Structure of Cinaciguat (BAY 58–2667) Bound to Nostoc H-NOX Domain Reveals Insights into Heme-mimetic Activation of the Soluble Guanylyl Cyclase\*\*

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Heme is a vital molecule for all life forms with heme being capable of assisting in catalysis, binding ligands, and undergoing redox changes. Heme-related dysfunction can lead to cardiovascular diseases with the oxidation of the heme of soluble guanylyl cyclase (sGC) critically implicated in some of these cardiovascular diseases. sGC, the main nitric oxide (NO) receptor, stimulates second messenger cGMP production, whereas reactive oxygen species are known to scavenge NO and oxidize/inactivate the heme leading to sGC degradation. This vulnerability of NO-heme signaling to oxidative stress led to the discovery of an NO-independent activator of sGC, cinaciguat (BAY 58–2667), which is a candidate drug in clinical trials to treat acute decompensated heart failure. Here, we present crystallographic and mutagenesis data that reveal the mode of action of BAY 58–2667. The 2.3-Å resolution structure of BAY 58–2667 bound to a heme NO and oxygen binding domain (H-NOX) from Nostoc homologous to that of sGC reveals that the trifurcated BAY 58–2667 molecule has displaced the heme and acts as a heme mimetic. Carboxylate groups of BAY 58–2667 make interactions similar to the heme-propionate groups, whereas its hydrophobic phenyl ring linker folds up within the heme cavity in a planar-like fashion. BAY 58–2667 binding causes a rotation of the εFe helix away from the heme pocket, as this helix is normally held in place via the inhibitory His\textsuperscript{105}–heme covalent bond. The structure provides insights into how BAY 58–2667 binds and activates sGC to rescue heme-NO dysfunction in cardiovascular diseases.

Heme is a key evolutionarily conserved cofactor involved in many processes ranging from signal transduction, gas transport, circadian rhythm, microRNA processing, and drug metabolism (1–3). Heme, as part of NO signaling through sGC,\textsuperscript{2} is critical for regulation of cardiovascular processes via cGMP-dependent pathways, making sGC a prime drug target in cardiovascular diseases (4). sGC is a heterodimeric protein, mostly in the α1β1 isoform, that stimulates the production of cGMP upon NO activation (5). Under normal physiological conditions, NO-activated sGC stimulates vasodilation and is required for platelet disaggregation as well as neurotransmission (6). During the pathophysiological conditions of oxidative stress common to vascular disease, the NO-sGC signaling pathway is disrupted due to sGC desensitization and degradation of sGC upon heme loss, thus limiting the extent to which traditional treatment methods such as nitroglycerin can increase sGC activity (7). BAY 58–2667 can overcome these defects by activating sGC and protecting heme-oxidized sGC from proteasomal degradation (4, 8). These beneficial characteristics contributed to the pharmaceutical potential of BAY 58–2667, and this compound is in clinical trials to treat acute decompensated heart failure (9–11). The initial steps of BAY 58–2667 activation of sGC are fundamentally distinct from activation by NO (Fig. 1). NO binds a 5-coordinated His\textsuperscript{105}–liganded heme, forming a brief 6-coordinated intermediate and subsequently causing breakage of the Fe–His\textsuperscript{105} bond to yield a 5-coordinated NO-bound heme in the activated state (Fig. 1B). In contrast, BAY 58–2667 activation of sGC is heme-independent yet takes place in the NO sensory H-NOX domain of sGC when it is heme-depleted (Fig. 1C) (12). This crucial H-NOX domain is the N-terminal domain of the sGCB1 subunit, which is adjacent to a Per-Arnt-Sim (PAS-like) H-NOXA domain, followed by a coiled-coil domain, and the C-terminal catalytic guanylyl cyclase domain. The sGCA1 subunit has a similar domain arrangement, except that its N-terminal domain does not bind heme. sGC has not been amendable to crystallization, and structural studies of sGC have therefore been limited to individual domains of homologous proteins (13–16). The H-NOX

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2 The abbreviations used are: sGC, soluble guanylyl cyclase; WT, wild-type; Ns, Nostoc sp.
domain used in these studies that is most homologous to the H-NOX domain of sGC is *Nostoc* sp. H-NOX (Ns H-NOX), which shares 35% sequence identity with sGC, having similar properties including its ability to bind CO and NO (supplemental Fig. 1) (13). We present here the 2.3-Å crystal structure of the Ns H-NOX/H18528 BAY 58–2667 complex and mutational results revealing insights into the molecular mechanisms of sGC activation by a heme mimetic.

**EXPERIMENTAL PROCEDURES**

**Preparation of the BAY 58–2667–H-NOX Complex**—We expressed and purified a C-terminally truncated version of *Nostoc* sp. H-NOX comprising residues 1–183 similarly as described previously for the full-length 1–187 protein (13). The heme was replaced by BAY 58–2667 by adding a 10-fold molar excess of the heme oxidizer NS-2028 (Alexis Biochemicals) and 5-fold molar excess of BAY 58–2667 (obtained from Dr J. P. Stasch, Bayer Schering Pharma AG) at 37 °C prior to Superdex 75 chromatography to remove the displaced heme and unbound BAY 58–2667.

**Crystallization and Structure Determination**—Colorless crystals of the Ns HNOX domain bound to BAY 58–2667 were obtained using sitting drop crystallization at room temperature with a protein concentration of ~10 mg/ml and a well solution of 1.8 M sodium malonate at pH 7.3. Crystals were cryoprotected in 3.0 M sodium malonate, pH 7.3, prior to dunking the crystal in liquid nitrogen. Data were collected at the Stanford Synchrotron Radiation Lightsource beamline 11-1 to 2.3-Å resolution and processed using HKL2000 (17). Crystals of BAY 58–2667 bound HNOX were in the same space group as for the heme-bound protein with two molecules in the asymmetric unit. Twinning analysis revealed a twinning fraction of close to 0.5 (18), which was refined in REFMAC (19) using the amplitude-based twin refinement with the H-NOX coordinates, without the heme, as the starting model (Protein Data Bank code 2O09; 13). The structure was subsequently refined using alternating cycles of fitting using COOT (20) and REFMAC. Heme density was absent, yet strong density for the two copies of BAY 58–2667 was present after the initial refinement. Subsequently, two BAY 58–2667 molecules were added in refinement using a stereochemistry library file that was generated with PRODRG (21). The structure was refined to a final R-factor of 0.150 with an R_free of 0.193 and validated using PROCHECK (22). Data collection and refinement statistics are listed in Table 1. Figures are generated using PyMOL.

**Site-directed Mutagenesis**—cDNAs encoding the α1 and β1 subunits of rat guanylyl cyclase, cloned into the mammalian expression vector pCMV5, served as the templates for site-directed mutagenesis (QuikChange, Stratagene) to generate sGC α1 R40A, β1 I111A, and β1 R116A mutations.

**Expression in COS-7 Cells of WT and Mutant sGC**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin (100 units/ml). Cells were transfected with SuperFect™ reagent using the protocol of the supplier (Qiagen). Cells (100-mm dish) were transfected with 10 μg of plasmid encoding each wild-type or mutated subunit. After 40 h, cells were washed twice with 5 ml of phosphate-buffered saline.
Cytosolic Preparation—After washes, cells are scraped off the plate in cold lysis buffer: phosphate-buffered saline buffer contained protease inhibitors, 50 mM HEPES (pH 8.0), 1 mM EDTA, and 150 mM NaCl. Cells were broken by sonication (three pulses of 3 s). The resulting lysate was centrifuged at 16,000 \( \times \) g for 10 min at 4 °C to collect the cytosol.

sGC Activity Assay—sGC activity was determined by formation of \([\alpha-^{32}P]\)cGMP from \([\alpha-^{32}P]\)GTP, as described previously (23). Reactions were performed for 5 min at 33 °C in a final volume of 100 \( \mu \)l, in a 50 mM HEPES, pH 8.0, reaction buffer containing 500 \( \mu \)M GTP, 1 mM dithiothreitol, and 5 mM MgCl₂. Typically, 40 \( \mu \)g of cell cytosol was used in each assay reaction. Enzymatic activity was stimulated with the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) (Calbiochem) at 100 \( \mu \)M. sGC activity is expressed in pmol/min mg.

RESULTS

To obtain the BAY 58–2667–Ns H-NOX complex, the heme of Ns H-NOX was first oxidized with the sGC-specific heme oxidizer NS-2028 (4\(H\)-8-bromo-1,2,4-oxadiazolo(3,4-d) benz(b)(1,4)oxazin-1-one); the heme was replaced by adding excess of BAY 58–2667 (cinaciguat; 4-((4-carboxybutyl)(2-((4-phenethylbenzol)oxy) phenethyl) amino)methyl(benzoic) acid). Subsequent crystallization and structure determination yielded the 2.3-Å resolution crystal structure of Ns H-NOX bound to BAY 58–2667 with two copies in the asymmetric unit (Table 1 and Fig. 2).

Electron difference density in the heme-binding pockets of each copy clearly revealed the trifurcated BAY 58–2667 ligand density (Fig. 2B). The two carboxylate groups of BAY 58–2667 make extensive interactions: the carboxy-butyl moiety interacts with the side chains of Tyr\(^{134}\), Ser\(^{136}\), and Arg\(^{138}\), whereas the carboxylate moiety of the benzoic acid interacts with Arg\(^{138}\) and the main chain nitrogen of Tyr\(^{2}\) (Fig. 2C). Both copies of BAY 58–2667 in the asymmetric unit have a similar binding mode, although one of the carboxylates is slightly rotated (supplemental Fig. 2). Electron density for the third moiety of BAY 58–2667 extending from the tertiary amine, the extended tri-benzylring containing moiety, is well defined except for the terminal benzyl ring (Fig. 2B); the orientations for this terminal benzyl ring also vary slightly between the two crystallographic copies (supplemental Fig. 2). This hydrophobic third branch of BAY 58–2667 makes extensive hydro-

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**TABLE 1**

Data collection and refinement statistics for Ns H-NOX complexed with BAY 58–2667

Values in parentheses are for the highest resolution shell.

| Data collection | Value |
|-----------------|-------|
| Wavelength      | 0.97945 |
| Space group     | P2\(_1\)3 |
| Cell dimensions (Å) | 123.27, 123.27, 123.27, 90, 90, 90 |
| Resolution (Å)  | 50–2.3 (2.36–2.30) |
| Total observations | 177,281 |
| Unique observations | 27,835 |
| \(I/\sigma I\)    | 25.0 (1.9) |
| Redundancy      | 6.4 (4.6) |
| Completeness (%)| 99.5 (97.0) |
| \(R_{\text{msd}}\) (%) | 5.8 (7.5) |

| Refinement | Value |
|------------|-------|
| Resolution (Å) | 50–2.3 |
| No. of protein atoms | 2866 |
| No. of solvent | 82 |
| No. of ligand atoms | 84 |
| R-factor (%) | 15.0 |
| \(R_{\text{free}}\) (%) | 19.3 |
| r.m.s.d. | 0.008 |
| For bond lengths (Å) | 1.13' |
| For bond angles | |

Ramachandran plot statistics (%)

- Residues in most favored regions | 94 |
- Residues in additional allowed regions | 5.1 |
- Residues in generously allowed regions | 0 |
- Residues in disallowed regions | 0 |

Average B-factors (Å\(^2\))

- Protein | 54.3 |
- Ligand | 57.8 |
- Solvent | 53.9 |

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**FIGURE 2. Structure of BAY 58–2667 bound to H-NOX.**

A. Ns H-NOX (gold ribbon diagram) bound to BAY 58–2667 (blue stick structure). Also shown is the side chain of His\(^{105}\) (stick) that is part of the \(\alpha\) helix. B. electron density of BAY 58–2667. Unbiased 2.3-Å resolution \(|F_o| - |F_c|\) omit density (green, contoured at 3σ) and the final refined \(2|F_o| - |F_c|\) density (dark gray, contoured at 1.25σ) are shown. C. stereo figure showing interactions of BAY 58–2667 with nearby H-NOX residues. Hydrogen bonds are shown as dashed lines.
phobic interactions with residues forming the rest of the heme cavity including Leu4, Trp74, Thr78, Lys83, Phe97, Leu101, Leu104, Val108, Leu148, and Leu152 (Fig. 2). Finally, the trifurcated tertiary amine is 4.1 Å from Trp71, apparently forming a cation/pi interaction of the positively charged amine and the pi clouds of the tryptophan. Despite having a shape dissimilar to heme, the trifurcated BAY 58–2667 folds up and occupies the heme cavity with its carboxylate groups superimposing on those of the heme propionate groups in the apo-Ns H-NOX structure (Fig. 3) published previously (13). BAY 58–2667, therefore, mimics heme in that it contains two hydrophilic charged carboxylates that interact with the YxSxR motif found to be crucial for heme and BAY 58–2667 binding (12, 24), yet, on the other hand, it is mostly hydrophobic and can “fold” in a somewhat planar heme-like fashion (Fig. 3C). A key functional difference between the two molecules is that BAY 58–2667 is a sGC activator, whereas heme is an inhibitor (that normally is broken via NO binding). The Ns H-NOX structure with BAY 58–2667 in the activated state reveals a 3° rotation of the αF helix, normally tethered by the heme via His105, as analyzed by the program ESCET (Fig. 4 and supplemental movie) (25). This rotation culminates in a ~1 Å shift of the carboxyl terminus of αF comprising residues 110–111 (see supplemental movie). NO activation of sGC would likely induce a similar conformational change of αF as NO–heme binding causes the breakage of the αF–His105–Fe bond (26) and would allow this helix to shift to alleviate the steric clashes between the heme and His105 due to van der Waals repulsion interactions. This conformational change distorts the surface region comprising residues 110–116 and nearby flanking residues 40–46 (Fig. 4, A and B) and highlights a region that is likely to be used by H-NOX to transmit its activation signal to the rest of sGC upon NO or BAY 58–2667 activation. We therefore probed the importance of this region for sGC activation via alanine-scanning mutagenesis of surface-exposed H-NOX residues Arg40, Ile111, and Arg116. WT and mutant versions of sGC were expressed in COS-7 cells, and their guanylyl cyclase activity was assayed (Fig. 5B), revealing that these residues affect NO-stimulated activity. Mutation of residues Ile111 and Arg116 have an ~4- and 3-fold, respectively, effect on max-

**FIGURE 3.** BAY 58–2667 binds to H-NOX heme cavity and mimics heme/protein interactions. A, binding of BAY 58–2667 (cyan sticks and transparent spheres) to heme cavity. B, stereo figure showing superposition of BAY 58–2667 (cyan) and heme (green) as bound to H-NOX structures. (Only the protein for the BAY 58–2667 complex structure is depicted for clarity.) Hydrogen bond interactions by the carboxylates of BAY 58–2667 with the YxSxR motif residues are shown as dashed lines. C, structural comparison of BAY 58–2667 and heme (in their conformation when bound to H-NOX).
imal sGC activation, whereas mutation of residue Arg^40 has the largest effect on NO-stimulated and basal sGC activity (Fig. 5B).

These results suggest that sGC residues Arg^40, Ile^111, and Arg^116 play a role in communicating H-NOX conformational changes to the rest of sGC and that there are likely some similarities between BAY 58–2667 and NO activation of sGC.

DISCUSSION

Our results regarding the 2.3-Å resolution structure of BAY 58–2667 bound to Ns H-NOX reveals the molecular details of the remarkable ability of BAY 58–2667 to activate heme-depleted sGC. By being able to mimic the heme yet avoid forming a covalent inhibitory bond with His^105, BAY 58–2667 is able to bind to the heme pocket and yet allow the conformational activation change involving helix αF and its His^105 residue. The largest shifts of the BAY 58–2667-bound Ns H-NOX structure compared with the heme-bound structure are in the C-terminal part of helix αF (Fig. 4). We postulate that this region, near residue 111, and the flanking region comprising residues 40–46 are therefore likely involved in communicating the activation conformational changes of the H-NOX domain to the rest of sGC. The importance of this region for sGC activation was probed by mutagenesis and indicated that the B1 H-NOX mutations R40A, I111A, and R116A negatively affect NO-stimulated cyclase activity (Fig. 5). Because these residues are mostly solvent exposed and not involved in direct heme binding or domain folding, these residues are likely involved in direct H-NOX/GC interactions (such a direct H-NOX/GC interdomain interaction had been previously observed (27)). In earlier work, residue Asp^45 also was found to be important in sGC activation (28); this residue also falls within this surface region providing additional support for the proposed activation relevance of this region near residues 110–116 and 40–46. Future structural studies are needed to indeed confirm such a direct interaction between these sGC domain and also what the differences and similarities of the NO-bound 5-coordinated sGC structure is compared with BAY 58–2667.

Structure-Activity Relationship of BAY 58–2667—BAY 58–2667 is the result of substantial structure-activity relationship optimization (9), and its critical features agree well with its H-NOX binding mode and induced conformational activation
why the butyl-carboxylate (Fig. 6, 5a) is more important compared with the methyl-benzoic acid (Fig. 6, 5b) because replacing the latter with a hydrogen leads to only a 63-fold IC$_{50}$ increase, whereas replacing the former leads to a drastic 4800-fold increase (9). The BAY 58–2667 H-NOX structure shows that the butyl-carboxylate makes key interactions with all three of the pivotal YxSxR motif residues known to be crucial for BAY 58–2667 activation (24, 28) holding this H-NOX region in place, whereas the benzoic acid carboxylate only interacts with Arg$_{138}$ and the backbone nitrogen of Tyr$_2$ (Fig. 2C). The dominance and importance of one of the carboxylates explains the potency of recently developed sGC activators with only one carboxylate, as described in a recent U.S. patent application (29). In summary, this analysis shows that two of the most critical features of BAY 58–2667 are its butyl-carboxylate moiety, which interacts with the YxSxR motif, and a long hydrophobic tail that can bend and occupy the mostly hydrophobic heme pocket. This analysis suggests that our BAY 58–2667 complex structure is a great tool for analyzing current analogs and likely also future analogs of BAY 58–2667.

In summary, the Ns H-NOX protein continues to be a great tool for understanding sGC. In addition to being the closest bacterial homolog to the H-NOX domain of sGC in terms of sequence and ligand binding (13), we show here that Ns H-NOX is, like sGC, capable of having its heme displaced by the sGC activator BAY 58–2667 and that the observed binding mode of BAY 58–2667 strongly correlates with the published sGC structure activity relationships (SAR) data on BAY 58–2667 analogs (Fig. 6). In addition, the BAY 58–2667 binding mode is in agreement and structurally explains previous mutagenesis results that pointed to the strong role for the YxSxR motif present in both sGC and Ns H-NOX (12, 24). And finally, the BAY 58–2667 induced shift of the His$_{105}$-containing αF helix and adjacent loop point to the importance of the surface patch comprised of regions 110–116 (and flanking residues 40–46), which we subsequently probed with sGC mutagenesis experiments (Fig. 5). These combined results point to the importance of this surface region for sGC activation by BAY 58–2667, which possibly shares some mechanistic similarities with NO activation of sGC. These cohesive results therefore aid in our understanding of sGC activation by a heme-mimetic and how BAY 58–2667 protects sGC from degradation by stabilizing the heme cavity and offer new structure-based design strategies to pharmacologically further target the sGC protein to treat certain cardiovascular diseases.

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