as a proxy for NO–heme dissociation reported a rapid deactivation rate with a half-life of 18 s [44]. In the off-rate studies of Brandish *et al.* [41], substrate had no effect on the rate of NO–heme dissociation and, when oxyhemoglobin was used as a trap for NO, the off-rate was found to be slow with a half-life of 2.5 min at 37 °C. Thus, although these experiments agree with the slow rates reported by some, they do not agree with data showing that off-rates in the presence of substrate are rapid. Nevertheless, these rates show that the affinity of the NO–heme complex is still high, ranging from the low to high picomolar range. By contrast, CO shows binding that is up to a million times weaker than that of NO, the Fe^{2+}–iron bond does not break, and the enzyme is activated only fourfold [33], suggesting that CO alone is not likely to be a physiological activator of sGC ([45]; reviewed in Ref. [46]).

The reasons for the discrepancies in the rates of NO–heme dissociation in the presence of nucleotide are not trivial and, as we discuss in the next sections, exploring the reasons for the differences in rates has led to a completely new understanding of the regulation of sGC by NO.

Regulation of sGC by non-heme NO

In an attempt to resolve the observed discrepancies in NO–heme off-rates, studies were undertaken in which the rate of NO dissociation from the heme was compared directly to the rate of enzyme deactivation measured in the same experiment [38]. These rates should be exactly the same if NO regulates sGC solely by binding to and dissociation from the heme. It was found, however, that the rate of dissociation of NO from the heme is ~150 times slower than the rate of enzyme deactivation. Isolation of the deactivated sGC enzyme showed that, although NO remained bound to the heme, this enzyme species had an activity of only 10–20% of that of the full NO-stimulated enzyme. Thus, additional NO – in excess of NO bound at the heme – is required for full enzyme activity. These unexpected results are clearly inconsistent with the binary model that NO regulates sGC solely at the heme.

Russwurm and Koesling [39] independently described isolation of a low-activity sGC species with NO bound at the heme. When they premixed sGC with GTP, cGMP or pyrophosphate before NO, however, they found that the activity of the Fe^{2+}–nitrosyl species was as high as when excess NO was used. Thus, for sGC to be fully activated with NO at the heme alone, substrate or product must bind to the enzyme before NO. The concentrations of products used were higher than would be expected under physiological conditions, so it is more likely that substrate is the physiologically relevant modulator of this effect. Russwurm and Koesling [39] also studied the effect of GTP on the binding of NO to the sGC heme. In the absence of GTP a six-coordinate intermediate was visible at 4 °C, but when GTP was premixed with sGC no six-coordinate species was evident, suggesting that conversion to the five-coordinate species (which is when the His105 bond to the iron breaks) occurs too rapidly for the intermediate to be observed. This study establishes that, if NO is bound to heme and there is no excess of NO, then GTP must be present to activate sGC fully at the heme. Furthermore, these results show that GTP affects NO–heme dynamics, accelerating formation of the final NO–heme complex.

On the basis of these observations, it seems that the order of GTP addition, through its effect on NO–heme dynamics in sGC, is crucial to the regulation of sGC by NO *in vivo* and is perhaps the reason for the wide discrepancies seen in the reported NO–heme off-rates. Intriguingly, some recent reports suggest that there is an allosteric nucleotide-binding site on sGC [47,48] (Box 1), and others indicate that physiological concentrations of ATP, an abundant cellular nucleotide, inhibit sGC activity via a mixed type of mechanism [49,50]. Thus, to investigate the regulation of sGC by NO in the context of cellular nucleotides, the effect of premixing sGC with GTP, ATP or both ATP and GTP on the binding and dissociation of NO to the heme has been studied [38]. Results from these experiments confirm that GTP accelerates formation of the final NO–heme complex, but demonstrate that physiological concentrations of ATP prevent GTP from accelerating this formation. Similar results were obtained on examination of the effect of GTP and ATP on

Box 1. A site for nucleotide modulation of soluble guanylate cyclase?

Recent data show that ATP can inhibit soluble guanylate cyclase (sGC) both competitively and non-competitively [49,50]. Although the location of an allosteric nucleotide-binding site has not been determined, homology between the catalytic domains of sGC and those of adenylate cyclase suggests that nucleotides might bind in a pseudosubstrate site that is symmetrically related to the catalytic site.

In adenylate cyclase, the pseudosubstrate site serves as the binding site for forskolin, an activator that causes conformational changes in the catalytic site (reviewed in Refs [64,65]). Accordingly, mutagenesis studies of the sGC pseudosubstrate site have implicated it as the region where ATP binds and affects catalysis [47]. Notably, some reports have suggested that YC-1, an allosteric activator of sGC that structurally resembles a purine nucleoside, might act analogously to forskolin. Mutation of residues in the putative pseudosubstrate site of sGC has provided indirect evidence that YC-1 signaling requires conserved motifs in this site [48], and it seems possible that YC-1 might be acting at a site that is also regulated by nucleotides.

Taken together, these studies suggest that a second nucleotide-binding site on sGC, possibly the pseudosubstrate site, binds to ATP, GTP and YC-1, although it remains possible that several such sites exist on the enzyme, increasing the complexity of regulation. Labeling studies using analogs of YC-1, ATP and GTP, in combination with careful competition studies, are required before a definitive assignment can be made on the mode of action of these molecules. In summary, nucleotides and purine-like molecules allosterically regulate the transmission of NO binding from the H-NOX domain to the catalytic site of sGC.

dissociation of NO from the sGC heme [38]. In these experiments, premixing GTP with sGC was found to accelerate dissociation of NO from the heme. Again, ATP blocked GTP from accelerating this dissociation. Surprisingly, although NO dissociation from the heme is slow when ATP and GTP are present, deactivation of the enzyme is rapid, reminiscent of the enzyme in the absence of premixed nucleotides.

The low-activity sGC species can be reactivated by the addition of more NO and, importantly, also by YC-1 (5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol), an allosteric activator of sGC (Box 2). In short, in the presence of physiological concentrations of ATP and GTP, NO partially activates the enzyme by binding to the heme, and full activation of sGC requires additional NO. These two NO-dependent activation states of sGC are temporally distinct: the low-activity species is long-lived because the NO–heme complex is highly stable; the high-activity species, which results from NO binding elsewhere on the enzyme, is transient and rapidly deactivates in the presence of a NO trap (Figure 3).

NO mediates two states of sGC activation: tonic and acute

These new results translate into a biochemical model with significant *in vivo* implications. Under normal resting conditions, during which basal levels of NO are synthesized in tissues, sGC in the presence of ATP and GTP is mostly likely to have NO stably bound at the heme and to be partially activated (Figure 4a). The picomolar affinity of the sGC heme for NO, coupled with the low nanomolar levels of basal NO in tissues, suggests that the sGC heme will be fully occupied. Only when a burst of NO synthesis occurs does the enzyme become fully activated, and deactivation occurs rapidly when levels of NO drop (Figure 4b). Intracellular globins, which react rapidly with NO, are likely to affect strongly the concentrations of NO that are available for activating sGC [51,52].

Since the early studies of Furchgott and colleagues [53], which identified NO as the endothelium-derived relaxing factor, a paradox has existed in the temporal signaling of NO. In the absence of endothelial stimulation, NO is

Box 2. Allosteric regulators of soluble guanylate cyclase

Several compounds have been identified that modulate soluble guanylate cyclase (sGC) activity. In a pharmacological screen, for example, the benzylindazole derivative YC-1 (5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol) was found to activate sGC in platelets [66]. In these studies, Wu *et al.* [66] claimed that YC-1 activated sGC independently of NO. However, YC-1 activates purified sGC only between three- and fivefold. Intriguingly, YC-1 synergizes with CO-bound sGC to activate the enzyme fully, and has only a minimal effect on the activation of sGC by excess NO [67,68].

In a recent study showing that NO at the heme activates sGC only partially, YC-1 was found to synergize significantly with this low-activity species to achieve full activity [38] (Figure I). Indeed, this result strongly suggests that the effects of YC-1 observed *in vivo*, which are typically described as NO independent, are in fact due to its synergy with the stable low-activity NO–heme sGC species, which effects full activity. The implications of this previously unobserved synergy are far-reaching: they imply that, *in vivo*, NO is constitutively bound to the heme of sGC because of the continual tonic activity of NOS and the high affinity of the NO–heme complex. YC-1 is, in effect, a reporter for basal NO and low-activity sGC. Furthermore, in this context the possibility of direct activation of sGC by CO becomes more problematic.

Recent resonance Raman and infrared spectroscopy studies with CO and YC-1 have attempted to explain why dissociation of the proximal axial histidine from the heme is not required to achieve full activation [69–72]. Although these reports do identify a possible five-coordinate CO–heme species, it is not abundant enough to account for the full activation of sGC. The non-essential nature of histidine dissociation with respect to YC-1 synergism has been further demonstrated in a recent study showing that YC-1 also synergizes with several nitrosoalkane ligands [73] (Figure I). In this report, nitrosoalkanes were shown to bind to the heme of sGC and to form six-coordinate complexes exclusively, regardless of the presence of YC-1. Despite having no effect on the coordination state of the heme–nitrosoalkane complexes, YC-1 nevertheless synergistically activated the enzyme, indicating that ligation of heme in the distal pocket is sufficient for synergistic activation of sGC by YC-1 and that histidine dissociation is not necessary for this activation.

The importance of sGC as a drug target has led to the identification of different modes of action through pharmacological screens. One mode of action seems similar to that of YC-1, which synergizes with an occupied heme (Figure I), whereas the other is apparently independent of heme [74,75]. In light of recent data describing two temporal states of NO activation of sGC, the characterization of these and other modes of action might provide important insights into the function and regulation of sGC.

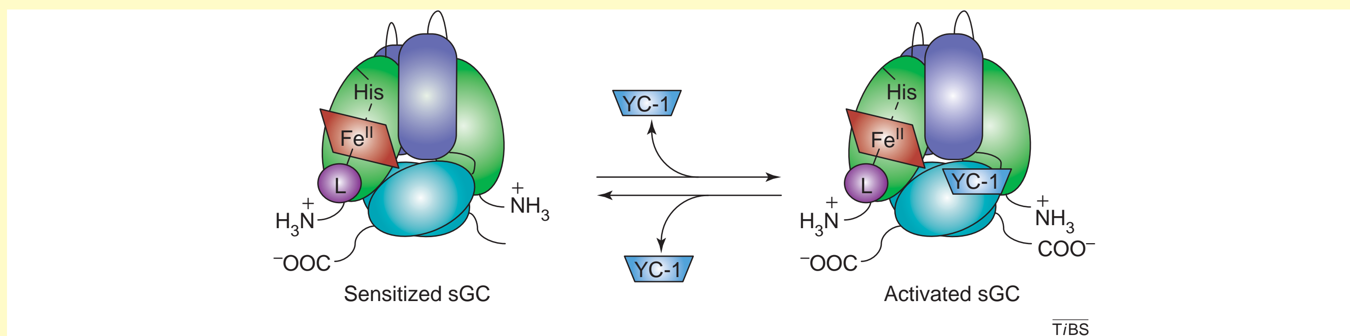

Figure I. Several molecules are now known to bind to the heme of sGC (L represents NO, CO or nitrosoalkanes). The NO–heme complex is five coordinate, whereas the CO and nitrosoalkane complexes are six coordinate (the dotted line to the histidine indicates the different coordination states). Assays of the NO–heme complex show that this species is stable and has low activity; the unstable CO–heme complex also has low activity. The nitrosoalkane complexes are stable and show almost no activity; however, YC-1 synergizes with each of these species to achieve much greater activation. Dissociation of the histidine bond is not required for YC-1 to activate sGC synergistically when ligands are bound in the distal heme pocket.

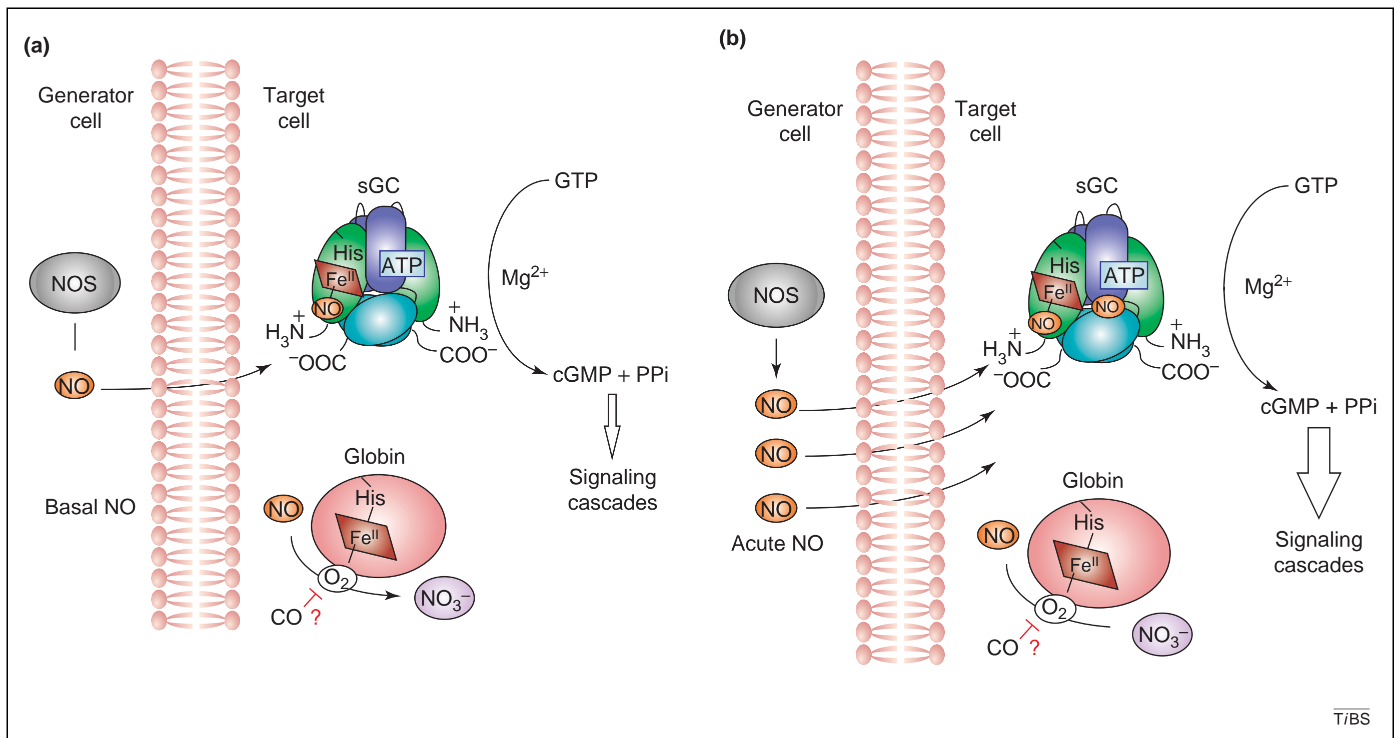

Figure 4. Tonic and acute NO signal transduction through guanylate cyclase (sGC). **(a)** Under resting conditions, basal levels of NO are tonically synthesized by NO synthase (NOS). In the presence of ATP, sGC has NO stably bound at the heme, generating a homeostatic cGMP signal. **(b)** On stimulation of the generator cell (e.g. by acetylcholine at the endothelial membrane), NOS is activated to produce a burst of NO. This acute signal fully activates the sensitized sGC, owing to NO binding at a non-heme site. The sharp rise in cGMP triggers large-scale responses such as vasodilation. Globins trap NO, reducing the amounts available for signaling. CO might inhibit these globins and prolong the NO signal.

spontaneously released and mediates a resting tonic state of the muscle [53]. If the endothelium is removed from a blood vessel, then contractile agonists become more potent. If hemoglobin is added to destroy NO in the vessel lumen, then, in the absence of contractile or relaxant agonists, the muscles contract. No rise in cGMP was detected in response to unstimulated release of NO in the Furchgott study [53], but subsequent work has shown that this low-level NO signal requires sGC and cGMP [54]. In short, NO seems to mediate a tonic resting state in smooth muscle through sGC. When acetylcholine or other relaxant factors stimulate NO synthase to produce acute levels of NO, then relaxation of the muscle is observed. This relaxation can be reversed rapidly as soon as NO synthesis ceases [55]. Addition of YC-1 to resting smooth muscle causes relaxation and this effect is slowly reversed, corresponding to the *in vitro* observations that YC-1 synergizes with the low-activity NO-bound sGC.

Similar observations have been made in other tissues affected by NO, including the cardiovascular system, lung, skin, intestine and hippocampal neurons involved in long-term potentiation (LTP; reviewed in Refs [6,56–58]). In fact, recent studies of LTP in hippocampal slices present the clearest evidence so far of tonic and acute NO/cGMP signaling [59]. Before and after the acute NO signal is triggered, low-level synthesis of NO is required to achieve LTP. Blocking the tonic signaling of NO through sGC, before and/or after the acute NO signal, prevents LTP formation. If tonic NO is inhibited in the hippocampus of conscious rats during a learning task, then the rats show amnesia [60,61]. Conversely, YC-1 enhances LTP formation, and its administration into the hippocampus during a learning task enhances learning [62,63]. In effect, physiological observations of the tonic and acute effects of NO in numerous contexts, ranging from the cardiovascular system to neurotransmission, can now be explained by the discovery that NO regulates two temporally distinct activity states of sGC.

It remains formally possible that the low-level activity of sGC in tissues is due to partial occupancy of the enzyme at low concentrations of NO, rather than to the low-activity species with full occupancy of the heme observed *in vitro*. However, the fact that the sGC NO–heme complex is picomolar *in vitro* and basal NO levels are nanomolar *in vivo* provides circumstantial evidence supporting full occupation of the sGC heme *in vivo*. Furthermore, YC-1 seems to synergize with sGC *in vivo* in the same way that it synergizes with the low-activity NO–heme species *in vitro*. It also strongly reactivates deactivated sGC both *in vitro* and *in vivo* (Box 2). YC-1 is, in effect, a reporter for the presence of low-activity NO–heme sGC in tissues. To test this idea further, cGMP levels could be quantified in tissues in which basal NO is produced and compared before and after the addition of excess NO or YC-1. If NO is truly mediating two distinct activities of sGC in cells, then excess NO and YC-1 should fully activate the low-activity NO–heme species to the same levels observed *in vitro*.

Because interpretations of the physiological data have often conflicted with the previous biochemical model of sGC regulation by NO, other factors have been called upon to account for the results. These interpretations must be

re-evaluated, especially because it now seems likely that sGC is constitutively bound to NO, forming a species that can be potently activated by YC-1 or acute levels of NO. The design and interpretation of future experiments in tissues should take into consideration the two NO-dependent activity states of sGC in addition to the effect of YC-1 and YC-1-like compounds on these states.

Concluding remarks

We have examined recent advances in the molecular basis of NO sensing in a newly defined 'H-NOX class' of gas sensors, one of which is the highly studied and important mammalian NO receptor sGC. NO discrimination is based on the fact that O_2 binding requires a hydrogen-bond donor in the distal heme pocket. Because sGC does not contain a hydrogen-bond donor in the distal pocket, it excludes O_2 from binding the heme. If a tyrosine is introduced into the distal pocket at the appropriate location, however, the heme domain of sGC becomes capable of binding O_2.

Ironically, recent detailed biochemical studies described in the second part of this review reveal that, although sGC has evolved to use a heme prosthetic group to sense NO in the presence of O_2, there is a second NO-binding site on sGC that seems to regulate acute activation of the enzyme. Physiologically, it seems that this non-heme site mediates vasodilation by acute NO signals, whereas the tight NO–heme complex mediates resting tone in the vasculature. Action of NO at the non-heme site occurs in the presence of GTP and ATP, suggesting that several allosteric nucleotide-binding sites are also present on the enzyme to regulate NO–heme dynamics and thereby catalytic activity. YC-1 seems to require a ligand bound to the heme in the distal pocket to activate sGC synergistically [73]; this requirement strongly suggests that the observed action of YC-1 and YC-1-like compounds in tissues depends on the existence of the stable NO–heme sGC species that is maintained by basal NO.

Identification of the non-heme NO-binding site on sGC, in addition to localization of the ATP and GTP sites, will be necessary to define further the mechanisms of regulation of this increasingly complex signal transduction enzyme. Interpretation of physiological studies of NO signaling should take into account the fact that NO has two distinct effects on sGC and that the concentration of NO, and the duration of its release, will have profound effects on the activation state of sGC.

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