PAS-mediated Dimerization of Soluble Guanylyl Cyclase Revealed by Signal Transduction Histidine Kinase Domain Crystal Structure*

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Signal transduction histidine kinases (STHK) are key for sensing environmental stresses, crucial for cell survival, and attain their sensing ability using small molecule binding domains. The N-terminal domain in an STHK from Nostoc punctiforme is of unknown function yet is homologous to the central region in soluble guanylyl cyclase (sGC), the main receptor for nitric oxide (NO). This domain is termed H-NOXA (or H-NOBA) because it is often associated with the heme-nitric oxide/oxygen binding (H-NOX) domain. A structure-function approach was taken to investigate the role of H-NOXA in STHK and sGC. We report the 2.1 Å resolution crystal structure of the dimerized H-NOXA domain of STHK, which reveals a Per-Arnt-Sim (PAS) fold. The H-NOXA monomers dimerize in a parallel arrangement juxtaposing their N-terminal helices and preceding residues. Such PAS dimerization is similar to that previously observed for EcDOS, AvNiFL, and RmFixL. Deletion of 7 N-terminal residues affected dimer organization. Alanine scanning mutagenesis in sGC indicates that the H-NOXA domains of sGC could adopt a similar dimer organization. Although most putative interface mutations did decrease sGCβ1 H-NOXA homodimerization, heterodimerization of full-length heterodimeric sGC was mostly unaffected, likely due to the additional dimerization contacts of sGC in the coiled-coil and catalytic domains. Exceptions are mutations sGCα1 F285A and sGCβ1 F217A, which each caused a drastic drop in NO stimulated activity, and mutations sGCα1 Q368A and sGCβ1 Q309A, which resulted in both a complete lack of activity and heterodimerization. Our structural and mutational results provide new insights into sGC and STHK dimerization and overall architecture.

The ability to sense small molecules is key for every life form and provides information about the extracellular milieu, monitors intracellular physiological status, or establishes cell-cell communication. Sensory signaling proteins are often modular in nature with distinct domains for ligand sensing and for output signals. A number of these domains are conserved in bacteria and animals. A striking example is the heme-nitric oxide/oxygen-binding (H-NOX) domain that can be a stand-alone protein in Nostoc cyanobacteria, or can be part of a multidomain protein such as in the mammalian soluble guanylyl cyclase (sGC) (1, 2) (Fig. 1A). An additional evolutionary relationship was detected between sGC and 2 other cyanobacterial signaling proteins (1, 2): the H-NOX associated H-NOXA (or H-NOBA) domain in sGC is also present at the N terminus of a cyanobacterial signal transduction histidine kinase (STHK) and 2-component hybrid sensor and regulator (2-CHSR) (Fig. 1) postulated to have a PAS-like fold (1). Both genes of these cyanobacterial proteins are adjacent to genes coding for stand-alone H-NOX domains (Fig. 1A) suggesting they might work in concert. This H-NOXA evolutionary link adds to the already complex and poorly understood regulation of sGC stimulating the need to study this ancient domain.

Upon NO activation, sGC increases its production of the second messenger cGMP (3–5) leading to vasodilatation, platelet aggregation, and induction of host defense mechanisms (6, 7). sGC is therefore a potential drug target for treating hypertension and erectile dysfunction (8). sGC is comprised of an α and β subunit with the α1/β1 isoform being the most ubiquitous (3). The H-NOXA domain is a central subdomain in both subunits: in sGCβ1, it is flanked by the N-terminal H-NOX domain and C-terminal predicted coiled-coil (CC) (9, 10) and catalytic guanylyl cyclase (GC) domain (Fig. 1A). sGCα1 has a similar subunit arrangement except that its N-terminal domain does not bind heme. As in sGCα1 and sGCβ1, the H-NOXA domains in STHK and 2-CHSR are followed by a CC domain further strengthening their evolutionary connection (1) (Fig. 1A).

Structural information on sGC is limited and comes mostly from the structures of the homologous adenyl cyclase catalytic domain (11–13) and bacterial H-NOX domains (14–16). Even less is structurally known about the H-NOXA/CC region

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The atomic coordinates and structure factors (code 2P04 and 2P08) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: H-NOXA, heme nitric oxide and oxygen binding associated; PAS, an acronym formed from three proteins: Per, Arnt, Sim; STHK, signal transduction histidine kinase; 2-CHSR, two-component hybrid sensor and regulator; sGC, soluble guanylyl cyclase; NO, nitric oxide; CC, coiled coil; SAD, single wavelength anomalous dispersion; r.m.s., root mean square.
except that it was found to contain two distinct regions key for dimerization: one comprises H-NOXA β1 residues 204–244, and the second contains CC residues 379–408 with the intervening sequences postulated to contribute to a functional binding region (17). Analogous regions in sGCα1 have also been shown to be important for sGC activity via deletion studies (9). These regions in sGCα1 and sGCβ1 have recently been further narrowed down (18). The α1 and β1 H-NOXA domains are 38% sequence identical and also share ~35% sequence identity with the H-NOXA domains in the STHK from *Nostoc punctiforme* PCC 73102 (Fig. 1B). Unlike the sGC subunits, the H-NOXA domain of the STHK was amenable to crystallographic analysis. We describe here the 2.1-Å crystal structure of the dimerized H-NOXA domain of NpSTHK revealing a PAS fold, a fold commonly found in sensory signaling proteins. We carried out additional structure-function studies in sGC and find that the H-NOXA subdomains of sGC could likely form a similarly arranged PAS-type heterodimer with an important role for functional sGC heterodimerization by the H-NOXA domains.

**EXPERIMENTAL PROCEDURES**

**Cloning of the H-NOXA Domain of NpSTHK**—The genomic DNA of *N. punctiforme* PCC 73102 was used as the template to PCR the 1–121 H-NOXA fragment of STHK *Npuntu*020000820 using the forward primer, 5′-ggaattcatgatctctccctcacttc-3′ and reverse primer, 5′-ccggaattctatgggtcctcctcacttc-3′. To obtain the 8–121 (Δ7) H-NOXA construct, the 5′-ggaattcatgatctctccctcacttc-3′ forward primer was used. The PCR-amplified fragments were inserted into a pET28a (Novagen) expression vector.

**Expression and Purification of NpSTHK H-NOXA Domains**—Both the 1–121 and truncated Δ7 H-NOXA domains were expressed as N-terminal His-tagged protein in *Escherichia coli* BL21(DE3) Star cells (Invitrogen) using isopropyl 1-thio-β-D-galactopyranoside for induction. The cells were pelleted and sonicated for 5 min on ice in the following buffer: 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol. The cell lysate was centrifuged at 16,000 × g for 10 min at 4 °C and the supernatant was incubated with nickel-nitrilotriacetic acid (Qiagen) beads. The beads were washed with the washing buffer: 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole. The protein was released from the beads by thrombin (Enzyme Research labs) digestion. Further purification was performed by gel filtration in a Sephadex-75 (GE Healthcare) column equilibrated with 5 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM β-mercaptoethanol.

**Subcloning, Expression, and Purification of the H-NOXA Domain of sGCβ1**—Residues 202–344 of the H-NOXA domain of rat sGCβ1 were subcloned into pET22b using the forward primer, 5′-ggatactggacgctcagctc-3′, and reverse primer, 5′-ggatactggacgctcagctc-3′. The C-terminal His-tagged sGCβ1 H-NOXA protein was expressed and purified similarly to the homologous NpSTHK counterpart except that the protein was eluted from the nickel-nitrilotriacetic acid beads by an imidazole gradient instead of thrombin digestion. The Ala-scanning mutants of the sGCβ1 H-NOXA domain were introduced into pET22b plasmid using the QuikChange site-directed mutagenesis kit (QuikChange, Stratagene) to confirm the sequence.

**Mutagenesis of Rat sGC and Transfection in COS-7 Cells**—Templates were cDNAs encoding the α1 and β1 subunits of rat sGC cloned into the mammalian expression vector pCMV5 (19). Mutations described in the text were introduced by PCR (QuikChange, Stratagene) and sequenced. 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 for 48 h with Superfect reagent using the protocol of the supplier (Qiagen).

**Cytosol Preparation and Western Blot Analysis**—COS-7 cells were washed twice with ice-cold phosphate-buffered saline and then scraped off the plate in cold lysis buffer: phosphate-buffered saline containing protease inhibitors, 50 mM HEPES, pH 8.0, 1 mM EDTA, and 150 mM NaCl. Cells were broken by sonication (3 pulses of 3 s) and centrifuged at 16,000 × g for 10 min at 4 °C to collect the soluble fraction (referred to as cytosol in the text). To determine the efficiency of transfection for wt sGC or mutants in COS-7 cells, 15 μg of cytosol was resolved on 10% SDS-PAGE and analyzed by immunoblotting with anti-sGC (anti-α1 subunit and anti-β1 subunit; Cayman Chemicals).

**sGC Activity Assay**—GC activity was determined by formation of [α-32P]cGMP from [α-32P]GTP, as previously described (20). Reactions were performed for 5 min at 33 °C in a final volume of 100 μl, in a 50 mM HEPES pH 8.0 reaction buffer containing 500 μM GTP, 1 mM dithiothreitol, and 5 mM MgCl2. Typically, 40 μg of COS-7 cytosol transfected with either wt or mutants were used in each assay reaction. All assays were done in duplicate and each experiment repeated twice. Enzymatic activity was stimulated with the NO-donor SNAP (Calbiochem) at 100 μM. sGC activity is expressed in pmol min⁻¹ μg⁻¹ and mean ± S.E.

**NpSTHK Crystallization**—Crystals of the NpSTHK-(8–121) protein construct were grown at 4 °C by sitting-drop vapor diffusion. Protein was concentrated to 20 mg/ml in 5 mM Tris, pH 7.5, 100 mM NaCl, 1 mM β-mercaptoethanol and was mixed with an equal volume of the reservoir solution: 1.7–1.9 M ammonium sulfate, 100 mM Tris-HCl, pH 7.7–9.0. Crystals were prepared for data collection by fast transfer into cryoprotectant solution containing 2.0 M ammonium sulfate, 100 mM Tris-HCl with 15% glycerol. The 1–121 NpSTHK protein was concentrated to 15 mg/ml after the gel-filtration step and mixed with an equal volume of 0.1 M HEPES, pH 7.5, and 1.5 M lithium sulfate monohydrate. Crystals appeared after 3 days at 20 °C. For data collection, the crystals were soaked in cryoprotectant containing 25% glycerol in addition to the crystallization solution and dunked into liquid nitrogen prior to data collection. Crystals of selenomethionine substituted 8–121 protein were used for single wavelength anomalous dispersion (SAD) phasing.

**NpSTHK Structure Determination and Refinement**—Due to great difficulty in obtaining diffraction quality crystals for the 1–121 NpSTHK construct, the initial structure determination
TABLE 1
Data collection, phasing, and refinement statistics for NpSTHK
Values for the highest resolution shell are listed in parentheses.

| Data collection | Native (8–121) | Se-peak (8–121) | Native (1–121) |
|-----------------|----------------|-----------------|----------------|
| Wavelength (Å)  | 1.2398         | 0.9790          | 0.97934        |
| Space group     | P6,22          | P6,22           | C2             |
| Cell dimension (Å) | a = b = 72.33 | c = 169.08     | a = b = 72.26  |
|                 | a = 95.21      | b = 44.36       | a = 95.21      |
|                 | c = 129.21     |                | c = 154.61     |
| Resolution (Å)  | 2.0            | 2.6             | 2.1            |
| Total observations | 104640         | 150704         | 213337         |
| Uniqueness      | 18435          | 8621            | 12364          |
| I/σ             | 12.2 (2.5)     | 16.2 (6.9)      | 10.0 (1.6)     |
| Redundancy      | 5.68 (4.05)    | 17.48 (19.27)   | 3.4 (1.9)      |
| Completeness (%)| 99.8 (99.2)    | 100.0 (100.0)   | 87.4 (59.8)    |
| Rfree (%)       | 8.0 (36.9)     | 9.3 (38.3)      | 11.9 (45.6)    |

SAD phasing
No. of sites: 6
Figure of merit: 0.63

Refinement
Resolution (Å): 2.0–42.3, 2.1–27.8
No. of protein atoms: 1773
No. of waters: 111
Sulfate ions: 0
Rmerge (%) (Np): 23.9, 19.9
Rfree (%) (Np): 27.8, 26.2
R.m.s. deviation for bond lengths (Å): 0.012, 0.011
R.m.s. deviation for bond angles (%): 1.37, 1.35
Ramachandran plot statistics
Residues in Most favored regions: 88.7
Additional allowed regions: 11.3
Generously allowed regions: 0
Disallowed regions: 0

was carried out using crystals of the shorter 8–121 construct. A native 2.0-Å resolution dataset for the 8–121 NpSTHK protein was collected at ALS (Beamline 4.2.2) and processed with D*Trek (21). The crystals belong to space group P6,22, with cell dimensions a = b = 72.3 Å, c = 169.1 Å and two molecules in the asymmetric unit. To obtain crystallographic phase information, a SeMet crystal of the 8–121 NpSTHK construct was used for SAD phasing. A 2.6-Å SAD data set was collected at the selenium peak wavelength at ALS. SOLVE/RESOLVE (22) was used for phasing and automatic model building. Manual model building was carried out in Coot (23) and REFMAC (24) for refinement. The final model includes NpSTHK residues 8–113 of molecule A and residues 10–119 of molecule B and 95 waters and 2 sulfate ions. The final model yielded an R/R_free of 23.9/27.8%.

For 1–121 H-NOXA, a native 2.1-Å resolution data set was collected at the Advanced Photon Source (Beamline 19ID) belonging to space group C2 with cell dimensions a = 95.2 Å, b = 44.4 Å, c = 59.7 Å and 2 molecules in the asymmetric unit. The structure of this larger construct was determined via Molecular Replacement using PHASER (25) using one of the truncated monomer structures from the P6,22 space group. Coot and REFMAC were used for model building and crystallographic refinement yielding a final model includes residues 1–107 of molecule A, 1–105 of molecule B, and 55 waters (R/R_free are 19.9/26.2%). The stereochemistry was checked using PROCHECK (26) (Table 1). Figures were generated using MOLSCRIPT and Raster3D (27, 28) and Pymol (pymol.sourceforge.net/).

RESULTS

NpSTHK Structure Reveals a PAS Fold—The structure of the H-NOXA domain of NpSTHK was solved via SAD of first a smaller Δ7 N-terminal-truncated fragment, followed by molecular replacement to solve the full-length NpSTHK H-NOXA domain structure (Table 1 and Fig. 1C). Two independent molecules in the asymmetric unit form a dimer. The final refined model of the NpSTHK dimer contains residues 1–107 of molecule A and 1–105 of molecule B.

The NpSTHK monomer structure is comprised of a 7-stranded anti-parallel β-barrel flanked by several α-helices (Fig. 1C). The structure of the NpSTHK monomer is similar to that of the PAS fold: its most similar structural neighbors are two heme containing sensors byFixL and EcDos (Protein Data Bank codes 1DRM and 1V9Z), followed by dPER (1W9A), HIF-2α (1P97), and photosynthetic yellow protein (3PYP) with DALI Z-scores (29) ranging from 8.3 to 7.6, respectively. A superposition of the NpSTHK and EcDos PAS domain (30) (Fig. 1D) details their close structural similarity (r.m.s. deviation of 2.2 Å for 70 Ca atoms). Their structure-based sequence alignment (Fig. 1B) reveals only 10% sequence identity. The canonical PAS fold contains 5 β-strands (31) but both NpSTHK and EcDos contain an additional short βC strand; NpSTHK contains even an additional 7th (βD) strand (Fig. 1D).

The region between the two N-terminal strands (βA/βB) and three C-terminal strands (βE/βF/βG) usually harbors 4 helices in PAS domains (31). Three of those helices are present in NpSTHK (Fig. 1B) although helix αD is in a somewhat different orientation (Fig. 1D). This multihelix containing region between βB and βE is structurally the most variable part of PAS domains, and can have as few as 2 helices such as Cita (32), possibly dictated by whether or not the PAS domain binds a ligand and if so, the type of ligand (31, 33) (Fig. 1D). The similarity between NpSTHK and EcDos is thus remarkable despite little sequence identity and extends beyond the PAS fold because they both contain an additional N-terminal helix, αA (Fig. 1D).

A structure-based sequence analysis of the homologous sGC subunits using the NpSTHK H-NOXA domain structure was used to probe sequence conservation and sites of insertions and deletions (Fig. 1B). Both sGC subunits have a large insertion between the βE and βF strands in their H-NOXA domain: a 9-residue insertion for α1 and 19 for β1. In addition, there are some smaller 1 residue insertions and deletions around the βC strand (Fig. 1B). To test whether the sGC subunit sequences are compatible with the PAS-fold of NpSTHK, MODELLER (34) was used to build homology models for the α1 and β1 H-NOXA domains using the alignment in Fig. 1B. The resulting models yielded no negative VERIFY3D structure validation (35) scores (or even scores with values lower than 0.1) indicating that both subunits can adopt the PAS fold of NpSTHK (the 19-residue insertion in β1 was omitted from the modeling due to its size and lack of template).

NpSTHK Dimer Organization—The two dimerized NpSTHK molecules make extensive interactions burying 2,395
Å² of surface (67% hydrophobic; surface calculations done using MSCON (36)) (Fig. 2, A–D). Dimer interface analysis programs ranked the significance of this interface very high yielding a maximal complexation significance score of 1.0 and a PITA score of 90 (37, 38). Truncated Δ7 NpSTHK forms a flipped dimer (Fig. 2E), buries less surface (1,888 Å²) with many water-mediated interactions, and is likely non-physiologically as indicative of the lower complexation significance score and PITA dimer evaluation scores of 0.44 and 47.1, respectively, the latter being below the threshold value of 67 (38). The two NpSTHK domains comprising the full-length dimer are related by an approximate 2-fold non-crys-
tallographic axis (178.5°) and are very similar in conformation (r.m.s. deviation of 0.45 Å for 105 Ca atoms). Dimerization involves juxtapositioning of the N-terminal helices and preceding residues and the face of the β-sheet (Fig. 2, A–D). The dimer interface contains a central cluster of hydrophobic residues (including Leu⁹⁹, Leu¹⁰³, Phe¹⁰⁷, Phe¹⁰⁹, and Leu¹⁰²) and hydrogen bonds (involving side chains of residues Thr⁷, Ser⁹, Glu⁹¹, Glu⁸⁹, and Gln⁹³ and main chain atoms of several other residues) (Fig. 2, B and C).

The resemblance of NpSTHK with EcDOS extends beyond the monomer fold because their dimer organizations are also similar involving N-terminal helix juxtapositioning (Fig. 3). The dimeric NpSTHK and EcDOS structures can be superimposed with r.m.s. deviations of 3.5 Å (for 2 × 70 Ca atoms), which is higher than the r.m.s. deviations of 2.2 Å for the monomer superposition yet low enough to indicate a similar dimer arrangement. Their similar dimer organization is attained because the hydrophobic nature of several residues at the core of the dimer interface is conserved (Leu¹³, Phe¹⁰⁰, and Leu¹⁰² in NpSTHK correspond to Leu²⁶, Leu¹²⁷, and Leu¹²⁹, respectively, in EcDOS; see Fig. 1B). The same fold and dimer organization are also present in another heme containing oxygen sensor, RmFixL (39), also an STHK protein (Fig. 3). Furthermore, such PAS dimerization was also recently observed in AνNifL (40), which led to the suggestion that this is a conserved dimerization motif for a subset of PAS domains (40).

Evidence for a NpSTHK-type Dimer for the H-NOXA Domains of sGC—The N-terminal region of the H-NOXA domain of NpSTHK provides most of the dimer contacts, which is in agreement with the corresponding sGCβ1 residues 204–244 found to be important for sGC dimerization (Fig. 1B) (17). Furthermore, the dimer interface region is largely conserved in sGCα1 and sGCβ1 (Fig. 4A), suggestive of a similar dimerization function in sGC. The hydrophobic nature of all 5 residues comprising the central cluster of the dimerization interface (Leu⁶, Leu¹³, Phe¹⁰⁷, Phe¹⁰⁹, and Leu¹⁰²) is conserved as well suggesting that sGCα1 and sGCβ1 H-NOXA domains could similarly heterodimerize, or homodimerize as observed in sGCα1 (Fig. 4A). To test whether a heterodimeric sGC indeed could adopt a similar NpSTHK dimer arrangement, a homology model was constructed for the αβ1 sGC H-NOXA heterodimer using MODELER, which resulted in similar (non-negative) VERIFY3D scores as was observed for the individual monomers. The modeled sGC heterodimer structure was additionally validated by carrying out dimer analysis calculations using PISA resulting in a ΔG for dimer formation of −16.9 kcal/mol and 2,156 Å² of buried surface (α2β1 yielded similar results). These values are very similar to that calculated for the experimentally determined NpSTHK dimer itself, whereas a MODELLER generated αβ1 heterodimer homology model based on the likely non-physiological ΔG NpSTHK dimer yielded only a ΔG of −7.9 kcal/mol (1,742 Å² of buried surface). These automated modeling and analysis results suggest that the sGC H-NOXA domain sequences are indeed compatible with formation of a NpSTHK-type heterodimer.

To further validate the presence of a NpSTHK-type dimer interface in sGC we mutated 8 putative H-NOXA interface residues to alanines (see Figs. 1B, 2, B and C, and 4 for the NpSTHK interface residue positions that were targeted for mutagenesis of the corresponding residue in sGC). We tested the effect of these mutations on dimerization of the isolated sGCβ1 H-NOXA domain and the dimerization and activity of full-length sGC. The 8 Ala mutations were made in sGCβ1; two of the mutations were also generated at the corresponding positions in sGCα1. All mutations had a negative effect, with varying degrees, on dimerization of the β1 H-NOXA domain (Fig. 4B). The β1 mutations that caused the largest effects on H-NOXA dimerization are Q231A, Q309A, and L322A, as more than half of the mutant protein became monomeric at 1 mg/ml (Fig. 4B). Even the mutations that were the least disruptive, S206A and F217A, still had a small but measurable negative effect on dimerization indicative of a role in dimerization (Fig. 4B). The latter F217A mutation also caused a widening of the dimer peak, perhaps suggesting an (additional) larger structural destabilization caused by this mutation (Fig. 4B).

The 8 sGCβ1 and 2 sGCα1 Ala-scanning mutations had in most cases less of an effect on dimerization of the full-length sGC as probed by immunoprecipitation and Western blotting (Fig. 4, C and D). Exceptions are the sGCβ1 mutations Q309A (and equivalent Q368A mutation in sGCα1), L322A, and to a lesser degree Q231A and I208A (Fig. 4, C and D). The Q309A (and sGCα1 equivalent) caused a complete loss of sGC heterodimerization as partnering subunits could not...
be pulled down with antibodies directed against either the α or β subunit (Fig. 4D). It is interesting to note that when this Gln mutation occurs in the sGCα1 subunit, the wt sGCβ1 subunit can be pulled down using a β-specific antibody at comparable levels, whereas when the mutation is introduced in the sGCβ1 subunit, wt sGCα1 itself cannot be pulled down with an α-specific antibody despite being present in the cell lysate probably as a result of aggregation. This differential sGC subunit behavior is similar at physiological receptor concentrations in mice with individual sGC subunits
knocked-out because only the sGCβ1 subunit could be detected in sGCα1 knock-outs (41) but not vice versa (42). A number of the mutations had a comparable effect on both sGCβ1 H-NOXA homodimerization and full-length sGC heterodimerization. sGCβ1 mutations Q309A, Q231A, and L322A caused a significant decrease in dimerization in both experiments, whereas F217A and S206A both had only a minor effect on H-NOXA homodimerization and little if any effect on full-length sGC dimerization (Fig. 4).

The effect of the Ala sGC mutations on guanylyl cyclase activity was also measured in mutant-transfected COS-7 cells. The effect on activity was most pronounced for the Q309A sGCβ1 mutant and the equivalent sGCα1 Q368A mutant, which both resulted in a complete loss of NO-stimulated and basal activity, in agreement with their inability to even form a heterodimer (Fig. 4D), the minimum needed for sGC basal activity (43). The β1 F217A and equivalent α1 F285A mutations caused a 3.6- and 6.1-fold drop in NO-stimulated activity, respectively (Fig. 4D). This result is somewhat unexpected because both Phe → Ala mutants had only limited effects on H-NOXA homo- and sGC heterodimerization (Fig. 4, B and D). Nevertheless, the substantial decrease in NO-stimulated activity by these Phe → Ala mutations could perhaps be explained by either a more global destabilization of the H-NOXA domains as mentioned above or perhaps an altering of the relative orientation of the H-NOXA dimer within the mutant sGC (described in more detail below). Except for the mutants shown in Fig. 4D, the remaining mutants had only a modest negative effect on guanylyl cyclase (Fig. 4C) yielding a maximal decrease of NO-stimulated activity of just under 3-fold. However, the basal activity of the cell lysate is also lower for these mutants such that the fold activity enhancement upon NO stimulation above for these latter mutants is similar to wt being around 15. The one exception is the L322A sGCβ1 mutant, which harbors a 29-fold increase in stimulated activity over basal activity (Fig. 4C).

The Ala-scanning mutagenesis revealed that some of the residues at the putative H-NOXA dimerization interface in sGC are critical for dimerization and/or activity. The most critical residues are Phe285 and Gln368 in sGCα1, and the equivalent Phe217 and Gln309 in sGCβ1, and correspond to Phe17 and Gln89 of the NpSTHK dimer (Figs. 1B and 2, B and C). Interestingly, Phe17 and Gln89 cluster and are both located at the bottom of the sGCα1 and sGCβ1, respectively) resulted in drastic loss of sGCβ1 H-NOXA homodimerization, loss of sGC heterodimerization, and loss of basal activity. This glutamine residue position is thus clearly crucial and, like Phe17, is positioned at the edge of the dimer interface toward the C-terminal CC + GC domains. Minor deviations from a perfect 2-fold axis have been observed to be as large as 7° in other dimer protein crystal structures (44) and we anticipate a symmetrical dimer when in solution.

Residue Gln89 of molecule A of NpSTHK forms a hydrogen bond and a water-mediated hydrogen bond with the backbone oxygens of Ala16 and Lys15, respectively, in addition to packing against residue Met91 being a key hydrophobic interface residue (Fig. 2, B–D). Note that due to the 1.5° deviation from the perfect 2-fold axis of the crystallographic NpSTHK dimer, residue Gln89 of the other subunit is located 1 Å more distant from the dimer interface although still involved in packing against Met91, a likely key interface residue. Minor deviations from a perfect 2-fold axis have been observed to be as large as 7° in other dimer protein crystal structures (44) and we anticipate a symmetrical dimer when in solution.

Of all the mutants generated in this study, mutations at the equivalent position of STHK:Gln89 in sGCα1 and sGCβ1, respectively) resulted in loss of sGCβ1 H-NOXA homodimerization, loss of sGC heterodimerization, and loss of basal activity. This glutamine residue position is thus clearly crucial and, like Phe17, is positioned at the edge of the dimer interface toward the C-terminal CC + GC subunits. We speculate that its mutation in the sGCβ1 H-NOXA homodimer affects its homodimerization via loss of 2 direct hydrogen bonds and 2 water-mediated hydrogen bonds across the interface, and reorientation of its neighboring inter-
PAS-mediated Dimerization of Soluble Guanylyl Cyclase and STHK

A

![Sequence identity diagram](image)

B

![Guanylyl cyclase activity graph](image)

C

![Western blot analysis](image)

D

![Guanylyl cyclase activity graph](image)

FIGURE 4. Probing the putative H-NOXA dimer interface in sGC by mutagenesis. A, conservation of surface residues of the NpSTHK H-NOXA monomer with the α1 and β1 sGC subunits. The surface facing the dimer interface (left) and the opposite side (right) are shown. Non-conserved surface H-NOXA residues are shown as white. Conserved residues at the dimer interface or in the vicinity are labeled. B, probing dimerization state of sGCβ1 H-NOXA domain mutants using size exclusion chromatogram. A Superdex 75 column (HR10/30, GE Biosciences) was used to test the oligomeric state of mutant H-NOXA proteins in which the following sGC residues were mutated to Ala (corresponding residues in NpSTHK are in parentheses): Ser206 (Leu6), Ile208 (Leu6), Phe217 (Phe17), Gln231 (Gln31), Gln309 (Gln89), Ile311 (Met91), Leu320 (Phe100), and Leu322 (Leu102). A mixture of molecular weight protein standards was also applied to the column for reference (gray). The theoretical molecular mass for the monomer of wt sGCβ1 H-NOXA domain is 17 kDa, which includes the His tag. For comparison, the crystallized NpSTHK, with a theoretical molecular mass of 14 kDa, elutes at 10.6 ml (not shown), which is similar to that of the wt sGCβ1 H-NOXA domain confirming the dimeric state for both proteins. The injecting volume for each run is 100 μl and protein concentration is 1 mg/ml. C, guanylyl cyclase activity and Western blot analysis of co-immunoprecipitated wt and 6 sGCβ1 mutants of sGC. Basal and NO-stimulated guanylyl cyclase activity of cell lysates with transfected sGC subunits are shown (upper panel). The Western blot (lower panel) includes co-immunoprecipitated sGC subunits pulled down with anti-sGCβ1 antibody (top gel) as well as the contents of the cell lysates (bottom). The Western blot was probed simultaneously with anti-sGCα1 and anti-sGCβ1 antibodies. D, guanylyl cyclase activity measurements and Western blot co-immunoprecipitation analysis of additional mutants of sGC generated in both α1 and β1 subunits. These mutations include the sGCβ1 F217A and Q309A mutations and are also generated at the equivalent positions in sGCα1 (α1 mutations F285A and Q368A, respectively). Data presentation are similar as in C but includes additional co-immunoprecipitation (IP) using an anti-sGCα1 antibody (top gel). This Western blot was also probed similarly as in C. The difference in specific activity observed between experiments C and D (as reflected in the wt activity) is probably due to the fact that two different batches of COS-7 cells were used in C and D. For each set of experiments, COS-7 cells were transfected three times and activity assays repeated three times with each measurement done in duplicate.

In summary, Ala scanning mutagenesis of the putative dimerization interfaces of the H-NOXA domains of sGCβ1 and sGCα1 can lead to loss of dimerization and/or guanylyl cyclase activity pointing to the role of H-NOXA in sGC dimerization and activity. The two mutations that were carried out in both sGCα1 and sGCβ1 subunits resulted in similar behavior (Fig. 4D) suggesting that they have roughly equally important roles. Together, these results are compatible with the postulate that the H-NOXA domains of sGC arrange themselves in a NpSTHK-like fashion. Such heterodimeric arrangement in sGC could be either rigid,
required to pivot/reorient, or be more transient during one of the stages of sGC activation. The implications of such a possible arrangement are discussed below.

**DISCUSSION**

The PAS fold and postulated NpSTHK-type dimer organization for the H-NOXA domains of sGC (Figs. 2–4) has interesting consequences regarding preferential sGC heterodimerization, possible allostery, and the overall domain architecture of sGC.

Possible Implications for Preferential Heterodimerization of sGC—Our structural results regarding the 1–121 and Δ7 truncated 8–121 NpSTHK constructs point to a critical role for the N terminus in dimer formation. Deleting the first 7 residues of NpSTHK resulted in a flipped dimer suggesting that these residues are critical for providing specificity for correctly orienting the monomers within the dimer. Within these first 7 residues, residues Pro6, Leu6, and Thr7 are involved in dimer interface interactions via either hydrogen bonds or van der Waals interactions (Fig. 2, B and C) in the 1–121 NpSTHK dimer structure. Residue Leu6 has a major role in that both its side chains makes interactions, with a relatively conserved hydrophobic region at the interface involving Ala22 and Val30, and its main chain N and O atoms make interface hydrogen bonds with the conserved Glu31 residue. N-terminal H-NOXA interactions thus likely provide the specificity for correct monomer:monomer juxtapositioning in NpSTHK yet could also have a role in preferential sGC heterodimerization as discussed next.

In addition to functional α1β1 and α2β1 heterodimerization, sGC homodimers are also observed for β1 (43), β2 (45), and α1 (46), the latter being less stable. Homodimers of sGC are inactive (43), except for the β3 subunit from Manduca sexta (47) and possibly the β2 sGC subunit although its weak activation needs non-physiological manganese, thus providing no conclusive evidence that its H-NOXA domains are dimerized under physiological conditions (45). A physiological equilibrium is thought to be present between homo- and heterodimeric sGC (43) although homodimeric β1 (41) and in particular homodimeric α1 are found to be very unstable in vivo (42). This balance is likely influenced by each of the three known interfaces involving the GC, H-NOXA, and CC domains (9, 17, 48). Such preferential heterodimerization tendency within these sGC domains is likely a consequence of complementary interface differences between α1 (or α2) and β1 subunits. Although speculative, sequence comparisons of interface residues and modeling of homodimeric and heterodimeric H-NOXA dimers suggests that sGC residues corresponding to NpSTHK residues Leu6 and Ala22 and Val30 could be, in part, responsible for preferential heterodimerization of sGC. These residues cluster (Fig. 2, B and C) and size variations across the interface suggest that a heterodimer might be more sterically compatible. In sGCα1 (and sGCα2), these residues are Leu274, Met290, and Leu298, respectively, yet are smaller in sGCβ1 being Ser206, Ile222, and Thr300, respectively, all within the stretch of residues 204–244 shown to be key for binding to α1 (17). Larger-sized interface residues at all these positions, such as in an α1 (or α2) homodimer, would likely cause steric hindrance between Met290 and Leu274 such that the latter cannot position itself to form key main chain hydrogen bonds (equivalent to NpSTHK residue Leu6). This disruption would favor such dimerization perhaps analogous to the disruptive effects of the Δ7 deletion in NpSTHK. sGCβ1 has smaller residues at these 3 positions likely permitting homodimer formation yet a heterodimeric α1β1 could possibly be favored via improved van der Waals packing by complementary combinations of small and larger residues at these 3 interface positions. Although it is interesting that the NpSTHK dimer structure could suggest a structural basis for a possible H-NOXA-contributing role in sGC heterodimerization, it remains speculative. Such a H-NOXA contributing role for sGC heterodimerization awaits future experimental validation especially in light of that residues preceding the N-terminal helix could possibly adopt different conformations such as a α-helical configuration in RmFixL (39).

Possible Role for PAS Dimer in Signaling or Allosteric Regulation in sGC and NpSTHK—The possibility of PAS-based allostery in sGC and NpSTHK is intriguing because the PAS domain is an important ancient sensory domain that can, for example, sense redox potential, small ligands such as oxygen, and light, in addition to maintaining protein-protein interactions (49, 50). PAS sensory domains are often linked with output domains such as a histidine kinase, phosphodiesterase, or adenylcyclase domains, and are remarkably abundant in cyanobacteria (51). Our results reveal a PAS dimer organization for NpSTHK indicating that it has the same subdomain architecture as RmFixL: a parallel dimerized (oxygen-sensing) PAS domain followed by a CC domain and histidine kinase domain. As noted earlier, such parallel oriented PAS domain dimers are also observed in EcDos and AvNiFL (Fig. 3), which lead to its recognition as a conserved PAS domain dimerization motif (41). RmFixL, EcDos, and AvNiFL either sense redox potential or oxygen each involving postulated signaling mechanisms that lead to changes at the PAS-domain interface (30, 40). The presence of a PAS domain in NpSTHK and sGC and the noted dimerization similarities raises the possibility that these PAS domains in NpSTHK and sGC are also used for signal transduction purposes or perhaps even sensory purposes. Obviously, the major sensory domain in sGC is the NO-sensing H-NOXA domain yet it is tempting to speculate that the PAS domain could harbor a second allosteric regulatory module in sGC. This module could be present as an evolutionary remnant, for perhaps heme binding, or as a site for an unknown regulator. Intriguing candidates could be the allosteric sGC regulators YC-1, as well as ATP and GTP, whose binding site, or sites (52), to sGC has not been unambiguously mapped (6, 53–56). Alternative to a small molecule, the pocket of a PAS domain is also capable of harboring a tryptophan side chain of a different subunit as observed in PERIOD (57). If such a PAS-mediated signaling or sensory regulation were indeed to occur in sGC or NpSTHK, some opening up of the PAS domain would be necessary for binding. Other possible PAS regulatory mechanisms have been observed as well and include N-terminal PAS helix unfolding (58), a speculated helix-swap event (59), or PAS pocket mediated inter-subunit interactions with a C-terminal helix (57) indicating that PAS-mediated contacts
that there is a direct interaction between domains need to form a catalytically active heterodimer and when combined with additional constraints in that the GC sequences for the overall architecture of the heterodimeric sGC

The locations of the N and C termini has interesting consequences for the systematic deletion mutagenesis analysis (18) in light of the above noted abundance of inter-subdomain interactions within sGC. The CC region is depicted as parallel CC segments as previously predicted (10), although we do not rule out other CC arrangements such as a 4-helix bundle. Despite some limitations, we generated a model for the entire sGC heterodimer (Fig. 5). In this modeled composite structure, part of the H-NOX domain is able to reach the GC domain (Fig. 5) with a stretch of 15 residues between the heme domain and the H-NOXA domain allowing such conformational flexibility. Although it is not known where and whether a protein:protein regulatory site is present on the sGC GC domains, such a site is present in the homologous adenylyl cyclase domains. The adenylyl cyclase activity is regulated by $\alpha_s$, binding to the AC2 domain, which is homologous to the $\beta_1$ cyclase domain (11). Assuming that sGC uses the same site on its catalytic domain for regulation, the H-NOX domain is oriented such that its region near loop L1 (containing Asp$^{44}$–Asp$^{45}$, speculated to be near the activation switch (14, 60) and shift up activation (16)), is closest to, and can be sensed by, the $\beta_1$ cyclase domain site that corresponds to the surface where $\alpha_s$ binds adenylyl cyclase. The above sGC model is speculative, in particular as it is in large part based on the NpSTHK-type PAS dimer organization and PAS domains can be capable of undergoing (mechanistic) reorganization such as in PilT (61). Nevertheless, this is a new global model of the sGC domain arrangement that provides a framework for future structure-guided experiments and can aid in the interpretation of a recent deletion mutagenesis study (18) as discussed below.

The above noted abundance of inter-subdomain interactions within sGC provides the possibility that some of these interfaces are more involved in the sGC activation mechanism than needed for sGC heterodimerization. A recent study detailing a systematic deletion mutagenesis analysis (18) in light of our structural results hints at such a possibility. This study first observed a roughly equal importance of the $\alpha_1$ and $\beta_1$ CC residues for overall sGC heterodimerization (18); the results from this study are also included in Fig. 1B. This study also points to a critical dimerization role for $\alpha_1$:363–372 and $\beta_1$:304–313, which are both located in H-NOX (18). This is in agreement with our structure-function results as these homologous stretches of sGC residues include the critical $\alpha_1$:Q368 and $\beta_1$:Q309 as well as $\beta_1$:I311, which also has a role in dimeriza-
tion (Fig. 4B). In addition to the H-NOXA interface, we had noted above that some of these residues might also provide key interactions with the downstream CC region. In further concord with our results, the deletion study found additional stretches of H-NOXA residues that either affected sGC dimerization and/or sGC activity (18) (Fig. 1B); all deletions contain residues that would correspond to the H-NOXA dimer interface or are between the H-NOXA and CC domains (deletions depicted in Fig. 1B). However, equivalent deletions in α1 and β1 have a different effect (except for the noted α1:363–372 and corresponding β1:304–313). Deletions in β1-H-NOXA caused loss of dimerization, and thus all activity, whereas α1-H-NOXA deletions Δ283–292 and Δ373–382 lead to an increase in cyclase activity and decreased EC50 for BAY41–2272 in the presence of 1 nM DEA/NO indicating that they are more readily and more potently activated (18). These differences from this deletion study are intriguing but need to be taken with the caveat that their system actually introduced an additional artificial dimerization interface as they likely disrupt not only the H-NOXA/H-NOX subdomains but also indirectly the adjacent H-NOX domain, loss of dimerization, and thus all activity, whereas deletions α1-H-NOXA residues are deleted likely provides new insights into the role of the H-NOXA domains for the activation mechanism of sGC. We propose that the CC domains provide the main, although not sole, driving force for sGC dimerization, whereas the H-NOXA subdomain heterodimer possibly serves as a regulatory interface needed to bring along and position the adjacent NO-regulated H-NOX domain such that it can interact and regulate the catalytic GC subunits (48). This could explain why deletions within the β1-H-NOXA domain are deleterious for heterodimerization as they likely disrupt not only the H-NOXA/H-NOX interface but also indirectly disrupt the H-NOX:GC interface because H-NOX can likely no longer be properly positioned by β1-H-NOXA. However, deletions of most stretches of residues within the α1-H-NOXA domain did not cause loss of dimerization but, unexpectedly, made sGC more active even at lower concentrations of BAY41–2272 + 1 nM DEA/NO (18). We therefore speculate that α1-H-NOXA is a likely negative regulatory subdomain that holds β1-H-NOXA, and thus indirectly also the adjacent H-NOX domain, in an inhibitory position/conformation prior to NO activation. Disruption of this interaction by deletions in α1-H-NOXA could possibly relax the β1-H-NOXA/H-NOX subdomains such that it is now more susceptible to BAY41–2272/NO activation yielding also higher cyclase activity. Further studies are needed to determine whether the H-NOXA domains in sGC, or PAS domain in STHK, are indeed used for such regulatory, or even sensory, mechanisms. Nevertheless, this structural interpretation of the Rothkegel et al. (18) deletion data using our structural results suggests that targeting the H-NOXA dimer interface or putative PAS-ligand binding pocket could lead to new therapeutic sGC stimulators and activators. It is worth noting that this approach of targeting a PAS domain with no known ligand was successful for the PAS kinase domain, whose pocket is mainly closed as well, using the SAR-by-NMR approach (62).

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