Synergistic activation of soluble guanylate cyclase by YC-1 and carbon monoxide: implications for the role of cleavage of the iron–histidine bond during activation by nitric oxide

James R Stone[1]* and Michael A Marletta[2]

Background: Nitric oxide (•NO) is used in biology as both an intercellular signaling agent and a cytotoxic agent. In signaling, submicromolar quantities of •NO stimulate the soluble isoform of guanylate cyclase (sGC) in the receptor cell. •NO increases the V_{max} of this heterodimeric hemoprotein up to 400-fold by interacting with the heme moiety of sGC to form a 5-coordinate complex. Carbon monoxide (CO) binds to the heme to form a 6-coordinate complex, but only activates the enzyme 5-fold. YC-1 is a recently discovered compound that relaxes vascular smooth muscle by stimulating sGC.

Results: In the presence of YC-1, CO activates sGC to the same specific activity as attained with •NO. YC-1 did not affect the NO-stimulated activity. The on-rate (k_{on}) and off-rate (k_{off}) of CO for binding to sGC in the presence of YC-1 were determined by stopped-flow spectrophotometry. Neither the k_{on} nor the k_{off} varied from values previously obtained in the absence of YC-1, indicating that YC-1 has no effect on the affinity of CO for the heme. In the presence of YC-1, the visible spectrum of the sGC–CO complex has a Soret peak at 423 nm, indicating the complex is 6-coordinate.

Conclusions: YC-1 has no effect on the affinity of CO for the heme of sGC. In the presence of YC-1, maximal activation of sGC by CO is achieved by formation of a 6-coordinate complex between CO and the heme indicating that cleavage of the Fe–His bond is not required for maximal activation of sGC.

Addresses: [1]Department of Biological Chemistry, School of Medicine, University of Michigan, Ann Arbor, MI 48109-1065, USA. [2]Howard Hughes Medical Institute, Interdepartmental Program in Medicinal Chemistry, Department of Biological Chemistry, College of Pharmacy, 428 Church Street, University of Michigan, Ann Arbor, MI 48109-1065, USA.

Present address: *Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA.

Correspondence: Michael A Marletta
E-mail: marle@umich.edu

Key words: allosteric, cGMP, forskolin, heme, stopped-flow

Received: **26 February 1998**
Revisions requested: **17 March 1998**
Revisions received: **3 April 1998**
Accepted: **7 April 1998**

Published: **5 May 1998**

Chemistry & Biology May 1998, **5**:255–261
http://biomednet.com/elecref/1074552100500255

Introduction

Living systems have evolved numerous ways to transmit information or signals from one cell to another, and one of the more unusual ways is the use of the free radical nitric oxide (•NO) [1,2]. At low concentrations (below 1 μM), •NO is used by cells as a signaling agent. •NO is synthesized in a cell from the amino acid L-arginine by a Ca^{2+}/calmodulin-dependent nitric oxide synthase [3,4]. The •NO then freely diffuses to another cell where it activates the soluble isoform of guanylate cyclase (sGC). Once activated by •NO, sGC synthesizes guanosine 3′,5′-cyclic monophosphate (cGMP) from guanosine 5′-triphosphate (GTP) [5,6]. The cGMP is, for the most part, retained within the second cell where it causes a cascade of biochemical reactions that results in a physical/functional change in the cell. For example, increased cGMP has been shown to cause platelet disaggregation, vascular smooth muscle relaxation, and intestinal epithelial cell secretion of ions and water [5,7,8]. •NO has no effect on the particulate isoform of guanylate cyclase (pGC) that is activated by small peptide hormones such as atrial natriuretic factor [6].

At concentrations above 1 μM, •NO is employed as a cytotoxic agent to kill invading organisms or malignant neoplastic cells. In recent years numerous mechanisms by which •NO might act as a cytotoxic agent have been described, such as nitrosylation and nitration of proteins [9,10], disruption of iron–sulfur centers [11], and direct oxidation of macromolecules via formation of peroxynitrite [12]. All these processes require relatively high concentrations of •NO (>1 μM), however. In contrast, the only known way for •NO to act as a signaling agent is by activating sGC, giving this enzyme an important role in cellular signal transduction.

sGC has been purified from mammalian sources and shown to be a heterodimer, consisting of an α subunit of 73–88 kDa and a β subunit of 70 kDa [13–16]. The two subunits of the heterodimer purified from mammalian sources so far have been cloned from several mammalian cDNA libraries and have been designated as α1 and β1 respectively [17–21]. Further screening of cDNA libraries along with genome studies have revealed sequences for other putative sGC isoforms [22,23] but the functional significance and native quaternary structure of these isoforms remain unclear. The purified enzyme from mammalian sources contains bound heme (iron protoporphyrin IX) [16,24]. As isolated, the heme is ferrous, but the heme can

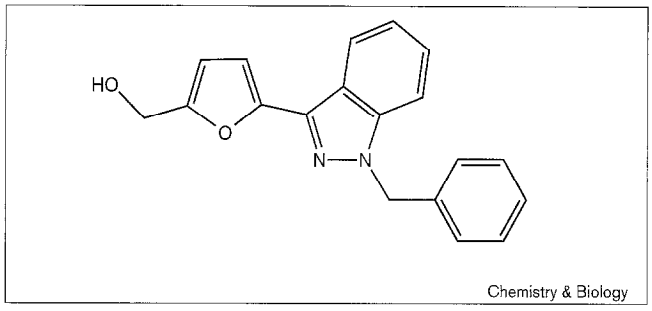
be oxidized with ferricyanide. Spectral studies have indicated that the heme is 5-coordinate high spin in both the ferrous and ferric states and a single histidine is the most likely axial ligand [16,25–27]. Unlike hemoglobin (Hb) and myoglobin (Mb), the 5-coordinate ferrous heme in sGC has the unusual property of not binding oxygen, even at millimolar concentrations [16].

It was first suggested two decades ago [28] that heme played a role in the activation of sGC by •NO. Partially purified sGC that was not responsive to •NO could be activated by •NO if heme was included in the reaction mixture. It was proposed later that activation of sGC by •NO was a direct result of the formation of a 5-coordinate nitrosyl–heme complex on the enzyme [29] on the basis of the observation that the ability of •NO to activate purified sGC was dependent on the presence of heme [30]. Enzyme containing heme was markedly activated by •NO and removal of the heme moiety resulted in the loss of the ability of •NO to activate the enzyme. Reconstitution of the enzyme with heme partially restored the ability of •NO to activate the enzyme. It was also demonstrated that purified heme-deficient enzyme could be activated by nitrosyl–heme complexes and also by protoporphyrin IX (lacking iron) [31]. This last observation led to the speculation that the nitrosyl complex formed on sGC was 5-coordinate and thus structurally resembled protoporphyrin IX. Upon binding of •NO to the heme iron, the *trans*-axial ligand was believed to dissociate from the heme. The release of this axial ligand and the subsequent changes in porphyrin structure were postulated to mediate a global conformational change in the protein, resulting in activation.

Although the heme in sGC does not bind oxygen, it does bind •NO. Electronic absorption, resonance Raman and electron paramagnetic resonance spectral studies have indicated that the nitrosyl complex is, in fact, 5-coordinate [16,25,32]. Furthermore, experiments simultaneously measuring cGMP formation and recording optical spectra have demonstrated that the 5-coordinate nitrosyl form of the enzyme is activated relative to the 5-coordinate resting state of the enzyme [33]. Also, CO, which forms a 6-coordinate complex with the heme, only activates purified enzyme to 1–3% of the levels achieved with •NO [16,34], supporting the idea that severing of the Fe–His bond is required for full activation of the enzyme.

YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole; Figure 1) is a recently discovered compound that relaxes vascular smooth muscle [35] and inhibits platelet aggregation [36–38]. These effects were found to be caused by elevation of cGMP levels. The target for YC-1 appears to be sGC, as YC-1 directly stimulates this enzyme but has no effect on pGC [38]. It has been reported recently that a much higher cyclase activity can be obtained with sGC in the presence of both CO and YC-1 than in the presence of either activator individually [39]. Furthermore, it has been reported that in the presence of YC-1, much lower concentrations of CO are required to activate sGC than are required in the absence of YC-1 [39], which has profound implications. For a number of years, it has been proposed that, by analogy to •NO generated nitric oxide synthase, CO generated by heme oxygenase may serve as an endogenous activator of sGC by binding to the heme moiety [40,41]. The main evidence contradicting this proposal is that the level of $\alpha 1\beta 1$ activation achieved with CO is marginal compared to that achieved with •NO and, furthermore, that nonphysiologically high concentrations of CO are required to observe this effect [16,34]. If YC-1 can both increase the activation by CO and decrease the concentration of CO required to produce this effect, however, then the possibility exists that there is a natural analog of YC-1 that may allow CO to function as an endogenous activator of sGC.

Figure 1



Chemical structure of 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1).

Here, it is shown that although YC-1 is synergistic with CO in activating sGC, YC-1 has no effect on the on-rate (k_{on}) or off-rate (k_{off}) of CO for binding to the heme. Additionally, it is demonstrated that in the presence of YC-1 CO forms a 6-coordinate complex with the heme and activates the enzyme to the same extent as does •NO. On the basis of these results, the requirement for cleavage of the Fe–His bond to achieve full activation of sGC is called into question.

Results

Activation of sGC by YC-1 and CO

YC-1 (100 μM) or CO (1 atm, 800 μM) added alone only activates sGC 5-fold (Table 1), whereas •NO (0.5%, 8 μM) activates the enzyme 178-fold. In the presence of both CO and YC-1, however, the specific activity was similar to that achieved with •NO, indicating a synergism between CO and YC-1 for activating the enzyme. The presence of YC-1 had no effect on the activity of enzyme activated with •NO. All activities were linear over 10 minutes.

Table 1

Activation of sGC with •NO, CO and YC-1.

Gas in headspace	YC-1 (100 μM)	Specific activity (nmole min^{-1} mg^{-1})	Activation (x-fold)
Argon	–	98 ± 7	
Argon	+	532 ± 25	5.4 ± 0.5
Carbon monoxide	–	471 ± 48	4.8 ± 0.6
Carbon monoxide	+	15,800 ± 1,200	161 ± 17
0.5% •NO in argon	–	17,400 ± 400	178 ± 13
0.5% •NO in argon	+	15,400 ± 600	157 ± 13

All errors represent one standard deviation. The errors for the specific activities correspond to the errors in the slopes from the linear regressions of the plots of product versus time. The errors in the activations were derived from propagation of the errors in the specific activities.

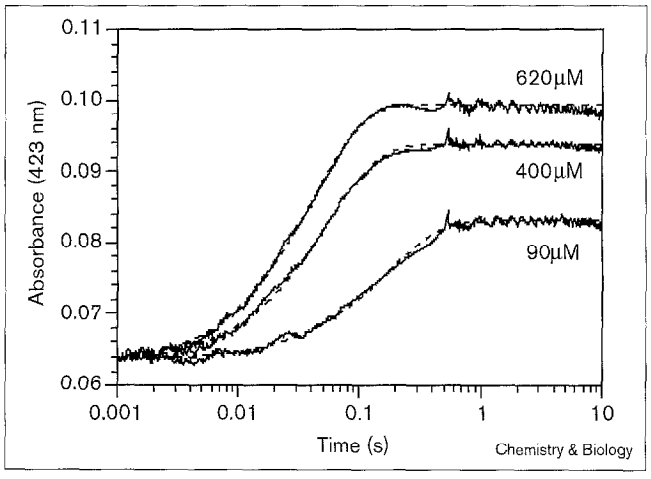
Stopped-flow kinetics

Representative traces for the binding of CO to sGC are shown in Figure 2. The total absorbance change was not the same for the different concentrations of CO because the binding site was not saturated. Each trace in Figure 2 has a single exponential fit superimposed on the data. From the single exponential fits of the data, pseudo first-order (observed) rate constants (k_{obs}) were obtained. A plot of these constants against the concentration of CO is shown in Figure 3. The plot was linear, consistent with a simple one-step binding mechanism. The slope of the line is the k_{on} for CO [$(3.63 \pm 0.15) \times 10^4$ $M^{-1}s^{-1}$], and the y intercept is the k_{off} (3.4 ± 0.5 s^{-1}). The k_{off} divided by the k_{on} (94 ± 14 μM) is a calculated value for the K_d for the binding process. These values are in good agreement with the values determined previously in the absence of YC-1 (Table 2), demonstrating that YC-1 has no effect on the affinity of CO for binding to the heme of sGC.

Measurement of the K_d of CO

Representative spectra taken in the stopped-flow spectrophotometer are shown in Figure 4. In the presence of 100 μM YC-1 and the absence of CO, the Soret peak is located at 431 nm, indicating a 5-coordinate heme complex with histidine as the axial ligand as determined previously [16,25]. Thus, YC-1 partially activates the enzyme without altering the heme coordination. In the presence of 0.62 mM CO and 100 μM YC-1, the Soret peak shifts to 423 nm, indicating a 6-coordinate CO complex [16,25,42]. Using values generated from difference spectra, a plot of the ΔΔAbs(423–440) versus the CO concentration is shown in Figure 5. Fitting the data with a typical saturation function, $\Delta\Delta Abs = \{\Delta\Delta Abs(max) \times [CO]\}/\{K_d + [CO]\}$, yields a K_d of 73 ± 18 μM for the binding of CO to the heme of sGC. This value is in good agreement with the value obtained from the rate constants and with the spectrally derived equilibrium constant obtained previously in the absence of YC-1 (Table 2).

Figure 2

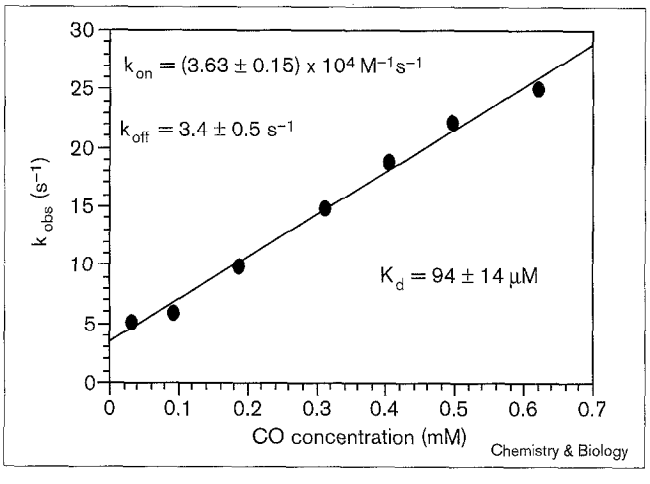


Stopped-flow monitoring of the binding of CO to sGC in the presence of YC-1. Original data (solid line), single exponential fit (dashed line). For all concentrations of CO, the data and the fit are essentially superimposable. Experimental conditions were as described in the Materials and methods section. The concentrations of CO shown are the final concentrations after mixing.

Discussion

The molecular details of •NO activation of sGC remain largely unanswered and are integral to the complete understanding of •NO signal transduction. Of great importance is the understanding of the unique properties of the heme moiety of the receptor that allow specific interaction with •NO in the presence of much higher concentrations of O_2. Perhaps even more important is the mechanism that leads to dissociation of •NO from the

Figure 3



Plot of k_{obs} against CO concentration. The pseudo first-order rate constants (k_{obs}) obtained from the single exponential fits of the stopped-flow data were plotted against the final concentrations of CO. The k_{on} is obtained from the slope, the k_{off} from the y intercept and the K_d from k_{off} / k_{on}.

Table 2

Comparison of kinetic and equilibrium binding constants for CO.

YC-1 (100 μM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (calc'd, μM)*	K_d (meas., μM)†
Absent‡	$(3.58 \pm 0.15) \times 10^4$	3.5 ± 0.5	98 ± 15	97 ± 9
Present	$(3.63 \pm 0.15) \times 10^4$	3.4 ± 0.5	94 ± 14	73 ± 18

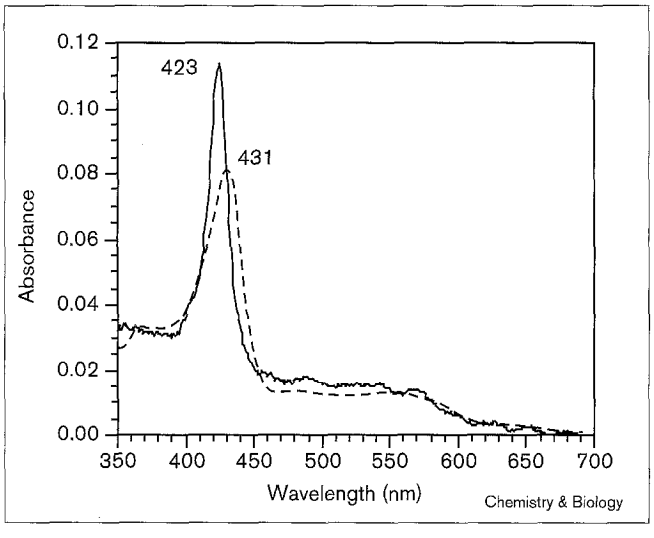
All errors represent one standard deviation. The errors for k_{on} and k_{off} correspond to the errors in the slopes and y intercepts respectively from the linear regressions of the plots of k_{obs} against CO concentration. The errors in K_d (calc'd) were derived from propagation of the errors in the k_{on} and k_{off}. The errors in K_d (meas.) correspond to the error derived in fitting the plot in Figure 5 with a standard saturation function. *Calculated from k_{off}/k_{on}. †Measured directly from spectra. ‡Previously reported [34].

heme, thereby returning the enzyme to the resting state. As •NO typically dissociates very slowly from ferrous heme complexes, unusual structural features must exist in the heme-binding site of sGC to allow the rapid dissociation of •NO required for cellular signaling.

The current working model of sGC activation includes a resting state of the enzyme that has low basal activity and a high-spin ferrous heme with a histidine residue as the axial ligand [16,25]. Our most recent studies with the native enzyme (unpublished observations, P.E. Brandish, S. Kim, J.R.S. and M.A.M.) and with an amino-terminal fragment of the β subunit [43,44] are consistent with a heme to heterodimer ratio of 1:1. Mutagenesis studies have implicated His105 in the β subunit as the likely heme ligand for the bovine lung isoform [43–45]. Upon •NO binding to the heme, the activity of sGC increases dramatically (up to 400-fold) and the heme is converted to a 5-coordinate complex with •NO bound to the distal side of the heme [27,33]. After treatment with •NO, the Fe–His bond is broken, a finding that has been confirmed using electronic, electron paramagnetic and resonance Raman spectroscopies [25,32]. It has been speculated, moreover, that •NO binding leads to a conformational change with a subsequent increase in activity, perhaps by rearrangement of essential active-site residues.

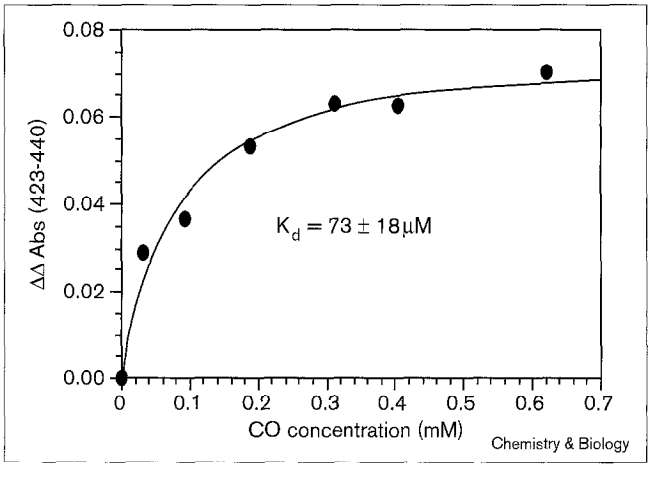
There are two major sources of movement in the regulatory portion of the protein brought about by •NO binding to the heme in sGC. First, in the porphyrin macrocycle, the porphyrin is likely to be puckered towards the distal histidine ligand in the resting state and puckered in the opposite way in the •NO complex. The significant movement of the porphyrin ring upon •NO binding could, in principle, provide the driving force for a global conformational change and the increase in catalytic activity. We have shown previously that CO is a weak activator of the enzyme (see below); furthermore, we have shown that the

Figure 4



Electronic absorption spectra of sGC. Spectra were recorded on the stopped-flow spectrophotometer after each kinetics measurement. Spectra in the presence of 100 μM YC-1 either in the absence (dotted line) or presence (solid line) of 620 μM CO are shown.

Figure 5



Plot of ΔΔAbs(423–440) against CO concentration. The ΔΔAbs(423–440) obtained from difference spectra were plotted against the final concentration of CO. The data were fit with a standard saturation function, $\Delta\Delta Abs = \{\Delta\Delta Abs(max) \times [CO]\}/\{K_d + [CO]\}$ to determine the K_d.

CO–sGC complex is 6-coordinate [25,34]. This complex would probably have a nearly planar heme with little puckering, resulting in a geometry in between that of the activated and unactivated enzyme. The second source of movement in the regulatory portion of the protein involves the breakage of the Fe–His bond and the subsequent movement of the protein backbone. The increase in catalytic activity coincident with •NO binding almost certainly involves either one or both of these movements, but there is no direct evidence to quantitatively support either mechanism of activation.

Our results with YC-1 shed some light on the mechanism of sGC activation. The results reported here clearly show that treatment of sGC with YC-1 and CO act synergistically, leading to an increase in activation that is essentially the same as that observed with •NO alone (Table 1). Significantly, as shown by our spectral results, the activation with YC-1 and CO occurs without breakage of the Fe–His bond (Figure 4). This result shows conclusively that full activation can be obtained without the cleavage of the Fe–His bond and, thus, without any movement of the protein backbone requiring the cleavage of this bond. In agreement with our results, Koesling and colleagues [39] have recently reported that YC-1 and CO act synergistically to activate sGC. Additionally, we both observe a small increase in activity with YC-1 alone (5.4-fold, Table 1; 12-fold [39]), similar to that seen with CO alone. They also reported that lower concentrations of CO were required to activate the enzyme when YC-1 was present. Analysis of their data reveals an EC_{50} for CO of ~37 μM in the presence of YC-1; the corresponding EC_{50} without YC-1 cannot be estimated from the data in the paper [39]. The value of 37 μM obtained with YC-1, however, is in excellent agreement with an analogous value determined by us previously in the absence of YC-1 ($\alpha K_d = 36 \pm 8$ μM) [34], indicating that YC-1 does not affect the EC_{50} of CO for activating the enzyme. In a very recent follow-up study by the same group [46] it was concluded that YC-1 decreased the k_{off} of CO from the heme, thus increasing the affinity of CO for the heme. In that study, occupation of the heme site with CO was assessed by enzyme activity measurements and not spectrally (as was done here), so multiple interpretations of the data are possible.

Under •NO-saturating conditions, Friebe *et al.* [39] observed an increase in the •NO-stimulated activity with the addition of YC-1, whereas we did not (Table 1). There were differences in enzyme preparation and experimental protocols, however, so it is not clear that this difference in our results is significant. They did show that YC-1 enhances the activation of the enzyme achieved with subsaturating concentrations of a •NO donor. Studies on organs measuring vascular smooth muscle relaxation also observed this synergism when low •NO-donor concentrations were used [35]. A possible explanation for the result is that YC-1 increases the affinity of •NO for the heme, even though this was not the case for CO. The rate limiting step in the dissociation of •NO from the heme of sGC has been proposed to be the reassociation of the axial histidyl ligand to the heme [33], a step that does not occur when CO dissociates from the enzyme. YC-1 may therefore increase the affinity of •NO for the heme by decreasing the k_{off} of •NO from the heme by decreasing the rate of the iron–histidine re-ligation.

From the work reported here we know the effect of YC-1 on the kinetic constants of association and dissociation and on the equilibrium constant with respect to CO. Our results also clearly show that the mechanism of YC-1 action does not involve altering either the k_{on} or k_{off} for CO and, hence, has no effect on the equilibrium constant (Table 2). We had previously determined the k_{off} for CO and found it to be significantly faster when compared to other ferrous-heme–CO complexes with a histidyl axial ligand, such as hemoglobin and myoglobin [34]. As YC-1 and CO act synergistically to activate sGC, one possibility was that YC-1 also enhanced the affinity of CO for the heme by decreasing the k_{off}, but clearly this is not the case.

How does YC-1 exert the observed effects on sGC? The answer to this question will come only after more studies with YC-1, such as detailed spectroscopy and determination of the YC-1-binding site. It is tempting to draw an analogy between the homologous catalytic domains of the adenylate cyclases and their activation by forskolin and the activation of sGC by YC-1. Two recent crystal structures, a type IIC2 homodimer by Hurley and colleagues [47] and a type VC1IIC2 heterodimer by Sprang and colleagues [48], have both shed light on how forskolin functions. It is clear from the structures, as well as from biochemical results generated by Gilman and colleagues [49], that forskolin acts to stabilize the dimer interface. As the active site involves residues from both subunits, it is clear how forskolin could act to increase activity. Forskolin only increases the apparent affinity of VC1 for IIC2 from ≥10 μM to ~1 μM, however, which is not enough to account for the level of activation observed with forskolin, and it has been suggested that forskolin also acts by altering the conformation of the protein at the active site.

Whether there is a natural analog of forskolin is not known. Regulation of adenylate cyclase *in vivo* is essential for the physiological processes controlled by this enzyme so the implications of a natural counterpart of forskolin are significant. The same significance with respect to sGC is true for sGC and a natural analog of YC-1. Specifically, it has been proposed that CO derived from heme oxygenase functions as a mediator of intercellular communication by activation of sGC [40,41] on the basis of the ability of high concentrations of CO to inhibit platelet aggregation and to relax vascular smooth muscle through an increase in cGMP

[50,51]. The difficulty with this hypothesis is that the concentrations of CO used were high and well outside of what would be expected to be generated physiologically. Furthermore, the resulting activation of sGC is only 5-fold with the purified bovine lung form of the enzyme [16]. Although it is possible that a unique CO-sensitive isoform exists, such an isoform has never been isolated. The results with YC-1 show that a high CO-stimulated activity can be obtained, although YC-1 did not alter the K_d for CO. A natural analog of YC-1 might both increase stimulation by CO and lower the K_d for CO; the existence of such an analog would support the CO-signaling hypothesis.

Significance

The soluble isoform of guanylate cyclase (sGC) is the only definitively characterized receptor for the intercellular signaling agent nitric oxide (•NO). The native enzyme is a heterodimer composed of α and β subunits. •NO is known to interact with a ferrous protoporphyrin IX heme on sGC, leading to up to a 400-fold increase in activity. The previously accepted model of activation, on the basis of spectroscopic studies, showed that the resting enzyme is a 5-coordinate complex with axial ligation to a histidine residue. Upon •NO binding the heme is converted to a 5-coordinate complex with •NO bound in the distal position and the Fe–His bond broken. Conformational changes speculated to occur with the formation of the •NO complex are thought to be involved in the activation of sGC. CO is a weak (approximately 5-fold) activator of the enzyme; the resulting CO–sGC complex was determined to be 6-coordinate with the Fe–His bond intact and CO bound on the distal side of the heme. The present studies with 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) and CO show that sGC can be fully activated without severing the Fe–His bond. These results require a new model to explain activation of sGC and, furthermore, suggest that a natural analog of YC-1 could induce dramatic changes in the activity and biological action of sGC.

Materials and methods

Materials

CO (99%) was obtained from Liquid Carbonic Specialty Gases (Chicago, IL). •NO (99.0%) was obtained from Matheson. sGC was purified from bovine lung using a scaled-up version (data not shown) of a previously reported procedure [27]. YC-1 was a generous gift from Professor Che-Ming Teng, National Taiwan University. Stock solutions were prepared in DMSO, and the YC-1 concentration was determined gravimetrically. All other materials unless otherwise stated were obtained from Sigma.

Stopped-flow kinetics

sGC (1.5 μM heme) in 25 mM TEA, 5 mM dithiothreitol (DTT), 50 mM NaCl, 100 μM YC-1, 1% DMSO, pH 7.4, under air was rapidly mixed with an equal volume of the same buffer containing varying concentrations of CO. The CO solutions were prepared by bubbling buffer for 30 min with a CO/N_2 mixture. The concentration of CO in solution was determined using a solubility of 0.003479g of CO per 100g H_2O at 10°C (assuming no effect of added solutes) when the partial pressure of CO in the headspace is 760 mm Hg [52]. The gas mixtures were prepared using a 150 mm gas proportioner equipped with high-resolution valves (Air Products). Data were acquired on a HI-TECH Scientific SF-61 stopped-flow spectrophotometer controlled by KISS software (Kinetic Instruments, Ann Arbor, MI) at 10°C. The binding of CO to the heme was monitored as an increase in the absorbance at 423 nm. For each CO concentration, the experiment was performed in triplicate, and the resulting traces were averaged. The final traces were then fit to a single exponential using KISS software.

Measurement of the K_d of CO for binding to the heme

Upon completion of each stopped-flow experiment, a spectrum was obtained of the equilibrium mixture. For each concentration of CO, the final spectrum had subtracted from it a spectrum of sGC mixed with buffer lacking CO. These difference spectra were used to determine the ΔAbs(440), ΔAbs(423), and subsequently the ΔΔAbs(423–440). A plot of the ΔΔAbs(423–440) versus the CO concentration was fit with a typical saturation function, $\Delta\Delta Abs = \{\Delta\Delta Abs(max) \times [CO]\}/\{K_d + [CO]\}$, to determine the K_d of CO for binding to the heme.

Activation of purified sGC with •NO, CO and YC-1

•NO was purified of other nitrogen oxides by bubbling through saturated KOH. Buffer containing 50 mM TEA pH 7.4, 2 mM DTT, 4.5 mM $MgCl_2$, 1.5 mM GTP, and 1% DMSO, with or without 100 μM YC-1 was placed in a gas-tight vial under 1 atmosphere of the appropriate gas and warmed to 37°C. Previously 100 μM YC-1 was found to be saturating (or near-saturating) for the activation of sGC [39]. The reaction was initiated by the injection of 4 μl of sGC with a gas-tight syringe to give a final reaction volume of 1.0 ml with an enzyme concentration of 0.80 μg/ml. At time intervals (0, 2, 5, 7, and 10 min), 100 μl aliquots were removed with a gas-tight syringe and quenched with 400 μl of 125 mM $Zn(CH_3COO)_2$ and 500 μl of 125 mM Na_2CO_3. The amount of cGMP generated was then quantitated *via* radioimmunoassay (Amersham).

Protein determination

Protein concentrations were determined using the Bradford microassay (Bio-Rad) using BSA as the standard.

Acknowledgements

We would like to acknowledge Professor Che-Ming Teng, National Taiwan University, for providing us with YC-1. This work was supported by the Hitchings Award of the Burroughs-Wellcome Fund.

References

1. Bredt, D.S. & Snyder, S.H. (1994). Nitric oxide: a physiologic messenger molecule. *Annu. Rev. Biochem.* **63**, 175-195.
2. Moncada, S., Palmer, R.M.J. & Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109-142.
3. Marletta, M.A. (1993). Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **268**, 12231-12234.
4. Marletta, M.A. (1994). Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* **78**, 927-930.
5. Waldman, S.A. & Murad, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.* **39**, 163-196.
6. Garbers, D.L. & Lowe, D.G. (1994). Guanylyl cyclase receptors. *J. Biol. Chem.* **269**, 30741-30744.
7. Goy, M.F. (1991). cGMP: The wayward child of the cyclic nucleotide family. *Trends Neurosci.* **14**, 293-299.
8. Warner, T.D., Mitchell, J.A., Sheng, H. & Murad, F. (1994). Effects of cyclic GMP on smooth muscle relaxation. *Adv. Pharmacol.* **26**, 171-194.
9. Beckman, J.S., Chen, J., Crow, J.P. & Ye, Y.Z. (1994). Reactions of nitric oxide, superoxide and peroxynitrite with superoxide dismutase in neurodegeneration. *Prog. Brain Res.* **103**, 371-380.
10. Roy, B., Lepoivre, M., Henry, Y. & Fontecave, M. (1995). Inhibition of ribonucleotide reductase by nitric oxide derived from thionitrites: reversible modifications of both subunits. *Biochemistry* **34**, 5411-5418.
11. Lancaster, J.R. & Hibbs, J.B. (1990). EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc. Natl Acad. Sci. USA* **87**, 1223-1227.
12. Rubbo, H., *et al.*, & Freeman, B.A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. *J. Biol. Chem.* **269**, 26066-26075.

13. Idriss, S.D., Pilz, R.B., Sharma, V.S. & Boss, G.R. (1992). Studies on cytosolic guanylate cyclase from human placenta. *Biochem. Biophys. Res. Commun.* **183**, 312-320.
14. Garbers, D.L. (1979). Purification of soluble guanylate cyclase from rat lung. *J. Biol. Chem.* **254**, 240-243.
15. Mulsch, A. & Gerzer, R. (1991). Purification of heme-containing soluble guanylyl cyclase. *Meth. Enzymol.* **195**, 377-383.
16. Stone, J.R. & Marletta, M.A. (1994). Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* **33**, 5636-5640.
17. Nakane, M., Arai, K., Saheki, S., Kuno, T., Buechler, W. & Murad, F. (1990). Molecular cloning and expression of cDNAs coding for soluble guanylate cyclase from rat lung. *J. Biol. Chem.* **265**, 16841-16845.
18. Nakane, M., Saheki, S., Kuno, T., Ishii, K. & Murad, F. (1988). Molecular cloning of a cDNA coding for 70 kilodalton subunit of soluble guanylate cyclase from rat lung. *Biochem. Biophys. Res. Commun.* **157**, 1139-1147.
19. Koesling, D., *et al.*, & Bohme, E. (1990). The primary structure of the larger subunit of soluble guanylyl cyclase from bovine lung. *FEBS Lett.* **266**, 128-132.
20. Koesling, D., *et al.*, & Frank, R. (1988). The primary structure of the 70 kDa subunit of bovine soluble guanylate cyclase. *FEBS Lett.* **239**, 29-34.
21. Giuili, G., Scholl, U., Bulle, F. & Guellaen, G. (1992). Molecular cloning of cDNAs coding for the two subunits of soluble guanylyl cyclase from human brain. *FEBS Lett.* **304**, 83-88.
22. Yuen, P.S.T., Potter, L.R. & Garbers, D.L. (1990). A new form of guanylyl cyclase is preferentially expressed in rat kidney. *Biochemistry* **29**, 10872-10878.
23. Harteneck, C., Wedel, B., Koesling, D., Malkewitz, J., Bohme, E. & Schultz, G. (1991). Molecular cloning and expression of a new α-subunit of soluble guanylyl cyclase. *FEBS Lett.* **292**, 217-222.
24. Gerzer, R., Böhme, E., Hofmann, F. & Schultz, G. (1981). Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS Lett.* **132**, 71-74.
25. Deinum, G., Stone, J.R., Babcock, G.T. & Marletta, M.A. (1996). Binding of nitric oxide and carbon monoxide to soluble guanylate cyclase as observed with resonance Raman spectroscopy. *Biochemistry* **35**, 1540-1547.
26. Stone, J.R., Sands, R.H., Dunham, W.R. & Marletta, M.A. (1996). Spectral and ligand-binding properties of an unusual hemoprotein, the ferric form of soluble guanylate cyclase. *Biochemistry* **35**, 3258-3262.
27. Stone, J.R. & Marletta, M.A. (1995). Heme stoichiometry of heterodimeric soluble guanylate cyclase. *Biochemistry* **34**, 14668-14674.
28. Craven, P.A. & DeRubertis, F.R. (1978). Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and hemeproteins. *J. Biol. Chem.* **253**, 8433-8443.
29. Ignarro, L.J., Wood, K.S. & Wolin, M.S. (1984). Regulation of purified soluble guanylate cyclase by porphyrins and metalloporphyrins: A unifying concept. *Adv. Cyclic Nucleotide Prot. Phosphor. Res.* **17**, 267-274.
30. Ignarro, L.J., Degnan, J.N., Baricos, W.H., Kadowitz, P.J. & Wolin, M.S. (1982). Activation of purified guanylate cyclase by nitric oxide requires heme: comparison of heme-deficient, heme-reconstituted and heme-containing forms of soluble enzyme from bovine lung. *Biochim. Biophys. Acta* **718**, 49-59.
31. Ignarro, L.J., Wood, K.S. & Wolin, M.S. (1982). Activation of purified soluble guanylate cyclase by protoporphyrin IX. *Proc. Natl Acad. Sci. USA* **79**, 2870-2873.
32. Stone, J.R., Sands, R.H., Dunham, W.R. & Marletta, M.A. (1995). Electron paramagnetic resonance spectral evidence for the formation of a pentacoordinate nitrosyl-heme complex on soluble guanylate cylcase. *Biochem. Biophys. Res. Comm.* **207**, 572-577.
33. Stone, J.R. & Marletta, M.A. (1996). Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry* **35**, 1093-1099.
34. Stone, J.R. & Marletta, M.A. (1995). The ferrous heme of soluble guanylate cyclase: formation of hexacoordinate complexes with carbon monoxide and nitrosomethane. *Biochemistry* **34**, 16397-16403.
35. Mulsch, A., Bauersachs, J., Schafer, A., Stasch, J., Kast, R. & Busse, R. (1997). Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. *Br. J. Pharmacol.* **120**, 681-689.
36. Wu, C., Ko, F., Kuo, S., Lee, F. & Teng, C. (1995). YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. *Br. J. Pharmacol.* **116**, 1973-1978.
37. Teng, C., Wu, C., Ko, F., Lee, F. & Kuo, S. (1997). YC-1, a nitric oxide-independent activator of soluble guanylate cyclase, inhibits platelet-rich thrombosis in mice. *Eur. J. Pharmacol.* **320**, 161-166.
38. Ko, F., Wu, C., Kuo, S., Lee, F. & Teng, C. (1994). YC-1, a novel activator of platelet guanylate cyclase. *Blood* **84**, 4226-4233.
39. Friebe, A., Schultz, G. & Koesling, D. (1996). Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. *EMBO J.* **15**, 6863-6868.
40. Marks, G.S., Brien, J.F., Nakatsu, K. & McLaughlin, B.E. (1991). Does carbon monoxide have a physiological function? *Trends Physiolog. Sci.* **12**, 185-188.
41. Verma, A., Hirsch, D.J., Glatt, C.E., Ronnett, G.V. & Snyder, S.H. (1993). Carbon monoxide: a putative neural messenger. *Science* **259**, 381-384.
42. Geibel, J., Cannon, J., Campell, D., & Traylor, T.G. (1978). Model compounds for R-state and T-state hemoglobins. *J. Am. Chem. Soc.* **100**, 3575-3585.
43. Zhao, Y., Schelvis, J.P.M., Babcock, G.T. & Marletta, M.A. (1998). Identification of histidine 105 in the β1 subunit of soluble guanylate cyclase as the heme proximal ligand. *Biochemistry* **37**, 4502-4509.
44. Zhao, Y. & Marletta, M.A. (1997). Localization of the heme binding region in soluble guanylate cyclase. *Biochemistry* **36**, 15959-15964.
45. Wedel, B., *et al.* & Koesling, D. (1994). Mutation of His-105 in the β1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl Acad. Sci. USA* **91**, 2592-2596.
46. Friebe, A. & Koesling, D. (1998). Mechanism of YC-1-induced activation of soluble guanylyl cyclase. *Mol. Pharmacol.* **53**, 123-127.
47. Zhang, G., Liu, Y., Ruoho, A.E. & Hurley, J.H. (1997). Structure of the adenylyl cyclase catalytic core. *Nature* **386**, 247-253.
48. Tesmer, J.J.G., Sunahara, R.K., Gilman, A.G. & Sprang, S.R. (1997). Crystal structure of the catalytic domains of adenylyl cyclase in a complex with $G_{s\alpha}$-GTPγS. *Science* **278**, 1907-1916.
49. Sunahara, R.K., Dessauer, C.W., Whisnant, R.E., Kleuss, C. & Gilman, A.G. (1997). Interaction of $G_{s\alpha}$ with the cytosolic domains of mammalian adenylyl cyclase. *J. Biol. Chem.* **272**, 22265-22271.
50. Graser, T., Vedernikov, Y.P. & Li, D.S. (1990). Study on the mechanism of carbon monoxide induced endothelium-independent relaxation in porcine coronary artery and vein. *Biomed. Biochim. Acta* **49**, 293-296.
51. Brüne, B. & Ullrich, V. (1987). Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol. Pharmacol.* **32**, 497-504.
52. Dean, J.A. (1992). *Lange's Handbook of Chemistry*. McGraw-Hill, New York.

Because *Chemistry & Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published via the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cmb – for further information, see the explanation on the contents pages.