

Biochemistry of Soluble Guanylate Cyclase

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Abstract Nitric oxide (NO) functions in biology as both a critical cytotoxic agent and an essential signaling molecule. The toxicity of the diatomic gas has long been accepted; however, it was not known to be a signaling molecule until it was identified as the endothelium-derived relaxing factor (EDRF). Since this discovery, the physiological signaling pathways that are regulated by NO have been the focus of numerous studies. Many of the cellular responses that NO modulates are mediated by the heme protein soluble guanylate cyclase (sGC). NO binds to sGC at a diffusion controlled rate, and leads to a several 100-fold increase in the synthesis of the second messenger cGMP from GTP. Other diatomic gases either do not bind (dioxygen), or do not significantly activate (carbon monoxide) sGC. This provides selectivity and efficiency for NO even in an aerobic environment, which is critical due to the high reactivity of NO. Several biochemical studies have focused on elucidating the mechanism of NO activation and O₂ discrimination. Significant advances in our understanding of these topics have occurred with the identification and characterization of the sGC-like homologues termed Heme-Nitric oxide and OXygen binding (H-NOX) proteins.

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1 The NO/cGMP Signaling Pathway

In eukaryotes nitric oxide synthase (NOS) produces NO from L-arginine (reviewed in (Alderton et al. 2001; Dudzinski et al. 2006; Stuehr 1997)). There are three isoforms of NOS, endothelial, neuronal and inducible (eNOS, nNOS, and iNOS). Both eNOS and nNOS are constitutively expressed and iNOS is induced with the appropriate immunostimulatory signals. The constitutive isoforms involved in signal transduction pathways (eNOS and nNOS) generate low nanomolar levels of NO and are regulated *in vivo* by the binding of calcium and calmodulin. NO produced by NOS can rapidly diffuse across a cell membrane to activate soluble guanylate cyclase (sGC), a hemoprotein that has evolved to selectively bind NO even in the presence of oxygen (μM) (Fig. 1). The sGC heme environment is unique when compared to the globins because it efficiently binds NO while having no affinity for O_2 coupled with an extremely slow rate of oxidation. sGC is a heterodimeric protein that consists of an α and a β subunit. NO binds to the heme of sGC and leads to a several 100-fold increase in the synthesis of cGMP from GTP. In addition to NO, cGMP production by sGC is regulated allosterically by substrate GTP and by ATP (Cary et al. 2005; Ruiz-Stewart et al. 2004) which are present at ~ 0.2 and ~ 1.7 mM, respectively, *in vivo* (Traut 1994). The efficient binding of NO by sGC allows for the rapid production of cGMP which then binds to phosphodiesterases (PDE), ion-gated channels, and cGMP-dependent protein kinases (cGK) to regulate several physiological functions including vasodilation, platelet aggregation and neurotransmission (Munzel et al. 2003; Sanders et al. 1992; Warner et al. 1994).

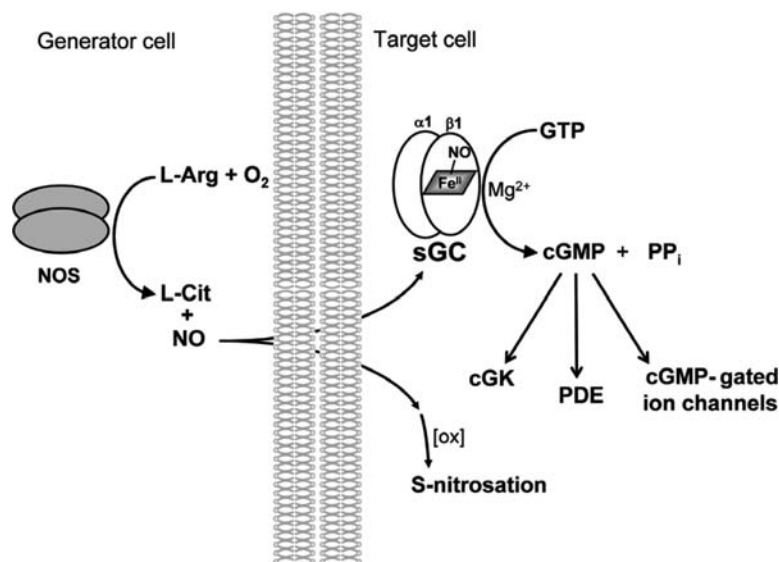


Fig. 1 Nitric oxide signal transduction pathway. NO synthesized by NOS diffuses across cell membranes to a target cell. NO activates sGC, which leads to an increase in cGMP synthesis. The oxidation products of NO can also react with protein thiols, which leads to protein S-nitrosation.

Both NOS and sGC are critical to the regulation of homeostasis, and dysfunction in the NO signaling pathway has been linked to several diseases (reviewed in (Bredt 1999)). The importance of the NO signaling pathway has also been demonstrated in mouse models where the triple NOS knock-outs exhibit characteristics consistent with nephrogenic diabetes insipidus (Morishita et al. 2005). Knock-outs of the sGC $\beta 1$ subunit exhibit elevated blood pressure, reduced heart rate and dysfunction in gastrointestinal contractility (Friebe et al. 2007), and studies on mice deficient of the sGC $\alpha 1$ subunit indicate that the protein is essential for NO-mediated pulmonary vasodilation (Vermeersch et al. 2007).

2 sGC Isoforms

sGC is a heterodimeric protein consisting of two homologous subunits, α and β . The most commonly studied isoform is the $\alpha 1\beta 1$ protein; however, $\alpha 2$ and $\beta 2$ subunits have also been identified (Harteneck et al. 1991; Yuen et al. 1990). The α subunits are highly homologous with 48% sequence identity, and β subunits have an overall sequence identity of 41%. Generally, a higher degree of variability is observed between α subunits of different species when compared to β subunits.

The localization of each subunit has been studied in mammals including humans, rats, and cows. Both $\alpha 1$ and $\beta 1$ are expressed in most tissues and it is well accepted that these proteins form a physiologically relevant heterodimer (Budworth et al. 1999). By Western blotting and quantitative PCR analysis the $\alpha 2$ subunit is found in fewer tissues compared to the $\alpha 1$ and $\beta 1$ isoforms, but is highly expressed in the brain, lung, colon, heart, spleen, uterus and placenta (Bellingham and Evans 2007; Budworth et al. 1999; Russwurm et al. 1998). Studies with the purified protein have shown that the $\alpha 2\beta 1$ heterodimer exhibits ligand binding characteristics identical to the $\alpha 1\beta 1$ heterodimer (Russwurm et al. 1998), but a splice variant of the $\alpha 2$ subunit ($\alpha 2i$) dimerizes with the $\beta 1$ subunit to form an inactive complex. $\alpha 2i$ contains an in-frame insertion of 31 amino acids within the catalytic domain and appears to function as a dominant negative protein (Behrends et al. 1995). The $\beta 2$ isoform is not very prevalent and is expressed primarily in the kidney (Yuen et al. 1990). While the $\beta 2$ isoform has not yet been purified and characterized, transient expression of the full-length protein in insect cells show that it is active as a homodimer, but exhibits no cyclase activity when expressed with $\alpha 1$ (Koglin et al. 2001). However, further experiments are necessary to determine if $\beta 2$ can indeed function as a novel homodimeric cyclase *in vivo*.

3 Architecture of sGC

The rat sGC $\alpha 1$ and $\beta 1$ subunits are 690 and 619 amino acids in length, respectively. These proteins are part of a large family of sGC subunits that are conserved in eukaryotes. Generally there is the highest sequence variability at the N-terminus

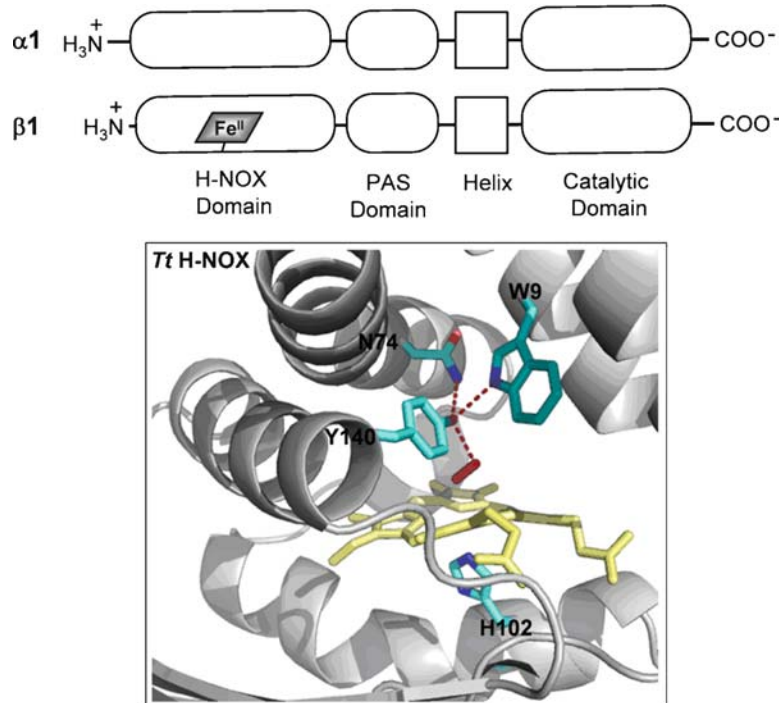


Fig. 2 Soluble guanylate cyclase. *Top panel*, domain architecture of sGC. sGC consists of two homologous subunits, $\alpha 1$ and $\beta 1$. Each subunit contains an N-terminal H-NOX domain, a central PAS domain, a putative amphipathic helix, and a C-terminal catalytic domain. Heme (grey parallelogram) binds to the H-NOX domain on the $\beta 1$ subunit. *Bottom panel*, crystal structure of *Tt* H-NOX bound to O_2 (1U55.pdb). Residues that stabilize O_2 -binding (W9, N74 and Y140) and coordinate to the heme iron (H102) are shown.

of α subunits and the greatest sequence identity at the C-terminus of both the α and β proteins. Each sGC subunit consists of four distinct domains. The $\beta 1$ subunit contains a N-terminal heme binding domain, a Per/Arnt/Sim (PAS) domain, a putative amphipathic helix, and a C-terminal catalytic domain (reviewed in (Cary et al. 2006)) (Fig. 2). While bioinformatics have sufficiently identified the PAS domain, the helix, and catalytic domain, experiments with sGC truncations and site-direct mutagenesis were necessary to localize the minimal heme binding domain of sGC. These experiments involved the systematic mutation of histidines (Wedel et al. 1994), expression of various truncations in *E. coli* (Zhao and Marletta 1997) and deletion of the $\beta 1$ N-terminus (Wedel et al. 1995). Taken together, these studies showed that the $\beta 1$ N-terminus constituted the heme binding domain and suggested that histidine 105 (rat numbering) was the proximal heme ligand.

The sGC heme binding domain was initially localized to residues 1–385 (Zhao and Marletta 1997); however, it is now clear that residues 1 to ~194 encode the minimal heme binding domain (Karow et al. 2005). While it was once thought that

this domain was exclusively associated with NO activated guanylate cyclases found in mammals, it is now known that the sGC heme domain is part of a conserved family of proteins found in prokaryotes and eukaryotes (Iyer et al. 2003). This family of proteins is termed the Heme-Nitric oxide and OXygen binding family (H-NOX) based on their ligand binding properties and are discussed in more detail in the next section (reviewed in (Boon and Marletta 2005)).

The central region of sGC contains two domains of unknown function. One domain includes residues ~200–350 of $\beta 1$ and ~270–400 of $\alpha 1$, and is predicted to adopt a PAS-like fold (Kelley et al. 2000). Typically PAS domains mediate protein-protein interactions and/or bind small molecules such as hemes, flavins and nucleotides. The other domain, a putative helix region, appears to be unique to sGC and shares no homology with any other protein in the NCBI protein database. Site-directed mutagenesis of residues in this region on the $\alpha 1$ subunit (Shiga and Suzuki 2005), as well as a bimolecular fluorescence complementation assay in cells suggest that the central regions of both sGC subunits are important for the formation of a functional heterodimer (Rothkegel et al. 2007).

The catalytic domains have been localized to the C-terminal 467–690 and 414–619 residues of the $\alpha 1$ and $\beta 1$ subunits, respectively (Winger and Marletta 2005). These domains must form a heterodimer for cGMP to be synthesized, and in the full-length protein the catalytic efficiency of the protein is dependent on the heme ligation state of the $\beta 1$ H-NOX domain. Interestingly, the activity of the isolated $\alpha 1_{\text{cat}}\beta 1_{\text{cat}}$ heterodimer is inhibited by the presence of the H-NOX domain (Winger and Marletta 2005). This shows that these domains interact in *trans*, and suggests that the NO mechanism of activation involves the relief of an inhibitory interaction between the H-NOX domain and the catalytic domains.

The sGC catalytic domains are highly homologous to both the particulate guanylate cyclase (pGC) and adenylate cyclase (AC) catalytic domains. There are crystal structures of AC catalytic domains (Sunahara et al. 1997; Zhang et al. 1997), but the structure of a guanylate cyclase has yet to be reported. However, key catalytic residues have been identified through multiple sequence alignments and homology models of the rat $\alpha 1$ and $\beta 1$ catalytic domains based on the AC structures (Winger 2004). From these models two conserved aspartate residues on the $\alpha 1$ subunit (D485 and D529 rat numbering) are predicted to bind two Mg^{2+} ions. These residues are critical to catalysis as the associated metals likely function to activate both the nucleotide 3'-hydroxyl and the α phosphate for the reaction, and stabilize the charge on the β and γ phosphates on both substrate and product. Additionally, β N548 is proposed to orient the ribose ring for the reaction. Residues thought to be responsible for base recognition include E473 and C541 on the $\beta 1$ subunit. Other residues on both α (R573) and β (R552) are thought to interact with the nucleotide triphosphate (Winger 2004). With the identification of these critical residues, predictions can be made about guanylate cyclase activity based on sequence analysis. This type of analysis would correctly predict that the $\beta 2$ isoform can function as a homodimer but that $\beta 1$, $\alpha 1$, and $\alpha 2$ need a partner to be active.

4 H-NOX Proteins

As mentioned earlier the sGC heme binding domain is now known to be part of a large family of heme binding proteins termed H-NOX proteins. To date all of the characterized bacterial H-NOX proteins bind heme, and were named H-NOX proteins to describe the ability of some of these proteins to bind O₂ in addition to NO and CO, while others, like sGC, discriminate against O₂ binding. H-NOX domains are found in both facultative aerobes and in obligate anaerobes. Interestingly, all of the isolated H-NOX domains from facultative aerobes do not bind O₂ while the H-NOX domains from obligate anaerobes form a stable O₂ complex. The variable ligand binding properties of these H-NOXs may have consequences for their ability to respond to different gases, and functional experiments will need to consider the possibility that the proteins may sense O₂ in addition to NO or CO.

In facultative aerobes the bacterial members of this family encode a single domain as a stand-alone protein, and genes that encode for either putative histidine kinases or diguanylate cyclases are found in the same predicted operon. This suggests that the domain has a role in two-component signaling in bacteria. In support of this hypothesis, an H-NOX domain and a predicted histidine kinase from *Shewanella oneidensis* were isolated and found to interact *in vitro*. Additionally, the functional interaction between the H-NOX and kinase was mediated by NO (Price et al. 2007).

In obligate anaerobes such as *Thermoanaerobacter tengcongensis* (*Tt*), the H-NOX domain is predicted to be part of a methyl-accepting chemotaxis protein. This would suggest that the H-NOX protein is involved in a chemotactic/signaling function, although this hypothesis awaits experimental verification. The isolation and characterization of the *Tt* H-NOX domain has significantly influenced our understanding of sGC since it was the first H-NOX domain to be structurally determined, and, moreover, it was crystallized bound to the diatomic ligand O₂ (Nioche et al. 2004; Pellicena et al. 2004) (Fig. 2). These structures showed that a distal pocket tyrosine (Y140) interacts with bound O₂ through a hydrogen bond, and provided the basis for the current molecular view of how sGC discriminates against O₂ binding, namely it lacks a hydrogen bond donor. This idea was further supported by the subsequent crystal structure of the O₂-excluding H-NOX from *Nostoc sp.* which shows that there is no hydrogen bond donor in the distal heme pocket (Ma et al. 2007). Additionally, comparison of the *Tt* H-NOX and *Ns* H-NOX structures in different ligation states (Fe^{II}-CO, Fe^{II}-NO and Fe^{II}-unligated states) provided evidence for a molecular mechanism of sGC activation. Specifically the differential pivoting and bending in the H-NOX heme upon NO or CO binding may account for the varying degree of activation induced by the two ligands (200-fold vs 4-fold, respectively) (Ma et al. 2007). The crystal structure of a domain from the *Nostoc punctiforme* signal transduction histidine kinase (STHK) was also recently determined. This domain has high sequence identity (35–38%) to the sGC PAS domain and the crystal structure showed that the domain dimerized and adopted a PAS fold (Ma et al. 2008). While structural studies of H-NOX proteins have facilitated our

understanding of sGC activation, the precise details about how movement in the sGC H-NOX domain affects the catalytic domain may remain unresolved until the full-length structure is elucidated.

Biochemical analyses on the sGC H-NOX domain, as well as the bacterial H-NOX proteins have also provided insight into the sGC heme environment and ligand binding characteristics – specifically discrimination against O₂ binding. If the *Tt* H-NOX distal pocket tyrosine is mutated to a leucine (Y140L), O₂ affinity is significantly reduced. Additionally, the introduction of a distal pocket tyrosine in O₂-excluding H-NOXs such as the *Legionella pneumophila* H-NOX (L2 H-NOX) and the sGC β 1(1–385) domain enables the proteins to bind O₂ (Boon et al. 2005).

5 sGC Activators

In addition to NO, a physiological sGC activator, CO can bind to the sGC heme and weakly activate the protein (Stone and Marletta 1994). The binding of CO leads to the formation of a 6-coordinate Fe^{II}-CO complex and a 2–4-fold increase in the rate of cGMP production. This activation is significantly lower than the 100–400-fold increase in cGMP production observed with NO. While the physiological relevance of activation of sGC by CO continues to be a matter of debate, CO has been used as a tool to probe the heme environment and activation properties of sGC. Other compounds that have been reported to activate sGC include organic nitrates (Artz et al. 2002) and protophorphyrin IX (Ignarro et al. 1982). Conversely, reported inhibitors of sGC include heme and hematin (Ignarro et al. 1982), LY83583 (Mulsch et al. 1988), methylene blue (Dierks and Burstyn 1998), and the heme oxidant 1H-[1, 2, 4]oxadiazolol[4, 3-*a*]quinoxalin-1-one (ODQ) (Garthwaite et al. 1995).

It has become clear that small molecules can modulate the activity of sGC and that new therapeutics might be developed for the treatment of various diseases. Several compounds were screened for the ability to increase cGMP levels in cell lysates to search for a novel sGC activator. Such a screen led to the identification of YC-1, a benzylindazole derivative that activates sGC without coordinating to the heme (Ko et al. 1994). YC-1 only activates the Fe^{II}-unligated sGC state 2–4-fold, but significantly increases sGC activity when a ligand is bound at the Fe^{II} heme (Friebe et al. 1996; Stone and Marletta 1998). This synergistic activation leads to a Fe^{II}-CO complex that is activated 100–400-fold and a Fe^{II}-NO complex that is activated 200–800-fold. The novel sGC activator YC-1 led several groups to carry out structure-activity relationships to improve both the solubility and efficacy of YC-1. This led to the identification of several other compounds including BAY 41–2272, BAY 41–8543, CMF-1571, and A-350619 (reviewed in (Evgenov et al. 2006)). Collectively these molecules constitute a novel class of sGC activators that require the presence of the heme moiety and have the ability to synergistically activate sGC with both NO and CO.

5.1 Activator Binding

The binding of both NO and CO to the sGC heme has been extensively studied with standard spectroscopic methods such as electronic absorption, resonance Raman (RR) and electron paramagnetic resonance (EPR) to understand how they activate sGC. sGC is isolated in the reduced unligated state which is characterized by an absorbance maximum at 431 nm. While sGC has the same histidine ligated heme cofactor as found in the globins, it has distinct ligand binding properties. Unlike the globin heme, the sGC heme is stable in the presence of molecular oxygen and is not susceptible to oxidation. Both CO and NO bind to the reduced sGC heme and form complexes that are characterized by an absorbance maximum at 423 nm and 399 nm, respectively. Importantly, with these spectroscopic techniques ligand binding can be probed independently of activity, which is important for assessing the mechanism of sGC activation.

CO binds to the sGC heme with a fast on-rate ($3.58 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) and a fast off-rate (3.5 s^{-1}) at 10°C . Experiments have shown that the binding of CO to the heme ($K_d = 97 \text{ }\mu\text{M}$) is directly correlated with sGC activation by CO ($K_A = 24 \text{ }\mu\text{M}$) (Stone and Marletta 1995). The binding and dissociation of CO from the sGC heme is increased in the presence of GTP and the allosteric activator YC-1 (Kharitonov et al. 1999). This effect shows that the binding of small molecules to a yet unknown site influences the heme site. This observation is also supported by experiments with RR spectroscopy which show that GTP and YC-1 have an effect on the RR spectrum of the sGC Fe^{II} -CO complex. Specifically, they cause a shift in both the Fe-CO and C-O stretching vibrations (Li et al. 2005; Makino et al. 2003; Martin et al. 2005). These effects are complicated as the small molecules change both the population of 6-coordinate Fe^{II} -CO and produce a small population of 5-coordinate Fe^{II} -CO. Some investigators have placed significant weight on the formation of a 5-coordinate Fe^{II} -CO complex and propose that the species is highly active. However, the Fe^{II} -CO complex in the presence of YC-1 and GTP is a mixture of three different species, and there is currently no evidence tying a specific activity with any one of these species.

Studies with NO have proven to be even more complicated. NO binds to the heme of sGC at a diffusion-controlled rate to form an initial 6-coordinate complex, which rapidly converts to a 5-coordinate ferrous nitrosyl complex (Stone and Marletta 1996). Initially it was thought to be a simple binding process, with a bimolecular formation of the 6-coordinate Fe^{II} -NO complex and a single exponential decay to the 5-coordinate Fe^{II} -NO complex. However, stopped-flow kinetics revealed that the NO association kinetics are best fit to a model that was bimolecular in NO concentration for both the formation of the 6-coordinate Fe^{II} -NO complex and, surprisingly, the conversion to the 5-coordinate Fe^{II} -NO complex (Stone and Marletta 1996; Zhao et al. 1999). The NO dependence of this second step suggests the existence of a second NO binding site (Stone and Marletta 1996; Zhao et al. 1999). Dissociation of NO from the heme is relatively slow (0.0007 s^{-1}) (Kharitonov et al. 1997) which would suggest that the sGC heme-NO complex has a 10–100 pM K_d . This is significantly lower than the measured K_d of NO activation

100–250 nM (Stone and Marletta 1996). This apparent contradiction was unexplained until recent work that showed NO coordination to the heme is not sufficient for full enzymatic activation (Cary et al. 2005; Russwurm and Koesling 2004). A low-activity Fe^{II} -NO complex can be formed in the presence of stoichiometric amounts of NO, and based on electronic absorption spectroscopy this species is identical to the highly active enzyme that is formed in the presence of substrate, products, YC-1, or excess NO. Based on these observations two mechanisms of NO activation have been proposed. One proposal is that excess NO activates the ferrous nitrosyl complex by binding to a nonheme site on the protein (Cary et al. 2005). The second proposal involves NO binding to the Fe^{II} -NO complex to form a transient dinitrosyl complex, which then converts to a 5-coordinate complex with NO bound in the proximal heme pocket (Russwurm and Koesling 2004) (Fig. 3). While both proposals involve the NO dependent conversion of a low-activity 5-coordinate Fe^{II} -NO complex to a high-activity 5-coordinate Fe^{II} -NO complex, the second proposal is dependent on NO binding to the heme cofactor. A recent study tested these proposals by using the heme ligand butyl isocyanide to block NO binding to the sGC heme. This study showed that NO activates the sGC Fe^{II} -butyl isocyanide complex without coordinating to the heme cofactor, and further supports the existence of a nonheme NO binding site (Derbyshire and Marletta 2007).

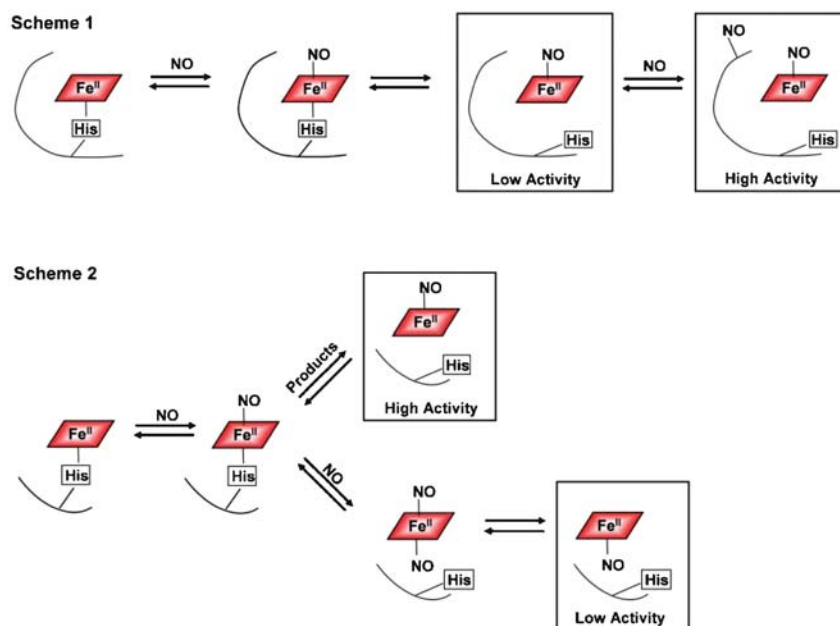


Fig. 3 Proposed mechanisms of NO activation of sGC. In Scheme 1, NO binds to the sGC heme to form a 5-coordinate ferrous nitrosyl complex. In the absence of excess NO this sGC species exhibits a low activity, which is activated by the binding of NO to a nonheme site. In Scheme 2, NO binding to the proximal heme pocket leads to a low-activity species, while NO binding to the distal heme pocket produces a high-activity species.

Recently the nitrosation of sGC in the presence of low levels of NO was reported. *S*-Nitrosation, the oxidative modification of cysteine residues, led to a reduction in NO-stimulated sGC activity (Sayed et al. 2007). This suggests that the modification may account for NO tolerance and desensitization. Additionally, this report highlights both the importance of cysteines to NO-induced activation, and shows that certain residues are accessible for binding NO.

As was observed for CO, the NO association and dissociation rates can be increased by the presence of GTP and YC-1 (Kharitonov et al. 1997; Russwurm and Koesling 2004; Winger et al. 2006). However, GTP only accelerates these rates if it is present before NO addition (Russwurm and Koesling 2004). This suggests there are multiple conformational changes that are dependent on the order of NO and GTP addition to sGC. Additionally, both RR and EPR spectroscopy confirm that the Fe^{II}-NO environment is influenced by the presence of GTP and YC-1. RR spectroscopy shows that the N-O stretch shifts from 1681 to 1700 cm⁻¹ in the presence of GTP (Tomita et al. 1997), and the EPR spectrum of the Fe^{II}-NO complex shifts to a unique signal with either GTP or YC-1 addition (Derbyshire et al. 2008; Makino et al. 2003). This indicates that there are at least two sGC Fe^{II}-NO conformations of differing activities, and that the abundance of each conformation is influenced by both substrate and activator binding. This proposal is further supported by NO dissociation experiments which show that the sGC ferrous nitrosyl complex adopts two 5-coordinate conformations that are influenced by the presence of GTP and YC-1; a lower-activity complex which releases NO slowly and a higher-activity complex which releases NO rapidly (Winger et al. 2006). Understanding these molecular details of activation are critical for the rational design of therapeutic agents to treat diseases involving the NO/sGC/cGMP pathway.

6 Ligand Discrimination in sGC

The ability of sGC to select against O₂ binding is important for it to function as a NO sensor in aerobic environments such as human tissue. Some of the earliest proposals about ligand discrimination in sGC were based on RR data and a wealth of experiments carried out with the globins. These proposals include negative polarity in the heme distal pocket (Deinum et al. 1996), a weak Fe-His bond (Deinum et al. 1996) and a sterically crowded distal pocket (Jain and Chan 2003). All of these factors could reduce O₂ affinity in a protein; however, it is unlikely that one of these proposals could alone prevent O₂ from binding to the sGC heme. From structure activity relationships with sGC and structural investigations on the H-NOX proteins, it appears that sGC has a relatively large, hydrophobic distal heme pocket (Derbyshire et al. 2005; Nioche et al. 2004; Pellicena et al. 2004). Additionally, a comparison of the Fe-His bond strength in various O₂-excluding and O₂-binding H-NOXs shows that there is no obvious correlation between Fe-His bond strength and the ability of a protein to bind O₂. Clearly, studies on H-NOX proteins have discredited many of the leading hypotheses on ligand discrimination in sGC, but structural studies on H-NOXs provided an alternative explanation.

The crystal structure of the O₂-binding *Tt* H-NOX shows that a distal pocket tyrosine (Y140) interacts with bound O₂ through a hydrogen bond (Nioche et al. 2004; Pellicena et al. 2004). Multiple sequence alignments of other H-NOXs with this protein suggest that O₂-excluding H-NOXs, like sGC, have hydrophobic distal heme pockets that lack a hydrogen bond donor. This prediction is supported by the recently solved crystal structure of the O₂-excluding H-NOX from *Nostoc* sp. (Ma et al. 2007). Additionally, *L2* H-NOX and the sGC heme domain β 1(1–385) were converted into O₂-binding proteins by introducing a single Tyr into their distal heme pockets (Boon et al. 2005). However, introduction of a single tyrosine in the full-length sGC did not stabilize O₂ binding, highlighting the importance of the other sGC domains in ligand binding (Martin et al. 2006). This work emphasizes the importance of a distal pocket hydrogen bond donor for ligand discrimination in H-NOXs, but suggests other factors must be critical for preventing O₂ binding in the mammalian sGCs. Recently, the identification of O₂-binding guanylate cyclases (*vide infra*) has allowed the importance of a distal pocket hydrogen bond donor in ligand discrimination to be more closely evaluated.

6.1 The Existence of O₂-Binding sGCs

As more genomes are sequenced an increasing number of organisms are found that contain predicted sGCs. Some sGCs are very similar to the well characterized rat α 1 and β 1 subunits while others vary significantly. For example, the *Drosophila melanogaster* genome contains five genes that code for sGCs. Two of these genes code for subunits that form a highly NO-sensitive sGC (Gyc α -99B and Gyc β -100B) while three code for subunits that are weakly stimulated by NO (Gyc-88E, Gyc-89Da and Gyc-89Db) based on lysate assays with overexpressed protein (Morton et al. 2005). These weakly NO responsive sGCs have been termed atypical sGCs (Morton 2004), and have been identified based on sequence analysis in organisms ranging from *Caenorhabditis elegans* (Gcy31-Gcy37) to *Oncorhynchus mykiss* to *Manduca sexta* (MsGC- β 3).

With the discovery that a distal pocket tyrosine is important for stabilizing O₂-binding in *Tt* H-NOX, the sequences of these predicted sGCs were examined to determine if they encode a tyrosine that aligns with *Tt* H-NOX Y140. Indeed, several of these atypical sGCs contain a tyrosine that is predicted to be in their heme distal pocket, which would suggest they bind oxygen. Experiments in mammalian cells did suggest that the *D. melanogaster* sGCs (Gyc-88E, Gyc-89Da and Gyc-89Db) were responsive to O₂ (Morton 2004), but this work did not directly examine ligand binding. This was only recently accomplished with the characterization of the purified Gyc-88E homodimer (Huang et al. 2007) which is the first and only report to show a Fe^{II}-O₂ complex in a guanylate cyclase. As expected, Gyc-88E also binds NO and CO. Interestingly, Gyc-88E is inhibited 2–3-fold by the binding of NO, CO and O₂, a property which is quite distinct from the ligand induced activation of the sGC α 1 β 1 heterodimer.

There is a contradiction to this current proposal on ligand discrimination in sGC. The Gcy-35 H-NOX domain from *C. elegans* does bind O₂, but does not encode a tyrosine that aligns with *Tt* H-NOX Y140 based on multiple sequence alignments or a homology model generated using the *Tt* H-NOX structure. However, there is no crystal structure of this protein and it remains possible that an analogous hydrogen bond donor in the distal heme pocket stabilizes O₂ binding. Importantly, deletion of *gcy-35* disrupts cGMP-dependent behavioral responses to hyperoxia in worms, suggesting the protein functions as an O₂ sensor *in vivo* (Gray et al. 2004). Thus, although significant progress has been made in our understanding of ligand discrimination in sGC there remain some unknown variables that may contribute to the specificity of these heme proteins.

7 Conclusions

Although several biochemical reports have emerged over the past several years about sGC structure and function, the mechanism of sGC activation by NO remains unresolved. Clearly, the sGC response to NO is very complicated, and involves both NO binding to the heme and a second NO binding site. Furthermore, sGC is regulated by allosteric interactions with ATP and GTP. There are likely to be other important regulatory factors, perhaps including S-nitrosation. Despite the complexity of sGC, the H-NOX protein family has illuminated many important structural features of the sGC heme binding domain, and this family is likely to continue to shed light on the complicated activation and deactivation pathways.

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