YC-1 activation of human soluble guanylyl cyclase has both heme-dependent and heme-independent components

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YC-1 [3-(5′-hydroxymethyl-2′furyl)-1-benzyl indazole] is an allosteric activator of soluble guanylyl cyclase (sGC). YC-1 increases the catalytic rate of the enzyme and sensitizes the enzyme toward its gaseous activators nitric oxide or carbon monoxide. In other studies the administration of YC-1 to experimental animals resulted in the inhibition of the platelet-rich thrombosis and a decrease of the mean arterial pressure, which correlated with increased cGMP levels. However, details of YC-1 interaction with sGC and enzyme activation are incomplete. Although evidence in the literature indicates that YC-1 activation of sGC is strictly heme-dependent, this report presents evidence for both heme-dependent and heme-independent activation of sGC by YC-1. The oxidation of the sGC heme by 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one completely inhibited the response to NO, but only partially attenuated activation by YC-1. We also observed activation by YC-1 of a mutant sGC, which lacks heme. These findings indicate that YC-1 activation of sGC can occur independently of heme, but that activation is substantially increased when the heme moiety is present in the enzyme.

ODQ | cGMP | nitric oxide

Soluble guanylyl cyclase (sGC) catalyzes the conversion of GTP to cGMP and pyrophosphate and functions as a receptor for NO. sGC is a heme-containing heterodimer, which is activated 200- to 500-fold on binding of NO to its heme moiety (1). Carbon monoxide also binds to the heme group of sGC and activates the enzyme to a lesser extent (2). Synthesized cGMP, in turn, regulates various cGMP-dependent effector proteins, such as protein kinases, phosphodiesterases, and ion channels. Through activation of these effectors sGC plays a critical role in smooth muscle contractility, platelet reactivity, central and peripheral neurotransmission, and other effects (3, 4).

sGC is a heterodimer composed of a 72-kDa α subunit (619 amino acid residues for the human homologue) and a 65-kDa β subunit (549 residues). It is believed that the enzyme has a modular structure comprising three hypothetical domains: (*i*) a catalytic domain in the C-terminal portion of both subunits, (*ii*) an ill defined and uncharacterized dimerization domain, presumably in the central part of the enzyme, and (*iii*) a regulatory domain composed of the N-terminal regions of both subunits. The heme prosthetic group of the enzyme is located in the regulatory domain of the enzyme, with the His-105 of the β subunit coordinating the heme iron (5, 6). The heme moiety is the target for NO-dependent regulation of sGC. Binding of NO and CO to ferrous heme activates sGC, whereas oxidation of heme to ferric heme results in attenuation or complete inhibition of the sGC response to NO and CO. Although 1H-(1,2,4)oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) compound oxidizes several hemeproteins (7), it displays a higher selectivity for the sGC heme (8). Nitrovasodilators, such as glyceryl trinitrate, isosorbide dinitrate, and isosorbide-5-mononitrate, can generate NO (9), which results in the formation of a nitrosyl heme of sGC and, thereby, activation of sGC. Although widely and successfully used for the treatment of congestive heart failure, angina pectoris, and myocardial infarction (10), nitrovasodilators can have a restricted application because patients may develop tolerance.

Although changes in the state of the sGC heme moiety apparently are the main mechanism of physiological activation and pharmacological inhibition of this enzyme, other regulatory mechanisms are possible. YC-1 [3-(5′-Hydroxymethyl-2′furyl)-1-benzyl indazole] is recognized as an activator of sGC that does not affect the state of the sGC heme, because no changes in the heme spectra are observed on addition of YC-1 (11, 12). Thus, YC-1 is regarded as a NO-independent allosteric regulator of sGC. The mechanism of YC-1-dependent activation of sGC is not completely understood because some aspects of it are unsettled. YC-1 alone activates the enzyme only 10-fold, but it potentiates the CO- and NO-dependent activation of sGC, resulting in stimulation of the highly purified enzyme that may be several hundred- to several thousandfold (1, 13). Whereas some reports indicate that YC-1 binding to CO-sGC results in a shift in the Soret absorption band by 4 nm to a shorter wavelength, increases the CO affinity by an order of magnitude, and induces a dramatic change in the kinetics of CO association (14, 15), others see no changes in the heme spectra of CO-sGC on binding of YC-1 (12). Raman resonance studies of YC-1 interaction with sGC also showed no changes in the Raman spectra on addition of YC-1 to basal sGC, but detected YC-1-induced changes the Raman spectra of CO-sGC (16, 17). Moreover, it was reported that heme-deficient sGC is not stimulated by YC-1 (12). Thus, some data suggest that YC-1 binding to sGC affects the state of the heme moiety and its surroundings, whereas other data demonstrate lack of any heme-YC-1 interaction.

The purpose of this study was to analyze the role of the heme moiety in the activation of sGC by YC-1. In this report we present evidence of a dual mechanism of sGC activation by YC-1. We found that YC-1 partially activates sGC with a fully oxidized heme group or a mutant recombinant enzyme lacking the heme, which suggests that YC-1 can activate sGC with both heme-dependent and heme-independent mechanisms.

Materials and Methods

Human sGC Expression and Purification. Human recombinant $\alpha_1\beta_1$ sGC was expressed by using the SF9/baculovirus system described (1). SF9 cells were coinfected with baculoviruses expressing independently α_1 and β_1 subunits, and cells were harvested 3 days after infection. To generate mutant sGC enzyme, the baculovirus-expressing $\beta_1^{\text{Cys-105}}$ subunit was used.

Abbreviations: sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; YC-1, 3-(5′-hydroxymethyl-2′furyl)-1-benzyl-indazole; ODQ, 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one.

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The cells were resuspended in lysis buffer [50 mM triethanolamine (TEA), pH 7.4/50 mM NaCl/10% glycerol/1 mM PMSF/5 mg/ml each of pepstatin A, leupeptin, aprotinin, and chymostatin]. Cells were disrupted by sonication and centrifuged at 100,000 × *g* for 60 min at 4°C. Expressed sGC contained a hexahistidine tag on the C terminus of the α_1 subunit. The enzyme was purified to homogeneity by using a 5-ml column of His-Bind resin (Novagen) as described (1). sGC-positive fractions determined by measuring the presence of a Soret peak (see below) were diluted three times with 10 mM TEA (pH 7.4), pooled, and loaded on a 2-ml Q-Sepharose column. The column was washed with 8 ml of 50 mM TEA (pH 7.4), and the enzyme was eluted with 150 mM NaCl. This step concentrated the sample and removed the imidazole from the sample after the Ni-affinity chromatography. Purified enzyme was stored in aliquots under argon and in the presence or absence of 5 mM DTT. All measurements were performed no later than 5 days after purification.

sGC Activity Assay. sGC activity was measured by the formation of [^{32}P]cGMP from [^{32}P]GTP as described (1). The reaction volume of 100 μl contained 50 mM TEA (pH 7.4), 1 mM EGTA, 10 mM 3-isobutyl-1-methylxanthine, 1 mg/ml BSA, 200 μM GTP, phosphocreatine, phosphocreatine kinase, 3 mM $MgCl_2$, 1 mM cGMP, and ≈1–2 × 10^5 cpm of [^{32}P]GTP. sGC enzyme (0.5 μg per assay) is used to measure basal activity, and 0.1 μg is used for sodium nitroprusside (SNP) or YC-1 stimulation. When necessary, DTT to a final concentration of 1 mM is added. The enzyme was activated with SNP (100 μM) or YC-1 (100 μM) or inhibited by the indicated concentration of ODQ. YC-1 and ODQ stock solutions were prepared in DMSO, but the DMSO concentration in the reaction mixture did not exceed 0.1%.

Spectroscopic Studies. All spectroscopic measurements were recorded with a dual-beam Cecil 9500 spectrophotometer equipped with a temperature controller. To measure the dose–response curve of sGC oxidation by ODQ we recorded the difference spectra of oxidized and reduced sGC heme at 25°C. Four matched cuvettes (Beckman Coulter) were used. Cuvettes 1 and 2 were placed in the sample beam, and cuvettes 3 and 4 were placed in the reference beam. Added to cuvettes 1 and 3 was 650 μl of 1.5 μM sGC, and to cuvettes 2 and 4 was added 650 μl of lysis buffer without protease inhibitors. Identical amounts of reagent were added to cuvettes 1 and 4 during titration to balance the spectral influence of ODQ. Changes in the Soret peak of sGC were monitored by recording the difference spectra between 370 and 550 nm after each addition of ODQ.

Mutagenesis. His-105 of the β_1 subunit was substituted with a cysteine by using a QuickChange mutagenesis kit (Stratagene) in pVL1392-β plasmid described (1). The following pair of primers was used for this purpose:

5′-CTACAGAACCTTGATGCTCTGCACGACCACC-TTGCTACCATCTACC-3′ and 5′-GGTAGATGGTAG-CAAGGTGGTCGTGCAGAGCATCAAGGTTCTGTAG-3′. The entire coding region of the β_1 subunit was sequenced after the mutagenesis procedure to ensure the presence of His to Cys substitution and the absence of potential aberrant mutations, and the coding region was recloned into *Eco*RI site of pVL1392 vector to avoid mutations in the vector. The obtained pVL1392-$\beta^{Cys\text{-}105}$ plasmid was used to generate the recombinant virus with BaculoGold DNA according to the manufacturer's protocol (PharMingen).

Results

ODQ-Dependent Oxidation of the sGC Heme in Reducing and Nonreducing Conditions. First, we investigated the characteristics of ODQ-dependent inhibition of the human sGC recombinant enzyme. Human sGC was expressed and purified as described in *Materials and Methods*. The changes in the heme state of human sGC were monitored in the absence of any reducing agents or in the presence of 1 mM DTT. We analyzed changes in the amount of ferrous heme sGC by monitoring time-dependent changes in the absorbance at 431 nm, corresponding to the maximum absorbance of the Soret peak (Fig. 1*A*). We observed that ODQ-induced oxidation of the heme group of 0.1 μM sGC was prevented by addition of 1 mM DTT, but in the absence of DTT, 100 nM ODQ time-dependently oxidized the heme iron. To analyze how reducing agents affect the oxidation of heme, we recorded the difference spectra of redox conversion of ferrous heme sGC to ferric heme sGC as a function of ODQ concentration, as described in *Materials and Methods*. A representative group of ODQ-dependent oxidation difference spectral scans for a DTT-free sample is presented in Fig. 1*B*. As expected, we observed an ODQ concentration-dependent decrease in the absorbance at 431 nm, an increase at 393 nm, and an isosbestic point at 409 nm. The differences between the absorbance values at 393 and 431 nm were calculated for each concentration of ODQ and plotted as a function of ODQ concentration (Fig. 1*C*). Comparison of the ODQ-dependent oxidation curves obtained in reducing and nonreducing conditions clearly indicated a right

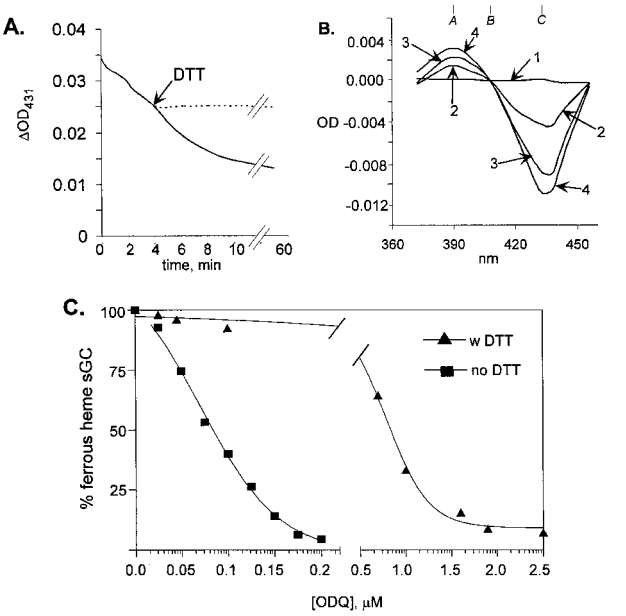

Fig. 1. ODQ-dependent oxidation of ferrous heme of human sGC. (*A*) Time course of ODQ-dependent oxidation of the sGC heme. sGC enzyme (0.1 μM) in 50 mM TEA (pH 7.4)/50 mM NaCl was incubated with 0.1 μM ODQ in the absence (solid line) or presence of 1 mM DTT (dotted line). Changes in the absorbance at 431 nm were monitored over time at 25°C. The arrow indicates the time when 1 mM DTT was added to the sample. (*B*) Representative difference spectra of redox oxidation of sGC heme at various concentrations of ODQ in nonreducing conditions. Difference scans were recorded at 25°C for various concentrations of ODQ as described in *Materials and Methods*. Representative difference scans are presented for 0.5 μM sGC enzymes in 50 mM TEA (pH 7.4)/50 mM NaCl supplied with 0 nM (spectrum 1), 15 nM (spectrum 2), 75 nM (spectrum 3), and 250 nM (spectrum 4) ODQ. Oxidation of the sGC heme results in the shift of the Soret band from 431 nm (line C) to 393 nm (line A) with an isosbestic point at 409 nm (line B). (*C*) Replotting of the data from difference spectra. The λ_{393} and λ_{431} values for each dose of ODQ were calculated. Data were plotted as the percent of reduced sGC as a function of ODQ concentration in both nonreducing (■) and reducing (▲) conditions. The fraction of reduced sGC was calculated as $\{1\text{-}[(\lambda_{393} - \lambda_{431}) - (\lambda^{o}_{393} - \lambda^{o}_{431})]/(\lambda^{max}_{393} - \lambda^{max}_{431})\}\cdot 100$, where λ_{393} and λ_{431} are corresponding absorbances at various ODQ concentrations, λ^{o}_{393} and λ^{o}_{431} are the absorbances of nontreated sGC and λ^{max} are the absorbances at the plateau of ODQ titration.

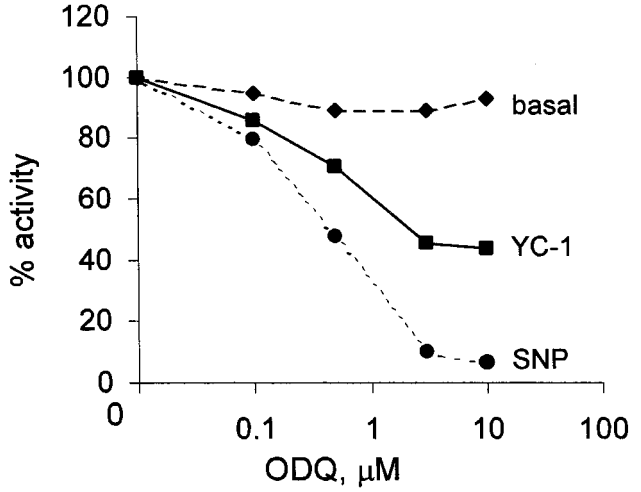

Fig. 2. ODQ-dependent inhibition of sGC activity. Human sGC was incubated in nonreducing conditions with various concentrations of ODQ for 3 min at room temperature, followed by the addition of 1 mM DTT. The enzyme's activity was measured as described in *Materials and Methods*. The effects of ODQ on basal sGC activity (dashed line, ◆) or sGC stimulated by YC-1 (solid line, ■) or SNP (dotted line, ●) are shown.

shift of effective ODQ concentrations in the presence of DTT. These analyses suggest that reducing agents in the activity assay attenuate the inhibitory effects of ODQ, probably by reducing some of the ODQ or partially reducing the oxidized heme.

sGC with Ferric Heme Is Activated by YC-1. Next, we investigated the effect of ODQ-dependent oxidation on the activity of sGC in nonreducing conditions. As expected, the basal sGC activity was not affected by ODQ (Fig. 2, dashed line), whereas the NO-dependent activation of sGC was inhibited in a concentration-dependent manner by ODQ (Fig. 2, dotted line). However, YC-1-dependent activation of sGC was only partially attenuated by ODQ treatment and reached a plateau at about 1–3 μM ODQ. Even at concentrations of 10 μM ODQ sGC enzyme was still activated by 100 μM YC-1, although only at 30–40% of nontreated levels (Fig. 2, solid line). At 10 μM ODQ the sGC heme was more than 90% oxidized, as suggested by spectral studies and nonresponsiveness to NO. The differences in the inhibitory effect of ODQ on NO- and YC-1-stimulated sGC suggest that YC-1 activation of sGC has two components. One component is heme-dependent, whereas another is heme-independent.

Heme-Deficient Mutant His-105 → Cys Is Activated by YC-1. To confirm the conclusion that YC-1 activation only partially depends on the heme prosthetic group, we generated a heme-deficient sGC enzyme. We substituted the heme-coordinating His-105 of the β subunit with a cysteine residue and coexpressed this mutant β subunit with the hexahistidine-tagged α subunit. As expected, the purified $\alpha_1\beta_1{}^{\text{Cys-105}}$ enzyme was heme-deficient and lacked the characteristic Soret band at 431 nm (Fig. 3). The mutant enzyme was not activated by SNP (Fig. 4*B*), whereas the wild-type enzyme showed a significant increase in cGMP production upon NO treatment (Fig. 4*A*). Analysis of the YC-1 effect on the mutant enzyme indicated that treatment with 100 μM YC-1 resulted in a 3-fold activation of the $\alpha_1\beta_1{}^{\text{Cys-105}}$ enzyme (Fig. 4*B*). In comparison, the wild-type enzyme showed a 7-fold activation under similar conditions (Fig. 4*A*). This YC-1 activation of the $\alpha_1\beta_1{}^{\text{Cys-105}}$ enzyme supports our conclusions that YC-1 binding and sGC activation can occur in the absence of a heme group. However, activation of the heme-deficient mutant enzyme was about 30% of the wild-type heme-containing enzyme. YC-1 activation of the mutant enzyme was not enhanced with SNP addition (Fig. 4*B*), supporting the evidence of heme deficiency.

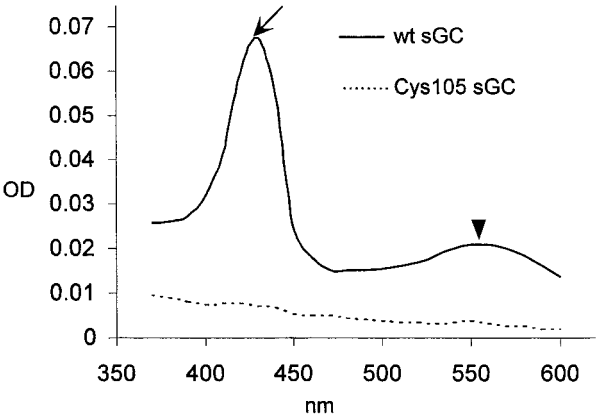

Fig. 3. $\alpha_1\beta_1{}^{\text{Cys105}}$ mutant enzyme is heme-deficient. UV-visible absorption spectra of 0.5 μM wild-type (wt; solid line) and mutant (dotted line) sGC enzymes in 50 mM TEA (pH 7.4)/50 mM NaCl were recorded. Arrow indicates the Soret peak at 431 nm, arrowhead indicates the wide α/β band at about 540–590 nm.

Discussion

YC-1 is an interesting NO-independent activator of sGC with some potential in pharmacological and biochemical studies of sGC. YC-1 treatment of preconstricted aorta results in vasodi-

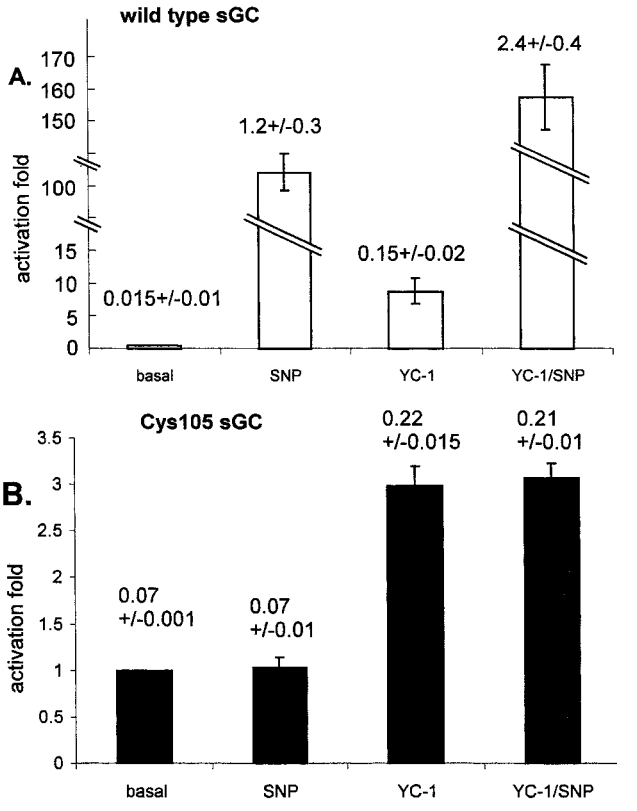

Fig. 4. $\alpha_1\beta_1{}^{\text{Cys-105}}$ mutant enzyme is partially stimulated by YC-1. Wild-type (*A*) or mutant (*B*) enzymes were stimulated with 100 μM SNP, 100 μM YC-1, or both. The data are presented as fold stimulation of basal enzyme activity. The numbers above each bar graph represent the specific activity of the enzyme in micromoles per milligram per minute ± SD of three independent measurements performed as described in *Materials and Methods*.

lation of aortic rings *in vitro* (18). Treatment of intact platelets with YC-1, in combination with CO or NO, elevated the levels of cGMP and decreased platelet aggregation (19). Administration of YC-1 alone or in combination with NO donors to whole animals resulted in inhibition of platelet-rich thrombosis (20) and a decrease of the mean arterial pressure (21). These effects correlated with increased cGMP levels in aorta. Cotreatment with YC-1 and nitrovasodilators could be of therapeutic value, because it would allow a reduction in the dosage of these drugs, thus potentially decreasing the development of tolerance.

Despite its potential as a pharmacological compound, the mechanism of YC-1-dependent activation is poorly understood. The chemical properties of YC-1 may be responsible for this lack of understanding. YC-1 is a highly hydrophobic compound with low aqueous solubility that is able to form, at best, a 100–200 μM solution, significantly hindering the investigation of the nature of the sGC/YC-1 interaction and the mechanism of sGC activation. The activation curve of sGC by YC-1 is linear in the 10–200 μM solubility range of the compound, but does not reach saturation (1, 13). Thus, the binding constant, the stoichiometry of enzyme YC-1 interaction, and whether there is any cooperative binding of multiple YC-1 molecules to sGC cannot be determined directly.

Several working models of YC-1 and sGC interaction can be deduced from reported studies. Binding of YC-1 to sGC may involve some interaction between the hydrophobic portions of YC-1 and the protoporphyrin ring of the heme moiety or the heme-binding pocket itself. Such an interaction could induce changes of the heme pocket perhaps similar to that induced by NO binding. This model presumes that the heme group is indispensable for YC-1 binding and YC-1-dependent activation. When purified sGC was depleted of heme by incubation with 0.5% Tween 20 and subsequent gel filtration, such a "heme-deficient" enzyme was not stimulated by YC-1 (12), which supports the hypothesis that YC-1 binding requires the presence of heme. However, the binding of YC-1 to sGC does not result in any changes in the Soret spectrum of the heme (12) and does not perturb the Raman spectrum of sGC (17), which almost certainly excludes a direct interaction between YC-1 and heme. Alternatively, YC-1 binding to sGC may not change the heme state and its immediate surroundings, but may induce structural changes in sGC that have functional effects downstream of the NO-induced perturbations of the heme pocket. However, evidence reported in the literature and in this study indicates that the mechanism of YC-1 action is more complex.

Studies presented in this article demonstrate that the heme dependence of YC-1 activation is not absolute. First, we probed the relationship between YC-1 activation and the heme group by changing the oxidation state of the heme with ODQ. ODQ oxidizes the sGC heme iron to a ferric state (22). sGC activity is optimal in the presence of reducing agents and, typically, 1 mM DTT is added to sGC assays. Because ODQ-oxidized sGC heme could be reduced back to the ferrous state by a strong reducing agent, such as dithionate (16), we investigated whether addition of 1 mM DTT in aerobic conditions to oxidized sGC would cause a significant reduction of heme (Fig. 1). Testing the time course of 0.1 μM sGC oxidation by equimolar 100-nM concentrations of ODQ, we found that addition of 1 mM DTT to the oxidized sGC heme prevented further oxidation of the heme, because the decline of 431 nm absorbance was prevented (Fig. 1*A*). However, in these conditions, 1 mM DTT did not reduce the oxidized heme back to the ferrous state (Fig. 1*A*), because no increase in 431-nm absorbance was observed. We initially analyzed the ODQ-dependent oxidation of sGC in nonreducing conditions by recording the difference spectra of ferrous and ferric sGC (Fig. 1*B*). We found that the IC_{50} of ODQ in nonreducing conditions ($\approx$75 nM) is at least one order of magnitude lower than if 1 mM DTT was present ($\approx$0.9 μM), as demonstrated in Fig. 1*C*. This

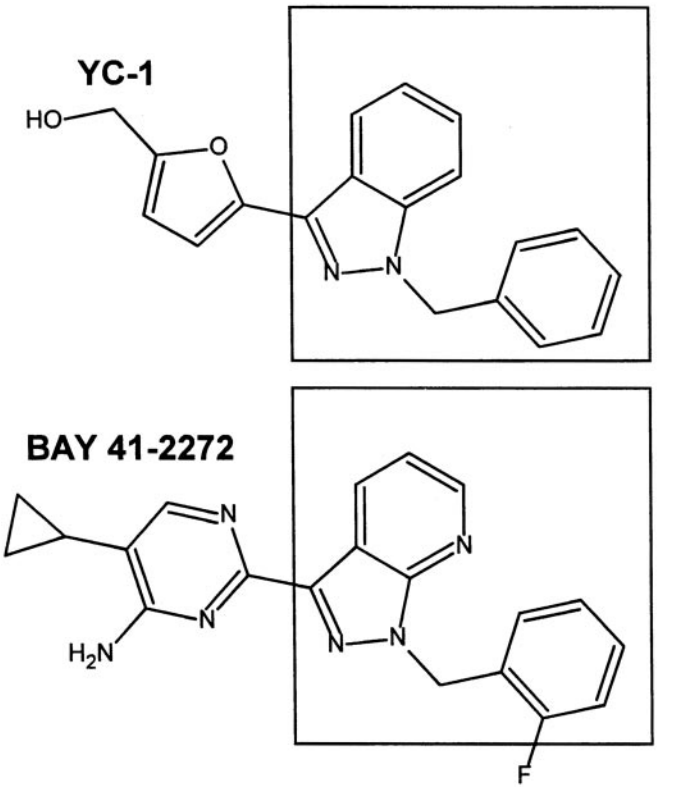

Fig. 5. Chemical structure of YC-1 and BAY41–2272. The boxed area indicates portions of the compounds that have similar structures.

optimization allowed us to evaluate the effect of heme oxidation on the activation of sGC by YC-1 and compare it with basal enzyme or enzyme activated by NO. As expected, oxidation of the heme iron only slightly affected the basal activity of the enzyme (Fig. 2). This 10–12% reduction of basal enzyme activity by ODQ was not observed if the assays were performed under argon with argon-saturated solutions, indicating that some environmental NO (or CO) was present in the aerobic assay. NO stimulation, however, was strongly inhibited by ODQ treatment. ODQ (0.5 μM) induced a more than 90% reduction of SNP-activated sGC activity (Fig. 2). The residual NO-stimulated activity at high 10-μM concentrations of ODQ probably indicates that some reduction of ferric heme occurs during the assay in the presence of DTT. We also observed an ODQ dose-dependent attenuation of the sGC response to YC-1 (Fig. 2). However, this attenuation was not as extensive as for NO stimulation and reached a plateau at about 1 μM ODQ (Fig. 2). YC-1-stimulated enzyme retained at least 40% of its original activation by YC-1 (Fig. 2), which suggests that YC-1 binding or its mechanism of action is not completely dependent on the sGC heme moiety. The existence of a plateau in ODQ-dependent inhibition of YC-1 activation also suggests that the binding of ODQ and YC-1 are not mutually exclusive processes and that their binding sites do not overlap.

To confirm our conclusions that YC-1 activation is at least partially independent of heme, we generated a mutant sGC with cysteine substitution of the His-105 of the β subunit. According to the UV-visible absorbance spectra of the mutant enzyme (Fig. 3), its unresponsiveness to NO activation, and lack of NO-dependent synergistic activation of YC-1-treated sGC, the $\alpha_1\beta_1^{Cys\text{-}105}$ enzyme was heme-deficient. This result corroborates previous findings that the mutation of His-105 residue affects the binding of heme (5, 6). We found that the mutant enzyme was stimulated by 100 μM YC-1 only 3-fold versus an 8- to 10-fold

stimulation of the wild-type enzyme (Fig. 4). Because heme-deficient enzyme was partially activated by YC-1, we conclude that YC-1 activation is not completely heme-dependent. The YC-1 activation of heme-deficient mutant $\alpha_1\beta_1^{Cys\text{-}105}$ was also not affected by addition of ODQ (data not shown), supporting the hypothesis that binding of YC-1 and ODQ occurs at non-overlapping sites.

The data presented in this report contradict previous reports on activation of the heme-deficient sGC by YC-1 (11, 12). We found only a partial attenuation of YC-1 activation in our mutant heme-free enzyme, whereas others demonstrated that sGC chemically depleted of heme with Tween 20 treatment is not responsive to YC-1 (11, 12). Such treatment with detergent may result in some irreversible changes in the enzyme's structure, affecting its responsiveness to YC-1. As mentioned above, YC-1 is a hydrophobic compound that, most probably, interacts with some hydrophobic surfaces or pockets of sGC. Tween 20 may also prevent these interactions by binding to the same hydrophobic moieties of sGC.

Our data indicate that the heme moiety is important but not necessary for YC-1-dependent activation of sGC. Previous studies of changes in Raman spectra upon YC-1 binding to a CO-sGC suggested that YC-1 binds near the heme-binding region of sGC (17). The partial attenuation of YC-1 activation by ODQ may indicate that there is more than one binding site for YC-1, and that at least one of these sites is affected by the oxidation of heme and resulting changes in the conformation of the heme pocket. However, there is some evidence against multiple YC-1-binding sites. Recently, a new allosteric compound, BAY41–2272, which affects the activity of sGC in a manner similar to YC-1, was described (23). This compound alone stimulates sGC without changing its Soret band characteristics and potentiates the effects of CO and NO in a manner similar to YC-1 (23). Moreover, YC-1 and BAY 41–2272 share some structural similarities (ref. 23; Fig. 5, boxed). ODQ oxidation of the heme strongly inhibited activation of sGC by 1 μM BAY 41–2272, but an increase in sGC activity is observed at higher concentrations of BAY 41–2272 (100 μM; ref. 23). These data corroborate our findings. The crosslinking derivative of BAY 41–2272 labeled only two closely located residues 238 and 245 of the α subunit of sGC, suggesting only one binding site for BAY 41–2272. The preservation of YC-1 responsiveness of the ODQ-oxidized sGC or mutant heme-free sGC suggests that the heme moiety or heme-binding pocket has an important, but not indispensable role in the interaction of sGC with YC-1. The binding of YC-1 may be facilitated by the protoporphyrin moiety of the heme, or by the hydrophobic residues that stabilize the heme in its pocket. Interaction of YC-1 with the heme pocket might be responsible for the sensitization of sGC to nitric oxide and carbon monoxide and explain changes in the dissociation constants of these gaseous activators (12, 14, 15). Oxidation of the heme iron and associated change in the charge in proximity to the heme or the complete removal of the heme group results in some structural changes in the heme pocket. These changes would result in a decreased affinity to YC-1, but not a complete disruption of sGC/YC-1 interaction. The existence of at least one additional contact region, which is not affected by the changes in the heme pocket, may be postulated. Cys-238 and -245 of the α subunit, identified as residues interacting with BAY41–2272, could be part of such a region. Mutagenesis of these and nearby residues in the α subunit could test this hypothesis.

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