Identification of Residues Crucially Involved in the Binding of the Heme Moiety of Soluble Guanylate Cyclase

Peter M. Schmidt, Matthias Schramm, Henning Schröder, Frank Wunder, and Johannes-Peter Stasch

From the Institute of Cardiovascular Research, Bayer AG, Aprather Weg 18a, D-42096 Wuppertal and the Martin-Luther-University, School of Pharmacy, Wolfgang-Langenberg-Strasse 4, D-06120 Halle, Germany

Soluble guanylate cyclase (sGC), a heterodimeric hemeprotein, is the only receptor for the ubiquitous biological messenger nitric oxide (NO), identified to date and is intimately involved in various signal transduction pathways. By using the recently discovered NO- and heme-independent sGC activator BAY 58-2667 and a novel cGMP reporter cell, we could distinguish between heme-containing and heme-free sGC in an intact cellular system. Using these novel tools, we identified the invariant amino acids tyrosine 135 and arginine 139 of the β-subunit as crucially important for both the binding of the heme moiety and the activation of sGC by BAY 58-2667. The heme is displaced by BAY 58-2667 due to a competition between the carboxylic groups of this compound and the heme propionic acids for the identified residues tyrosine 135 and arginine 139. This displacement results in the release of the axial heme ligand histidine 105 and to the observed activation of sGC. Based on these findings we postulate a signal transmission triad composed of histidine 105, tyrosine 135, and arginine 139 responsible for the enzyme activation by this compound and probably also for transducing changes in heme status and porphyrin geometry upon NO binding into alterations of sGC catalytic activity.

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† To whom correspondence should be addressed. Tel.: 49-202-368738; Fax: 49-202-368009; E-mail: Johannes-Peter.Stasch.JS@bayer-ag.de.

‡ The abbreviations used are: sGC, soluble guanylate cyclase; DEA/NO, diethylamine NONOate; NO, nitric oxide; ODQ, 1H-[1,2,4]-oxadiazolo[4,3-e]quinoxalin-1-one; PTPX, 3,18-divinyl-2,7,13,17-tetramethylporphine-8,12-dipropionic acid; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; BAY 58-2667, 4-[(1-carboxybutyl)2-[(4-phenethylbenzyl)oxy][phenethylamino]methyl] benzoic acid; BAY 41-2272, 5-cyclopropyl-2-(1-[2-fluorobenzyl]-1H-pyrazolo[3,4-d]pyrindin-3-y1)pyrimidin-4-ylamine; CHO, Chinese hamster ovary cells; RLU, relative light unit(s); WT, wild type.

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Residues Involved in the Binding of the sGC Heme

EXPERIMENTAL PROCEDURES

Compounds—BAY 58-2667 (4-(1[(4-carboxybutyl)2-[(4-phénylbenezyl)(oxy)phénylethyl]amino]methyl] benzoic acid) and BAY 41-2272 (5-cycloprenyl-2-[(2-fluorobenzyl)-1H-pyrálzol[3,4-b]pyridin-3-yl]pyrimidin-4-yIamîne) were synthesized as described previously (20, 21).

DEANO (2-[N,N-diethylamino]diazonolate-2-oxide), ODQ (1H-(1,2,4)-oxadiazole[4,3-a]quinazolin-1-one), and PPIX (3,18-divinyl-2,17,13,17-tetramethylporphine-8,12-dipropionicoacid) were purchased from Alexis Biochemicals (San Diego, CA). All other chemicals of analytical grade were obtained from Sigma (Tokyo, Japan) and Merck (Darmstadt, Germany).

Mutagenesis—The mutagenesis was performed using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocols. The following primers were used to perform the desired mutations: βH105F: 5′-GCAGAACCTGCGACGGCTCTCTGCAGAAGAAGGCGGC-3′; βY135F: 5′-GCCAAGGGCTTCATCGCAGCTCTCCGCAAGGAGCGGCG-3′; βY135A: 5′-GCCAAGGGCTTCATCGCAGCTCTCCGCAAGGAGCGGCG-3′; βR139A: 5′-CATTCTGCACTACTTCGGAACCTAGGGGCTTCGACAGATTG-3′; βR139A: 5′-CATTCTGCACTACTTCGGAACCTAGGGGCTTCGACAGATTG-3′; βR139A: 5′-GCAAAGGGCTTCATCGCAGCTCTCCGCAAGGAGCGGCG-3′; and βY135F/R139A: 5′-GCAGAACCTGCGACGGCTCTCTGCAGAAGAAGGCGGC-3′. The accuracy of the mutations was verified by sequencing (Invitrek, Berlin, Germany).

Generation of a cGMP Reporter Cell—To characterize sGC mutants in an intracellular environment, a cGMP reporter cell was constructed based on a method reported earlier (22). Briefly, a CHO cell line expressing cytosolic aequorin was stably transfected with a plasmid coding for the cGMP-gated ion channel CNG2 under a zeatin resistance. Thereafter, zeatin-resistant clones were characterized for channel expression, and active clones were subcloned by the limited dilution technique. Selected clones were cultured in Dulbecco’s modified Eagle’s medium/F-12 with L-glutamine (Invitrogen, Carlsbad, CA), 1 mM sodium pyruvate, and 0.075% sodium bicarbonate, supplemented with 5% (v/v) serum-free medium/F-12 with L-glutamine (Invitrogen), 1 mM and 10 mM stock solutions of BAY 58-2667 were prepared in MeSO and added to the samples to obtain a final concentration of 10 and 100 μM. ODQ was prepared as a 100 μM stock solution in MilliQ H2O and diluted to a final concentration of 10 μM. Samples containing 15 μg of sGC were incubated at 37°C for 10 min in the absence or presence of BAY 58-2667 (10 and 100 μM) alone or combined with ODQ. Before the UV-visible spectra were recorded, the enzyme was separated by ion exchange chromatography from BAY 58-2667, ODQ, or free heme that might interfere with the measurement.

Separation of sGC from Detergent—Separation of sGC from detergent and unbound heme was performed as previously described (26). Briefly, sGC-containing samples were loaded onto ion exchange columns and washed once to remove any traces of detergent. Bound sGC was eluted with 60 μl of elution buffer (300 mM NaCl), and UV-visible spectra were recorded as described above.

RESULTS

Sequence Alignments—The N-terminal 200 amino acids of the β-subunit of sGC are sufficient to bind the prosthetic heme moiety with spectral characteristics comparable to those of the native sGC (27, 28). Based on this information, we initiated a BLAST search with this putative heme-binding domain. Various sGC α- and β-subunits from different species as well as predicted proteins of unknown function were found. A subsequent alignment of sequences known to bind heme identified invariant or conserved amino acids (Fig. 1). From these residues, the positively charged and polar amino acids βH105, βY135, and βR139 were chosen for site-directed mutagenesis as indicated by the asterisk.

Screening sGC Mutants—For screening sGC mutants we constructed a novel cGMP reporter cell line based on a CHO cell stably transfected with a cGMP-dependent cation channel (CNG2) and cytosolic aequorin. After maximum activation of this reporter cell in a 96-well microtiter plate, a signal of about 2,500,000 RLUs in each well could be measured. To ensure that the measured sGC activity was within the linear range of the readout system the experimental conditions were chosen to obtain a maximum signal between 500,000 and 1,000,000 RLUs after maximal sGC activation (see Supplemental Material to Fig. 2). The different mutants of the sGC β-subunit were constructed according to the results of the alignment (Fig. 1), verified by sequencing and expressed in the cGMP reporter cell line.

WT-sGC—As shown in Fig. 2A CHO cells cotransfected with WT α1- and β1-cDNA exhibited the activation profile of heme-containing sGC. DEA/NO activated the transiently transfected reporter cells to a maximum of 26.7-fold with an EC50 of 27 nM (Fig. 3B; see Supplemental Material to Fig. 2). In addition, a concentration-dependent stimulation of 15.9-fold could be achieved by the NO-independent but heme-dependent sGC-stimulator BAY 41-2272 (Fig. 2A and Table I). The presence of 10 nM DEA/NO, a concentration that exhibits only negligible effects on sGC activation (2.2-fold; Fig. 3A), the maximal activation of BAY 41-2272 was increased 60% (Fig. 2A and Table I). The NO- and heme-independent sGC activator BAY 58-2667 showed a concentration-dependent activation of the transiently transfected enzyme of 7.4-fold that was potentiated up to 25.6-
fold in the presence of the sGC inhibitor ODQ (Fig. 2A and Table I).

βH105F—The mutant βH105F was generated as a control that causes the expression of heme-free sGC. Indeed, the mutant βH105F exhibited the activation pattern expected of heme-free sGC: no measurable activation of the enzyme in the presence of DE/NO (Fig. 3, A and B), a negligible activation (2.1-fold) after incubation with BAY 41-2272, not further elevated when combined with NO (Fig. 2B and Table I), and activation by heme-independent BAY 58-2667 within the tested concentration range (Figs. 2G, 3A, 3B, and Table I). Incubation with 10 μM BAY 58-2667 led to an activation of 13.2-fold increasing to 16.0-fold upon addition of ODQ (Fig. 2G). A saturable activation could not be reached within the tested concentration range. According to the calculated concentration-response curves, EC50 values of about 8 μM and a maximal stimulation factor of 25 were assumed (Table I). The exchange of both residues with alanine abolished any sGC activation by NO, BAY 41-2272, and the combination of both (Figs. 2G, 3A, 3B, and Table I). Incubation with 10 μM PPIX led to an activation of 16.8-fold increasing to 18.7-fold in the presence of ODQ (Fig. 2F). Based on the extrapolated concentration-response curves, EC50 values of about 7 μM were assumed.

βY135F plus R139L and βY135A plus βR139A—The construct with both Y135F and R139L resulted in an enzyme that was insensitive to NO, BAY 41-2272, and the combination of both (Figs. 2G, 3A, 3B, and Table I). Incubation with 10 μM BAY 58-2667 led to an activation of 13.2-fold increasing to 16.0-fold upon addition of ODQ (Fig. 2G). A saturable activation could not be reached within the tested concentration range. According to the calculated concentration-response curves, EC50 values of about 8 μM and a maximal stimulation factor of 25 were assumed (Table I). The exchange of both residues with alanine abolished any sGC activation by NO, BAY 41-2272, and PPIX (30). Furthermore, BAY 41-2272 was added to the incubation buffer to amplify the sGC activation upon PPIX reconstitution (23, 24). WT-sGC represents a useful tool to investigate sGC reconstitution (19, 24, 29). Reconstitution was performed in the presence Tween 20 for both the removal of the native heme moiety and to facilitate the subsequent reconstitution with PPIX (30). Due to its activating effect PPIX represents a useful tool to investigate sGC reconstitution (19, 24, 29). Reconstitution was performed in the presence Tween 20 for both the removal of the native heme moiety and to facilitate the subsequent reconstitution with PPIX (30).
required to achieve even a slight increase (3.1-fold) in the activity of the βR139L mutant. The βY135F exchange was not responsive even at the highest applied concentration of PPIX (Fig. 3C).

**Spectroscopic Studies**—To explore potential interactions between the heme moiety of sGC and BAY 58-2667, the enzyme was incubated with BAY 58-2667 in the absence and presence of ODQ and separated subsequently by ion exchange chromatography. The native ferrous sGC showed the Soret band at 431 nm that was shifted to 392 nm after oxidizing the heme by addition of ODQ (Fig. 4A). Incubation of the native sGC with 10 μM BAY 58-2667 did not result in any shift of the Soret peak (Fig. 4B). Incubation of sGC with 10 μM BAY 58-2667 in the presence of ODQ led to the removal of the prosthetic heme moiety as did incubation with 100 μM BAY 58-2667 without additional ODQ (Fig. 4B).

**sGC Activity Assay**—Purified WT-sGC was incubated with increasing concentrations of BAY 58-2667 from 100 pM to 200 μM in the absence and presence of 10 μM ODQ. As shown in Fig. 4C, a biphasic activation of sGC was observed in the absence of ODQ. The first step displayed a concentration-dependent activation of the enzyme from 1 nM to 50 nM with an EC50 of 3.6 nM followed by a phase of slight increase of sGC activation with increasing concentrations of BAY 58-2667 up to about 3 μM. Thereafter, a second phase of sGC activation started from 3 μM until the maximal solubility of this compound was reached (200 μM). In the presence of ODQ a sigmoidal concentration response curve with an EC50 of 9.6 nM was observed reaching a maximal specific activity of 19,050 nmol·min⁻¹·mg⁻¹.

**DISCUSSION**

Here we report the identification of amino acids crucial for binding of the prosthetic heme moiety to sGC as well as for NO-independent sGC activation through BAY 58-2667. Based on these findings, we propose a model for the BAY 58-2667-induced activation of sGC summarized in Fig. 5. This model might also be useful to understand the NO-driven activation of the enzyme via a signal transmission triad consisting of the histidine 105, tyrosine 135, and arginine 139 located within the β1-subunit of sGC. These residues might be involved in the
transduction of heme status and porphyrin geometry upon NO binding into alterations of sGC catalytic activity.

In the early 1980s, studies with different porphyrin derivatives suggested that the propionic acid groups of the porphyrin interact with basic residues of the enzyme (19). More than two decades later our knowledge of the heme binding domain and the intramolecular signal transduction has advanced little due to the lack of any crystal structure of the enzyme (31). Moreover, elucidating the function of this domain by mutagenesis studies failed due to the lack of compounds capable of activating the heme-free enzyme. Using a novel cGMP reporter cell line that obviated the need to purify sGC mutants together with the newly discovered heme-independent sGC activator BAY 58-2667 (14), the heme-dependent sGC stimulator BAY 41-2272, NO and the sGC inhibitor ODQ, it was possible for the first time to distinguish between heme-containing and heme-free sGC within their cytosolic environment. This cGMP reporter cell, stably transfected with a cGMP-gated cation channel and aquorin, transduces intracellular cGMP concentrations via Ca\(^{2+}\) influx into bioluminescence that can be easily measured through a charge-coupled device camera.

A starting point for our work was the hypothesis of Ignarro and coworkers (19), who postulated positively charged or polar amino acids of sGC as counterparts interacting with the propionic acid groups of the heme moiety. The N-terminal 200 residues of the \(\beta_1\)-subunit form a domain capable of binding heme with spectral characteristics comparable to that of the native enzyme, suggesting that the postulated amino acids probably reside in this subunit (27, 28). Consequently, we initiated a BLAST search and included in the subsequent multiple-sequence alignment all sequences of proteins known to bind heme. Very recently this approach was supported by Iyer and coworkers (32) who postulated an ancient heme-NO binding domain in the N-terminal region of the \(\beta_1\)-subunit of sGC that could be found even in prokaryotic organisms (32). Two identified invariant amino acids in this alignment that fit the prediction of Ignarro and coworkers (\(\beta Y^{135}\) and \(\beta R^{139}\)) were exchanged by alanine, and the ability of these muteins to bind heme was evaluated by transfection into the cGMP readout cell.

As controls for the analysis we used the WT-sGC, which contains heme, and \(\beta H^{105} F\), which does not (16–18, 30). The cGMP readout cell transiently transfected with WT-sGC displayed an activation profile similar to that of the isolated enzyme responding to DEA/NO and/or BAY 41-2272 (13, 25, 33). Additionally, incubation with BAY 58-2667 led to an increase in sGC activity that was potentiated in the presence of ODQ as described for the purified enzyme (14, 15). Conversely, the cGMP readout cell transfected with the \(\beta H^{105} F\) mutant behaved as expected for the heme-free apo-enzyme in that it responded to neither NO nor BAY 41-2272 (16–18, 30). Finally, the lack of potentiation of the BAY 58-2667-induced activity by ODQ is further indication of the loss of the heme moiety, which is essential for the oxidizing effect of ODQ (14, 34–36). Basal activity of this mutant was slightly higher compared with the WT-sGC resulting in lower activation factors in agreement with findings of Martin and coworkers (18).

Having established that the cellular detection system is a sensitive and reliable method to distinguish between heme-containing and heme-free sGC in its cytosolic environment, we evaluated the various novel muteins. The replacements of the tyrosine 135 (\(\beta Y^{135} A\) and \(\beta Y^{135} F\)) and arginine 139 (\(\beta R^{139} A\) and \(\beta R^{139} L\)) resulted in enzymes that were not responsive to NO, BAY 41-2272, or the combination of both. BAY 58-2667-mediated enzyme activation was observed, but was not further potentiated by ODQ. Therefore, it can be assumed that the removal of the hydroxyl-group of the tyrosine 135 or the positive charge of the arginine 139 led to the expression of heme-free sGC. Interestingly, substituting the conserved aromatic amino acids surrounding the tyrosine 135, such as histidine 134 and tyrosine 136 with alanine had no effect on heme binding to sGC (data not shown). This is in agreement with findings described by others (16, 17). The importance of tyrosine 135 and arginine 139 for heme binding was also confirmed by PPIX reconstitution studies. PPIX shares the propionic acid moieties with the native heme and might be expected to share also the requirement for the two identified residues. In contrast to the heme-depleted WT-sGC, the mutants \(\beta Y^{135} F\) and \(\beta R^{139} L\) could not be reconstituted with PPIX even at micromolar concentrations. These observations are summarized in Fig. 5. The sGC prosthetic heme is embedded in a hydrophobic binding pocket between the histidine 105, tyrosine 135, and the arginine 139. Comparable interactions were described for various other heme binding proteins such as cytochrome P-450, cytochrome b, or FixL (37–39).

Interestingly, the exchange of tyrosine 135 and arginine 139 not only caused the loss of heme binding by sGC but also reduced the potency of BAY 58-2667. In contrast, although the heme was lost from \(\beta H^{105} F\), the potency of BAY 58-2667 was undiminished. In our recent work (14) we postulated a model with two binding sites for BAY 58-2667 at the sGC: one high affinity binding site independent from the presence and oxida-
tion state of the heme and a second one, which changes its affinity for BAY 58-2667 from low to high upon oxidation or removal of the heme moiety of sGC. For the putative heme-independent binding site a $K_D$ value of 3.2 nM was determined and a saturation of binding was reached at concentrations of about 50 nM (14). These results are in agreement with the first step of the biphasic activity profile showing an $EC_{50}$ value of 3.6 nM and a saturable activation of the enzyme at nanomolar concentrations. The second postulated binding site for BAY 58-2667 changes its affinity from low to high upon oxidation or removal of the heme moiety (14). Under the latter conditions there was also a doubling of maximal binding in the receptor binding study, an increase in sGC activation, and a shift in the photoaffinity labeling pattern of a derivative of BAY 58-2667 from the $\alpha_1$ to the $\beta_1$-subunit (14). At the time these observations were first reported, they were difficult to interpret. The results reported in this work may complete the puzzle.

TABLE I

Stimulation of different sGC mutants

Maximal stimulation factors (x-fold) and $EC_{50}$ values in nM of WT-sGC and mutant-sGC after incubation with BAY 41-2272 (1 nM to 10 $\mu$M) and BAY 58-2667 (1 nM to 10 $\mu$M) in the absence and presence of DEA/NO (10 nM) or ODQ (10 $\mu$M). Data are mean ± S.E. of 5–16 independent experiments performed in quadruple.

|            | BAY 41-2272 |            | BAY 58-2667 |
|------------|-------------|------------|-------------|
|            | $EC_{50}$   | x-fold     | $EC_{50}$   | x-fold     |
| WT         | 607 ± 59    | 15.9 ± 0.4 | 386 ± 44    | 25.4 ± 0.5 |
| $\beta$H105F | ND          | 2.09 ± 0.08| ND          | 2.44 ± 0.15|
| $\beta$Y135F | ND          | 1.63 ± 0.03| ND          | 3.04 ± 0.10|
| $\beta$Y135A | ND          | ND         | ND          | ND         |
| $\beta$R139L | ND          | 1.75 ± 0.08| ND          | 2.91 ± 0.21|
| $\beta$R139A | ND          | ND         | ND          | ND         |
| $\beta$YR→FL | ND          | ND         | ND          | ND         |
| $\beta$YR→AA | ND          | ND         | ND          | ND         |
| $\beta$YR→FL | ND          | ND         | ND          | ND         |

$^a$ ND, not determined.
$^b$ Extrapolated from concentration response curve.

FIG. 4. UV-visible spectra of 15 $\mu$g of the native or oxidized form of sGC in the absence (A) and presence (B) of BAY 58-2667. sGC was incubated with the indicated compounds and chromatographed before recording the UV-visible spectra. Curves are the mean of three independent records smoothed by the weighted average of the nine nearest neighbors method. C shows the activation profile of purified WT-sGC after incubation with increasing concentrations of BAY 58-2667 in the absence and presence of ODQ (10 $\mu$M). sGC activation is displayed as specific activity (nmol/min/mg). Data are mean ± S.E. from four independent experiments performed in duplicate.

FIG. 5. Proposed model for the interaction of the prosthetic heme moiety and BAY 58-2667 with sGC. The heme of the native sGC complexed via its central iron by histidine 105 as axial ligand and by interaction of tyrosine 135 and arginine 139 with the propionic acid groups is shown in A. Formation of the nitrosyl-heme complex with the breaking of the histidyl-heme bond is displayed in C. sGC activation by displacement of the heme with PPIX or BAY 58-2667 is shown in B and D.
Because a space-filling electronic model of BAY 58-2667 closely resembles that of the heme moiety (Fig. 6), we hypothesize that the second binding site of BAY 58-2667 is in the heme pocket and that the carboxylic groups in BAY 58-2667 interact with tyrosine 135 and arginine 139 in place of the heme propionic groups. To test this hypothesis, sGC was incubated with BAY 58-2667 in the presence and absence of ODQ. Heme that became dissociated from sGC was removed by ion exchange chromatography before UV-visible spectra were recorded. The Soret peak remained unchanged after incubation with BAY 58-2667 (10 𝜇M), however, in the presence of additional ODQ a loss of the heme moiety was observed. Interestingly, a further increase of the concentration of BAY 58-2667 up to 100 𝜇M rendered the enzyme heme deficient even in its ferrous state. Oxidation of the heme by the sGC inhibitor ODQ is known to change the secondary structure of sGC (40, 41) and weaken the binding of the heme moiety (5) as described for other heme proteins such as myoglobin (42–44). Therefore, the ferric heme could be displaced at lower concentrations of BAY 58-2667, whereas for the displacement of the tightly bound ferrous heme higher concentrations of BAY 58-2667 were needed.

These findings are consistent with the biphasic activation curve of BAY 58-2667 on the ferrous form that might be explained by a model of sGC activation with two high affinity binding sites for BAY 58-2667: one that is saturable at nanomolar concentrations and shows no direct interference with the heme moiety, and a second one that exhibits a direct competition between BAY 58-2667 and the prosthetic group for the tyrosine 135 and the arginine 139. Within this model the biphasic activation profile observed for BAY 58-2667 at the native ferrous sGC can be easily explained. At concentrations up to 50 nM BAY 58-2667 the putative heme-independent high affinity binding site may be saturated in agreement with the findings of the receptor binding studies reported earlier (14), whereas the second binding site remains occupied by the heme moiety of sGC. Total displacement of the heme finally happened at high micromolar concentrations in agreement with the spectroscopic studies. In contrast, in the presence of ODQ the weakly bound ferrie heme was displaced immediately by BAY 58-2667 resulting in the observed strong one-step activation of the enzyme. However, until this model is confirmed by cocrystallization studies, it cannot be excluded that the activation of the native heme-containing enzyme contributes to a small percentage of heme-free sGC in the enzyme preparation.

The competition between the heme and BAY 58-2667 together with the observed involvement of the tyrosine 135 and arginine 139 in the sGC activation by this compound implicate the possibility that the heme propionate interaction with these residues may be more than a simple rigid coordination. It might be that this interaction is part of intramolecular signal transmission cascades involved in translating changes of the porphyrin geometry and heme status into structural changes of the protein as observed for other heme proteins (38). This proposed signal transmission triad consisting of histidine 105, tyrosine 135, and arginine 139 may also explain why sGC is activated by PPIX or CO to a lesser extent than by NO, namely due to differences in porphyrin geometry.

**CONCLUSION**

Using the novel cGMP reporter cell and the recently described heme-independent sGC activator BAY 58-2667, we were able to disentangle between heme-free and heme-containing sGC in an intact cellular system. In addition to the established histidine 105, we identified two additional residues, tyrosine 135 and arginine 139, to be crucially important for heme binding to sGC. Moreover, the heme-dependent high affinity binding site of BAY 58-2667 was characterized, exhibiting a direct competition between this compound and the heme moiety of sGC. Based on these findings we postulate a signal transmission triad composed of histidine 105, tyrosine 135, and arginine 139 in the β subunit of sGC, responsible for the BAY 58-2667-induced sGC activation and probably involved in transducing changes in the heme geometry upon NO binding into alterations of the catalytic rate of the enzyme. These results await confirmation via crystallization- and cocrystallization studies with sGC and BAY 58-2667. Meanwhile, the use of different sGC activators, such as BAY 58-2667 and BAY 41-2272, with sequence alignments and mutagenesis studies, seems to be a promising approach to elucidate mechanisms of sGC activation and intramolecular signaling.

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