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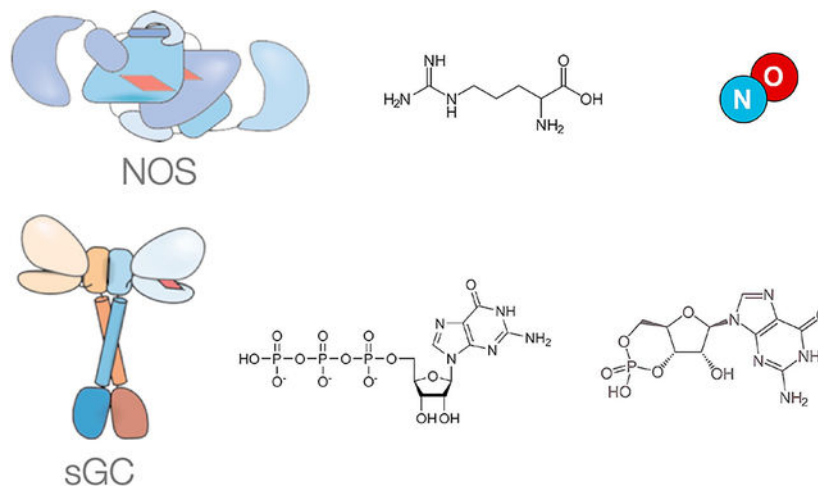# Revisiting Nitric Oxide Signaling: Where Was It, and Where Is It Going?

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Abstract

Nitric oxide (NO) has long been known to be an intermediate in bacterial pathways of denitrification. Only in the middle to late 1980s was it found to play a central role in a much broader biological context. For example, it is now well established that NO acts as a signaling agent in metazoans, including humans, yet NO is toxic and very reactive under biological conditions. How is the biology in which NO plays a role controlled? How is NO used and the inherent toxicity avoided? Looking back at the initial discovery time, to the present, and on to the future provides many answers to questions such as those listed above.

Graphical Abstract

As it turns out, 1988 was an eventful year in the world of nitric oxide (NO) function in biology. This Perspective looks forward from a *Biochemistry* paper published in that year.[1] Some context will be helpful to appreciate the field and thinking at that time and where we are today. The key early discoveries, including the serendipity involved, have recently been reviewed.[2] Our work from the early 1980s to the publication of the 1988

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paper was directly influenced by Tannenbaum and colleagues at the Massachusetts Institute of Technology, who showed in metabolic balance studies that humans were capable of nitrite (NO_2^-) and nitrate (NO_3^-) biosynthesis.[3] At that time, the interest in NO_2^- and NO_3^- exposure concerned the potential for the these anions (specifically NO_2^- under acidic conditions) to engage in the endogenous formation of carcinogenic *N*-nitrosamines. Prior to the Tannenbaum work, exposure was expected to be environmental, for example, in drinking water; however, mammalian NO_2^- and NO_3^- biosynthesis changed the thinking about endogenous *N*-nitrosamine formation. The mammalian source and pathway were unknown, and it was in that context that we began our studies.

EARLY STUDIES

Two important clues came from continued studies in the Tannenbaum lab. One comparing normal and germ-free rats provided unambiguous evidence that NO_3^- biosynthesis (actually NO_3^- urinary excretion) was a mammalian pathway,[4] and the other showed that the nitrogen atom in excreted NO_3^- was enriched with ^{15}N when rats were fed $[^{15}N]NH_3$ and the amount of excreted NO_3^- was enhanced following treatment of the rats with *Escherichia coli* lipopolysaccharide (LPS).[5] The LPS experiment was prompted by continued metabolic balance studies in humans and the serendipitous observation of highly elevated NO_3^- levels in a subject who became ill during data collection.

With this information, we were able to outline a rational experimental approach toward the formation of NO_3^- in mammals; thus, we showed that immunostimulated macrophages[6] and macrophage cell lines formed NO_2^- and NO_3^- in response to LPS and IFN-γ.[7] Interestingly, but a puzzle at the time, NO_2^- and NO_3^- were produced in a defined ratio suggestive of a common intermediate. With a cellular source of NO_2^- and NO_3^- identified, the question of the chemical precursor could addressed in a direct set of experiments. Because $[^{15}N]NH_3$ enriched NO_3^-, the hypothesis was that NO_2^-/NO_3^- was a product of protein catabolism, so a protocol for testing this hypothesis involved activation of macrophages with LPS/IFN-γ followed by a change in medium and resupplementation with individual amino acids. With this approach, the NO_2^-/NO_3^- precursor was found to be L-arginine and the sole amino acid product to be citrulline.[8] Although never determined, the stereochemistry at the α-carbon has been assumed not to change so the citrulline formed should have the L stereochemistry. With these findings, the question of why activated macrophages would convert L-arginine to NO_2^-/NO_3^- naturally arose. Speculation rested on the fact that immunostimulated macrophages were activated with respect to killing. It had been known for many years that NO_2^- was toxic to bacteria,[9] so was it possible that this metabolically derived NO_2^- was being used as a bacteriostatic or even killing agent. A further, key important tie of this pathway was made by Hibbs and co-workers when they showed L-arginine was required for activated macrophages to kill co-cultured tumor cells.[10] Hibbs also reported on the formation of NO_2^- and citrulline from L-arginine.[11]

ENDOTHELIUM-DERIVED RELAXING FACTOR (EDRF)

Independently and in parallel, pharmacologists were in search of EDRF (endothelium-derived relaxing factor). Two important contributors to the EDRF story were Robert

Furchgott and Lou Ignarro.[12] Along with Ferid Murad, Furchgott and Ignarro shared the 1999 Nobel Prize in Physiology or Medicine. EDRF was known to involve cyclic GMP as a second messenger formed via the action of the soluble isoform of guanylate cyclase (sGC). You could say that the search for EDRF began in the days of Alfred Nobel and the headache-inducing molecule nitroglycerin. Subsequent studies showed that many simple organic nitrates were vasodilators.[13] It was Murad and colleagues who showed that NO itself could activate sGC leading to cGMP formation.[14] Though hindsight descriptions differ, at the time, NO activation was viewed an anomaly of sorts because surely something as toxic as NO could not be the physiological activator, i.e., Nature's EDRF. However, that indeed turned out to be the case. Furchgott first suggested EDRF was NO, but it was a 1987 paper published by Moncada and colleagues that definitively showed EDRF was NO.[15]

EDRF–MACROPHAGE CONNECTION

The Moncada report was a galvanizing paper. We recognized that the instability of NO in aerobic, aqueous solution would lead to the formation of NO_2^- and NO_3^-, so we looked to see if cytosol from LPS/IFN-γ-activated macrophages could form NO from L-arginine; indeed, this was the case and led to the 1988 *Biochemistry* paper.[1] Moncada and colleagues looked to the literature for a lead to NO formation and found the macrophage NO_2^-/NO_3^- story that prompted them to look for NO from L-arginine in endothelial cells and found this was the case.[16] Therefore, as mentioned above, 1988 was an eventful year for NO biochemistry. These papers showed the common biochemical thread of NO formation in endothelial and activated macrophages, albeit toward very different biological end points. In that same year, Garthwaite and colleagues reported on a role for NO in neuronal signaling.[17] The key discoveries culminating with the papers in 1988 focused intense research efforts in the ensuing years up to the present. There were several key papers published simultaneously or close in time to those mentioned above, including one from Hibbs and another from Stuehr and Nathan.[18,19]

In addition to showing that NO was an intermediate in the L-arginine to NO_2^- and NO_3^- reaction, our *Biochemistry* paper reported that the enzymatic activity was cytosolic and had a strict requirement for NADPH. This requirement for NADPH coupled with previous work on the NO_2^- and NO_3^- to citrulline pathway[8] showing that the N in NO_2^- and NO_3^- was derived from one of the two chemically equivalent guanido N's in L-arginine led to the speculation that the first step must involve N-hydroxylation (Figure 1). It should be noted that the pathway in the figure was developed without any knowledge of how many enzymes might be involved in the overall transformation or, for that matter, any intermediates. Of course, it turned out to be one enzyme, nitric oxide synthase (NOS), and N-hydroxylation did indeed turn out to be the first step in the overall reaction (Figure 2).[20] The rest of the pathway outlined in Figure 1 was based simply on reasonable chemical steps to get to NO and citrulline.

NITRIC OXIDE SYNTHASE AND SOLUBLE GUANYLATE CYCLASE

Progress on NOS was rapid, including purification of the constitutive and inducible isoforms,[21–23] general characterization, mechanism,[24] and structures.[25] The constitutive

isoforms involved in signaling are strictly controlled by Ca^{2+} and calmodulin. Ca^{2+} and calmodulin were found to co-purify with the inducible NOS isoform and thus, once expressed, are constitutively active. All NOS isoforms contain an oxidase domain where the chemistry on L-arginine takes place and a reductase domain where reducing equivalents from NADPH are shuttled into the oxidase domain. The P450-type heme in the oxidase domain was consistent with the hypothesis that the first step of the reaction is the hydroxylation of arginine.[26] The oxidase domain was also found to contain 6(*R*)-tetrahydro-L-biopterin.[27,28] The not so obvious role for this cofactor took some time to sort out.[29] Many have contributed to our current mechanistic understanding. A review by Groves is an excellent summary of that understanding.[30] Crane and others have characterized bacterial oxidase homologues of NOSs with intriguing biological roles.[31]

Once made, NO function ensues. If signaling, the firmly established target is, as mentioned above, sGC (Figure 3). When involved in the response of the host to infection, the indiscriminate reaction chemistry of NO comes into play acting as a general inhibitor of cell growth, sometimes leading to cell death. As described above, decomposition of NO to NO_2^- and NO_3^- involves electrophilic intermediates capable of nitrosation. Cysteine residues are particularly susceptible, and the role of S-nitrosation in signaling remains an active area of research, though key details of control remain to be established.[32]

It may be obvious that using a toxic molecule like NO as a signaling agent places stringent demands on generation (NOS must be tightly controlled and levels kept below toxic), and then it follows that the sGC receptor must be capable of sensing those same low levels of NO (made lower through NO decomposition as it traverses from one to cell to another, e.g., from an endothelial cell to a smooth muscle cell). Molecular aspects of NO signaling have recently been reviewed.[33] Strict control over Ca^{2+} leads to sensitive control over constitutive NOSs; therefore, how is the receptor built to sense low NO levels, and what is the mechanism of NO activation?

SGC STRUCTURES

Attempts to obtain a full length heterodimeric sGC structure were a tortuous path that finally reached an end point in 2019 with two groups reporting structures, both using cryo-electron microscopy.[34,35] Prior to that, structures of domains and single-particle EM work painted an intriguing picture but one that lacked the NO activation details that could be seen only in a full length structure. The NO binding site in sGC is a ferrous heme with histidine ligation, exactly what it is in the globins, yet sGC showed no measurable affinity for O_2. This ligand discrimination against O_2, molecular engineering if you will, had to take place if you were to take advantage of the very tight affinity of NO for a Fe(II)-heme but doing so in the presence of a much higher concentration of O_2. While the solution to this problem was not obvious, it was research with bacterial heme domain homologues of sGC (termed H-NOX domains) that provided the answer.[36] H-NOX domains have proven to be very important in understanding ligand binding in sGC,[37] including their function in bacteria.[38,39]

The full length structures provided the long sought after view of NO activation. Chen and colleagues determined the structure of the human enzyme,[35] and we determined a structure

of sGC from the tobacco horn worm *Manduca sexta*.[34] Both papers reported structures of the unactivated (basal activity) and NO-activated forms. The resolution obtained by Chen and colleagues was higher than that obtained by us; however, there was excellent agreement between the two papers. We have reported previously on the three activity states of sGC: (1) unactivated, (2) one NO, and (3) excess NO.[40,41] However, because a mechanism for activation by excess NO is lacking, some skepticism about this point remains. There is also an activity state (4) that is NO plus the sGC stimulator YC-1. In fact, YC-1 activates the one-NO form of sGC to the fully active state obtained without excess NO (Figure 4). YC-1 was the initial hit in a screen that led to Bayer's development of the 2013 FDA-approved Adempas (riociguat) used to treat pulmonary hypertension. Termed sGC stimulators, Adempas and others in development act directly on sGC. Interconversion between the one-NO activity state (~20% maximal) and excess NO (fully active) is reversible, so to be certain sGC was captured in the fully active state, we added both NO and YC-1 to samples for structural determination. Hence, the YC-1 binding site is visible in that structure (Figure 5c) and provides a rationale for the action of stimulator molecules related to the YC-1 to keep the enzyme in the highest-activity state. In addition to the very significant conformational changes that take place upon activation, perhaps the most remarkable feature is the bent coiled coil that links the regulatory (NO binding) domain with the catalytic domain that straightens upon activation (Figure 5b). The culmination of all of the movement leads to an open active site that can now bind GTP. Accumulated evidence points toward a physiological model in which sGC toggles between the one-NO state and fully activated. In a model such as this, it is easy to see how drugs like Adempas or the precursor YC-1 functions.[34]

THE PRESENT AND FUTURE

Remaining questions involve NOS, though they are more focused on mechanism and thus more nuanced. Full length structures would help to decipher aspects of electron transfer and mechanism. Drug development around NOS was primarily directed toward inhibition to prevent the damaging effect of high levels of NO under conditions of immune stimulation. Structures of the oxidase domains of the three isoforms, some with inhibitors bound, were used to develop isoform-specific inhibitors. Significant success was realized[25] but has not matched thus far with clinical success.

sGC structures reported in 2019 herald a new beginning to understand this complex protein as they open the door to a molecular view of sGC that has long been sought. Interaction of NO with the heme is certainly a key focal point in the activation process; however, as noted, excess NO is required for full and sustained activation. The mechanism and structural changes that take place under those conditions will be an important focus. Adempas was approved in 2013, and one of the reported sGC structures observed the YC-1 binding site. Is this the Adempas binding site? It is reasonable to assume so, but it will have to be proved. Montfort and colleagues have studied the binding of a stimulator to bacterial H-NOX domains and related those findings to the YC-1 binding site observed in full length sGC.[42] Bayer continues to develop sGC stimulators with new and diverse structures. Astellas, Cyclerion (spun out of Ironwood), and Merck are also developing sGC stimulators.[43,44] Also under development are the so-called sGC activators. These molecules act on apo-sGC

and are thought to stabilize apo sGC through occupation of the heme binding site.[45] Development of these molecules has not yet advanced beyond preclinical studies.

There has been much attention devoted to protein S-nitrosation as a mechanism of NO signaling. The debate continues and is complicated by the promiscuous reactivity of NO and intermediates in the pathway of decomposition to NO_2^- and NO_3^-. A literature search will produce many papers devoted to this topic. Gusarov and Nudler wrote a "preview" piece on two papers that appeared in that same issue of *Cell*.[46] This "preview" nicely summarizes the range of views on this topic and the key questions that remain to be answered.

ACKNOWLEDGMENTS

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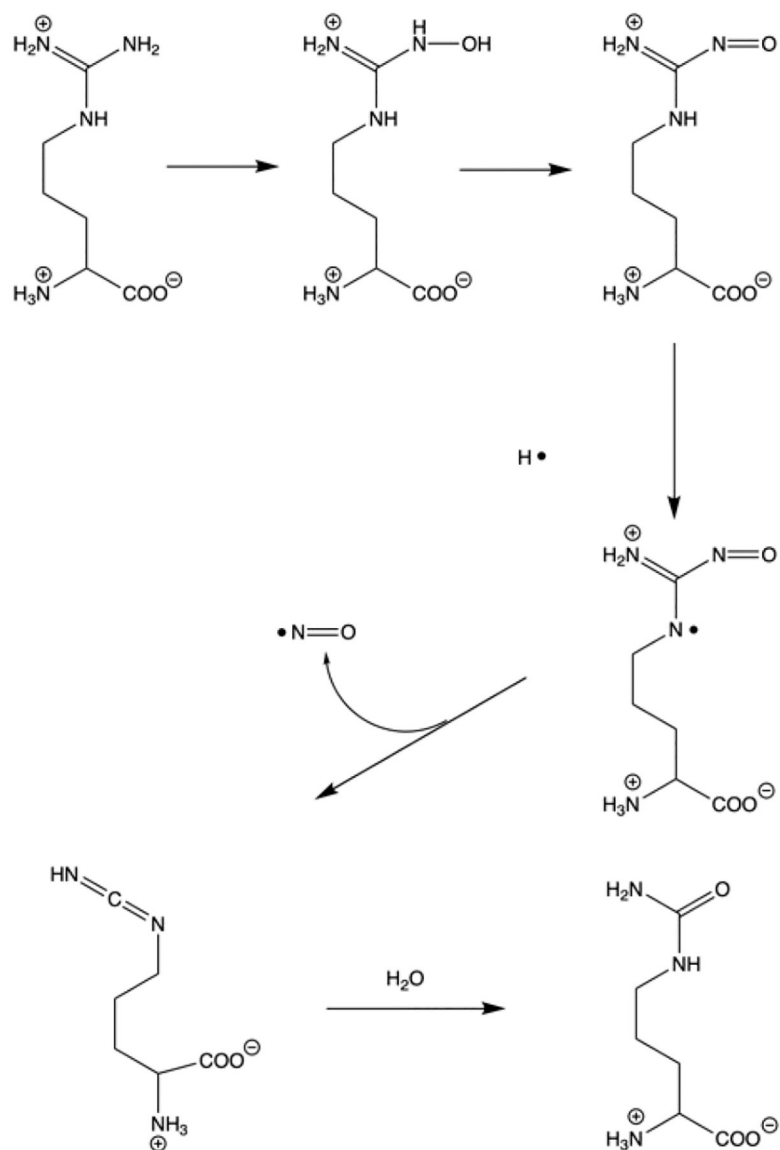
Figure 1.
Scheme for the formation of NO from L-arginine. The reaction scheme is reproduced from Scheme II in ref 1. As noted in the text, the scheme was developed without knowledge of the enzymology (one enzyme or more), cofactors, and co-substrates. The first step, N-hydroxylation, was subsequently verified.
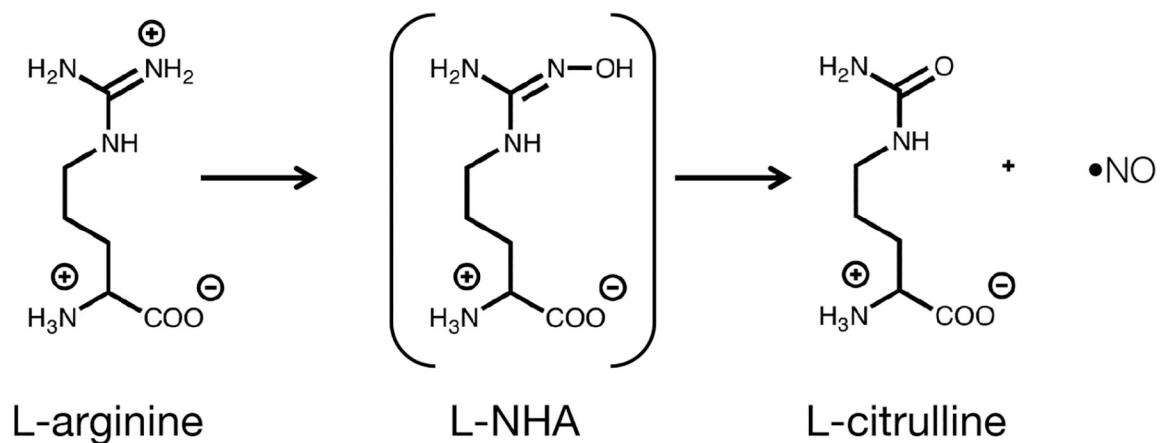
Figure 2.
NOS reaction. NOS reaction depicting L-NHA as an intermediate and the end products of the reaction, L-citrulline, and •NO. Note that the stereochemistry of the citrulline product has never been determined, but because no chemistry takes place at the α-carbon, the assumption is that the product is L-citrulline.
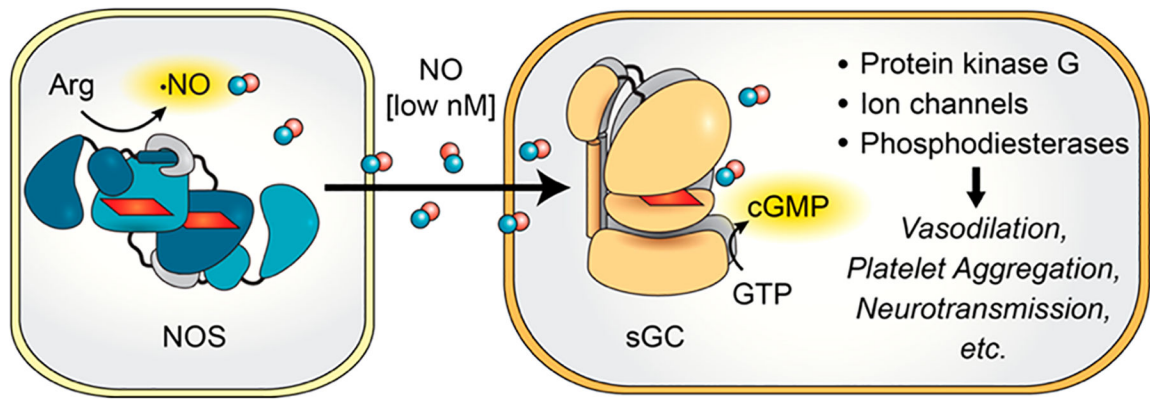
Figure 3.
Schematic of NO signaling. Depicted is paracrine (cell to cell) NO signaling. The activity of NOS in the NO generating cell is tightly controlled by Ca^{2+} and calmodulin (not shown), typically leading to low nanomolar NO concentrations. NO then diffuses into the target cell that contains sGC. Activation of sGC leads to the conversion of GTP to cGMP. The second messenger cGMP through a cascade of events leads to biological outcomes such as vasodilation.
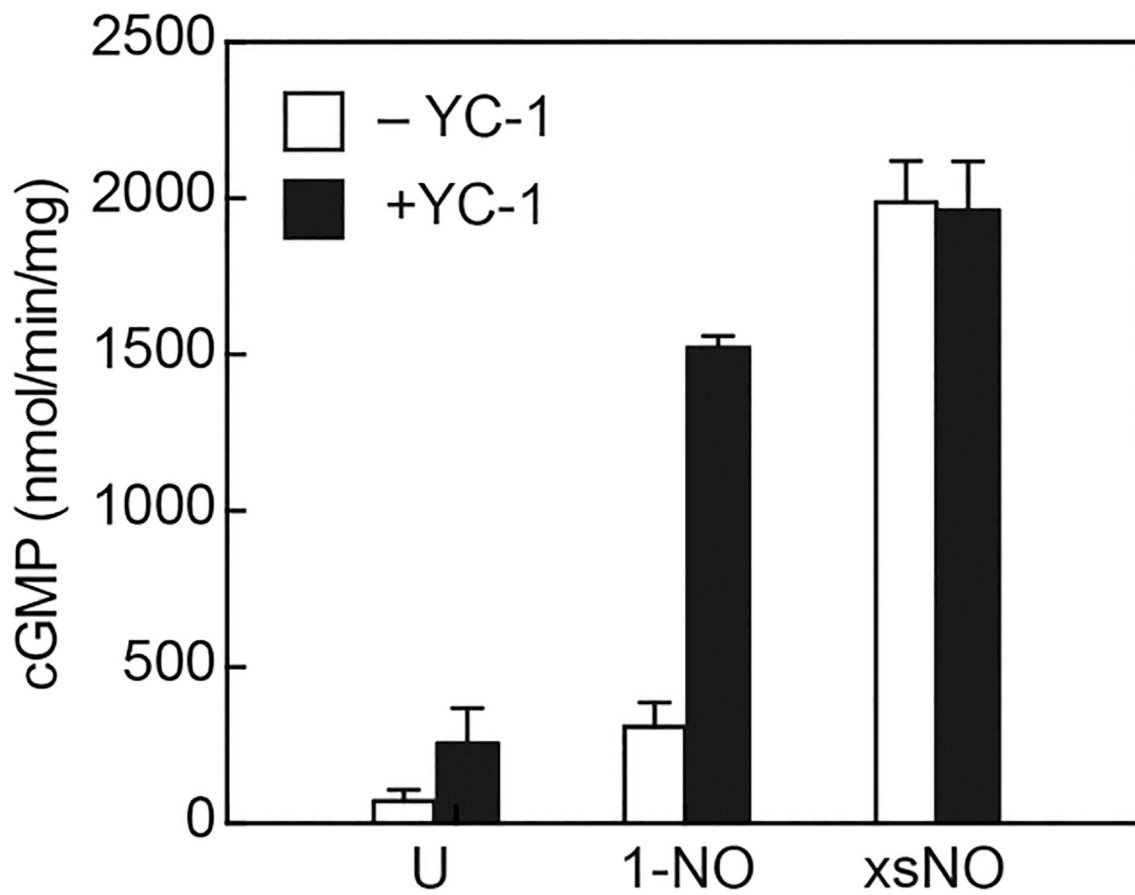
Figure 4.
sGC activity states. Discontinuous cGMP activity assay for *M. sexta* sGC with various activation conditions: one-NO, excess NO (xsNO), and YC-1 ligands. Initial rates were obtained from the data. The average initial rate is plotted, and the error bars reflect one standard deviation ($n = 4$). Figure reproduced from ref 34. Creative Commons Attribution license.
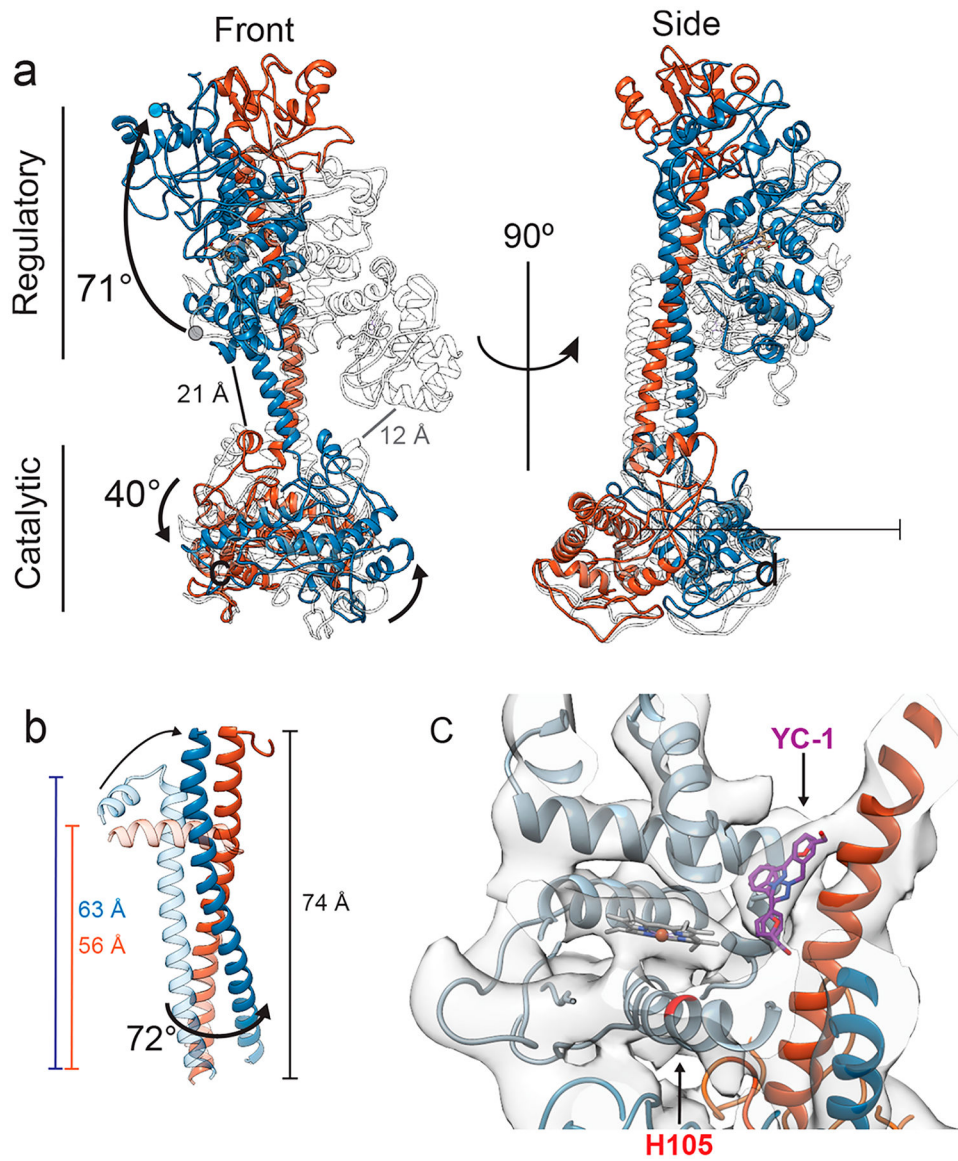
Figure 5.
sGC structures. Conformational rearrangements of *M. sexta* sGC upon activation. (a) Overlay of the inactive (transparent, gray) and active (*α*, orange; *β*, blue) states shown in two views. Rotation of the regulatory domain and CAT dimer is shown with arrows and labeled with a degree of rotation. Distances between the *β* H-NOX domain and CAT dimer are labeled for inactive (gray) and active (black). (b) Overlay of the inactive (transparent) and active (colored) CC domains when aligned with the active CC domain. Dimensions of the CC and rotation are labeled in color for the inactive and black for the active. (c) View of the *β* H-NOX domain with heme colored gray and *β* H105 colored red. Two fits for the stimulator, YC-1, are colored purple. Figure reproduced from ref 34. Creative Commons Attribution license.