

Interfacial Residues Promote an Optimal Alignment of the Catalytic Center in Human Soluble Guanylate Cyclase: Heterodimerization Is Required but Not Sufficient for Activity

Franziska Seeger,^{†,§} Royston Quintyn,[‡] Akiko Tanimoto,[‡] Gareth J. Williams,[§] John A. Tainer,^{§,||} Vicki H. Wysocki,[‡] and Elsa D. Garcin*,^{†,‡}

[†]University of Maryland Baltimore County, Baltimore, Maryland 21250, United States

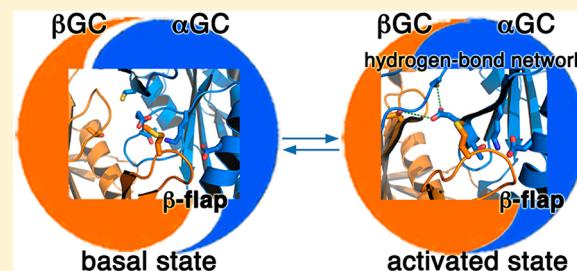
[‡]The Ohio State University, Columbus, Ohio 43210, United States

[§]Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

^{||}The Scripps Research Institute, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: Soluble guanylate cyclase (sGC) plays a central role in the cardiovascular system and is a drug target for the treatment of pulmonary hypertension. While the three-dimensional structure of sGC is unknown, studies suggest that binding of the regulatory domain to the catalytic domain maintains sGC in an autoinhibited basal state. The activation signal, binding of NO to heme, is thought to be transmitted via the regulatory and dimerization domains to the cyclase domain and unleashes the full catalytic potential of sGC. Consequently, isolated catalytic domains should show catalytic turnover comparable to that of activated sGC. Using X-ray crystallography, activity measurements, and native mass spectrometry, we show unambiguously that human isolated catalytic domains are much less active than basal sGC, while still forming heterodimers. We identified key structural elements regulating the dimer interface and propose a novel role for residues located in an interfacial flap and a hydrogen bond network as key modulators of the orientation of the catalytic subunits. We demonstrate that even in the absence of the regulatory domain, additional sGC domains are required to guide the appropriate conformation of the catalytic subunits associated with high activity. Our data support a novel regulatory mechanism whereby sGC activity is tuned by distinct domain interactions that either promote or inhibit catalytic activity. These results further our understanding of heterodimerization and activation of sGC and open additional drug discovery routes for targeting the NO-sGC-cGMP pathway via the design of small molecules that promote a productive conformation of the catalytic subunits or disrupt inhibitory domain interactions.



The enzyme soluble guanylate cyclase (sGC) plays a key role in the cardiovascular system and is a validated drug target for the treatment of cardiovascular diseases.^{1–5} It catalyzes the formation of the cardioprotective signaling molecule cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP).⁶ Nitric oxide (NO) binds to the N-terminal regulatory domain, thereby inducing the transition from basal to activated sGC, resulting in an increased level of cGMP production. Under conditions of oxidative stress, decreased NO bioavailability^{7,8} and increased levels of oxidation of sGC both lead to impaired sGC activation and decreased levels of cGMP production.¹ Additional physiological regulators of sGC are being discovered, such as the thrombospondin-1/CD47 signaling pathway,⁹ that are capable of further decreasing sGC activity.^{10,11} Restoring healthy levels of cGMP in the diseased state thus requires alternative ways to activate sGC. Understanding the molecular events controlling sGC activity may lead to additional classes of cGMP-modulating therapeutics. However, despite great progress in the field, sGC regulation is largely enigmatic. With this goal in mind, we

aim to characterize structural changes that occur at the catalytic center during the activation process.

The sGC enzyme is a heterodimer of α and β subunits¹² sharing a similar modular organization: an HNOX regulatory domain, an HNOXA domain and a coiled-coil (CC) domain involved in dimerization, and a catalytic guanylate cyclase (GC) domain, from the N- to C-terminus. Crystal structures of independent sGC domains and homologues have been determined,^{13–20} and recent low-resolution electron microscopy data suggest how these domains might assemble in the full-length enzyme.²¹ However, the high-resolution three-dimensional structure of full-length sGC is still missing. Both long-range domain–domain interactions and local short-range conformational changes were proposed to account for sGC activation. Several studies point to the inhibitory interaction of

Received: January 29, 2014

Revised: March 12, 2014

Published: March 14, 2014

the β HNOX domain with the cyclase domain.^{22–25} Furthermore, the α HNOX and α HNOXA domains were shown to be located near the β HNOX domain to keep it in an inhibited conformation that is released upon NO or YC-1 binding.^{26,27} From these results, a collective model in which the N-terminal regulatory domains autoinhibit sGC activity was developed. If this model is correct, then isolated cyclase domains should display high levels of activity.

Here we combined X-ray crystallography, activity measurements, and native mass spectrometry analysis of the wild-type human heterodimeric catalytic domains of sGC (hereafter termed $\alpha\beta$ GC) to characterize the structural features that modulate the orientation of the catalytic subunits leading to sGC activity and to propose a novel model for sGC regulation.

■ EXPERIMENTAL PROCEDURES

Materials. All chemicals were obtained from Sigma-Aldrich and purification columns from GE Healthcare unless otherwise indicated.

Mutagenesis, Expression, and Purification of the $\alpha\beta$ GC Heterodimer, α GC, and β GC. Entry clones for human α_3 GC GUCY1A3 (amino acids 466–690, GenBank accession number JX420281) with an N-terminally His-tagged thioredoxin tag in a pNH-TrxT vector and mutant β_1 GC GUCY1B3 (amino acids 407–619, GenBank accession number JX420282) with a C-terminally His-tagged Flag tag in a pNIC-CTHF vector were kind gifts of Dr. Allerston (Structural Genomics Consortium). The β GC G476C/C541S double mutant was changed back to the wild type using standard site-directed mutagenesis (Stratagene) with the following primer pairs: forward primer (5'-cgttactgcctgtccggc-3') and reverse primer (5'-gccaacaggcgttaacg-3') for C476G and forward primer (5'-cgaaactgttgcgcataatgtatcg-3') and reverse primer (5'-tcataacttatcgccaacatgttcg-3') for S541C.

We also designed a shorter construct for α GC, named α GC661 (residues 466–661) by inserting a stop codon at position 662 by site-directed mutagenesis with the following primer pairs: forward primer (5'-gatcgatcgtatcgtatgtaccaactcaa-3') and reverse primer (5'-tttgatgttgttactactgtatcgcatc-3'). All mutations were confirmed by DNA sequencing (Genewiz).

Each catalytic subunit was independently co-expressed with the GroEL-ES chaperone system from a pACYC-derived plasmid (Takara Inc.) in *Escherichia coli* BL21(DE3) cells (Life Technologies). Cells were cultured overnight at 37 °C. Protein expression was induced with 1 mM IPTG and L-arabinose (2 g/L) when OD₆₀₀ reached 1. Cells were grown for 20 h at 15 °C, pelleted, and frozen at –80 °C until they were used. To purify the $\alpha\beta$ GC heterodimer, cell pellets from each subunit were mixed and lysed in buffer A [50 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 30 mM imidazole, 0.1% (v/v) Tween 20, 0.1 mg/mL DNaseI, 0.5 mg/mL lysozyme, and protease inhibitor cocktail (Roche)] via sonication. The first chromatography step was a nickel HisTrap affinity column, and α GC and β GC co-eluted with a 15 to 100% gradient of buffer B [50 mM sodium phosphate (pH 7.4), 0.5 M NaCl, and 0.3 M imidazole]. Both proteins were cleaved overnight [in 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 5% glycerol] using a His-tagged tobacco etch virus (TEV) protease yielding α GC(466–690) and β GC(407–626). For β GC, the seven extra C-terminal amino acids are part of the linker for TEV cleavage of the C-terminal His-Flag tag. A second Ni affinity step was performed to remove the cleaved tags and the TEV protease. The proteins were further purified using a HiTrap Q HP anion

exchange column and Superdex 200 or Superdex 75 gel filtration in a final buffer consisting of 20 mM Hepes (pH 7.5) and 0.15 M NaCl. The protein was aliquoted, flash-frozen in liquid nitrogen, and stored at –80 °C. The same protocol was used to purify all catalytic constructs. The final yields are 3.5 mg of α GC, 0.75 mg of β GC, 0.25–1 mg of copurified $\alpha\beta$ GC, 0.8 mg of α GC661, and 0.5–0.8 mg of copurified α GC661 β GC per liter of cell culture.

Crystallization, Data Collection, and Refinement.

Crystals of wild-type $\alpha\beta$ GC catalytic domains were grown at 20 °C using a vapor diffusion sitting drop setup containing 0.2 μ L of 14 mg/mL $\alpha\beta$ GC with 0.2 μ L of a reservoir solution composed of 20% (w/v) PEG3350, 50 mM HEPES (pH 7.0), and 1% Tryptone. The drops were set up using an Art Robbins Phoenix crystallization robot. Before data collection, the crystal was cryo-protected in mother liquor supplemented with 20% (v/v) ethylene glycol and flash-frozen in the nitrogen cryostream. X-ray diffraction data were collected on beamline 8.3.1 at the Advanced Light Source at Lawrence Berkeley National Laboratory. Data processing and reduction were conducted with MOSFLM.²⁸ Phasing by molecular replacement was conducted with PHASER²⁹ using the mutant $\alpha\beta$ GC structure [Protein Data Bank (PDB) entry 3UVJ] as a starting model. The final model was obtained with iterative cycles of refinement with PHENIX³⁰ and rebuilding with COOT.³¹ The final model and structure factors were deposited in the PDB (entry 4NI2).

Activity Assay. The cyclase activity reaction was performed using ~5 μ M full-length sGC (based on the absorbance at 431 nm) and ~10 μ M catalytic constructs (based on the absorbance at 280 nm). The reaction assay was conducted in 40 mM HEPES (pH 7.4), 0.5 mM dithiothreitol, 0.3 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, and 3 mM MgCl₂ or MnCl₂. The reaction mixture was incubated at 15 °C for 20 min and the reaction stopped with the addition of EDTA (final concentration of 10 mM). The reaction mixture was stored at –80 °C until cGMP was quantified using a cGMP enzyme immunoassay kit (R&D) following the manufacturer's protocol. Activity was measured in duplicate, and the experiments were repeated three times to ensure reproducibility.

Multiangle Light Scattering. The KW 403 gel filtration column was equilibrated with buffer A [50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 5% glycerol, 1 mM MgCl₂, and 1 mM TCEP]. Bovine serum albumin was used as a reference. We injected 50 μ L of $\alpha\beta$ GC at 30 μ M.

Mass Spectrometry (MS). The protein samples were buffer exchanged into 0.1 M ammonium acetate (AA) buffer (pH 7.4) using either size-exclusion chromatography spin columns (Bio-Rad) or Amicon Ultra-4 centrifugal filter units (EMD Millipore). Subsequently, the concentrations of the buffer-exchanged proteins were calculated by measuring the A₂₈₀ and a solution of 0.1 M triethylammonium acetate (TEAA) was added to the protein samples in a 1:4 (TEAA:AA) ratio to produce charge-reduced catalytic dimers, unless otherwise stated. In the K_D analysis of the catalytic dimers, various protein concentrations were obtained by serial dilution starting from the stock solution of the buffer-exchanged protein sample. For all other mass spectrometry analyses, the concentration of catalytic dimers used was 10 μ M, unless otherwise stated.

Nano-electrospray ionization mass spectrometry (nano-ESI/MS) analysis was conducted by utilizing a modified Quadrupole Ion Mobility Time of Flight (Q-IM-TOF) instrument (Synapt G2, Waters Corp., Manchester, U.K.) with a customized

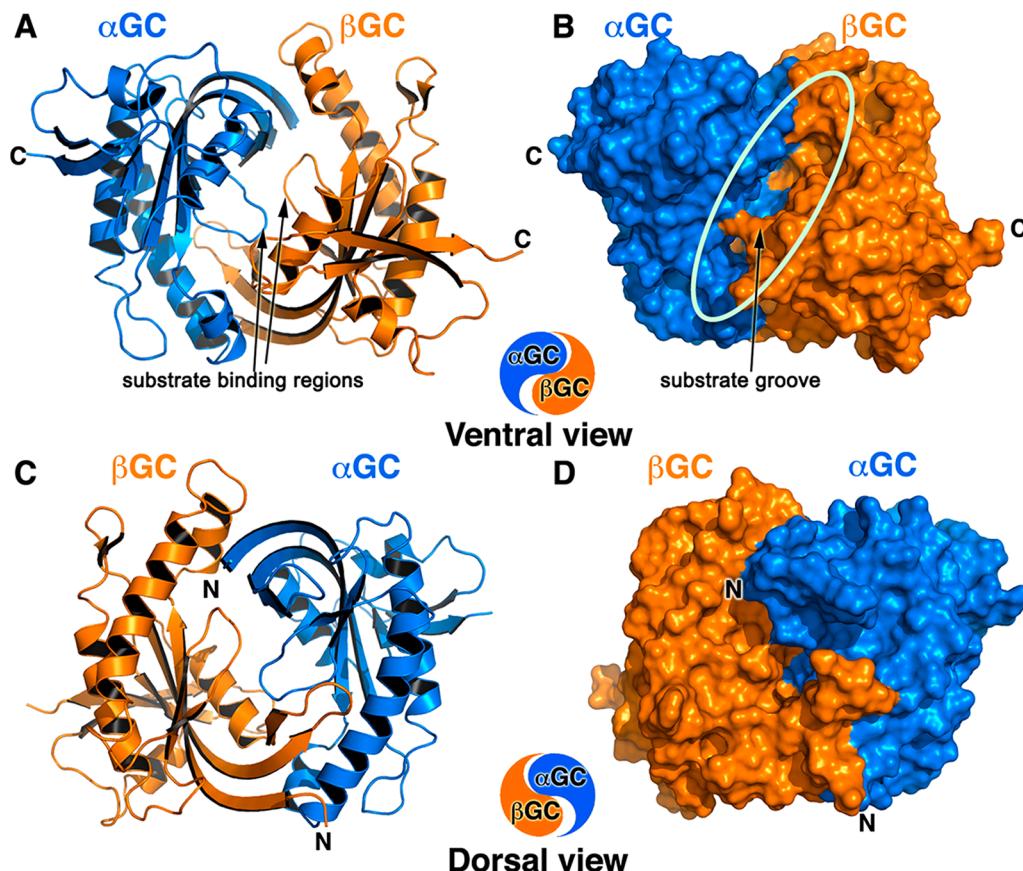


Figure 1. Overall structure of heterodimeric wild-type $\alpha\beta$ GC catalytic domains that resembles the Chinese yin-yang symbol with both subunits arranged in a head-to-tail conformation. (A and B) Ventral face of the heterodimer as a cartoon (A) and a solvent accessible surface representation (B). The deep extended substrate groove (shown by the oval in panel B) bisects the ventral face, which contains the C-termini of both subunits (denoted with C). The α GC subunit is colored blue, and the β GC subunit is colored orange. The substrate binding regions of α GC (residues 523–534) and β GC (residues 470–480) adopt an extended conformation in the absence of substrate and metals (arrows). (C and D) The dorsal face of the heterodimer as a cartoon (C) and a solvent accessible surface representation (D) is flatter than the ventral face and contains the N-termini of both subunits (denoted with N).

surface-induced dissociation (SID) device installed before the IM chamber as previously described.³² All experiments were conducted using a nanoelectrospray source, using a capillary voltage of 1.0–1.5 kV and a cone voltage of 50–75 V. No heating was applied to the cone. Nano-ESI glass capillary and surface preparation procedures can be found elsewhere.³³ The following instrumental conditions were used: 5 mbar for the source/backing pressure, 2 mbar for the nitrogen gas pressure in the IM cell, rate of 120 mL/min for the flow of gas into the helium cell, $\sim 6 \times 10^{-7}$ mbar in the TOF analyzer, and a wave velocity and a height of 300 ms⁻¹ and 20 V, respectively, for IM experiments. Nano-ESI/MS is a gentle method of ionization in which salts and solvent are often retained, giving *m/z* values higher than those calculated from sequence, especially for oligomers. This problem is sometimes eliminated in nano-ESI-MS/MS measurements of fragments, if the MS/MS approach unfolds the monomers causing a loss of salts, solvents, and ligands.

RESULTS

Overall Structure of Wild-Type Human Heterodimeric $\alpha\beta$ GC and Comparison with Mutant Heterodimeric $\alpha\beta$ GC. To identify the structural determinants for sGC catalytic activity, we determined the X-ray structure of the wild-type heterodimeric $\alpha\beta$ GC catalytic domains of human sGC in the

apo form at 1.9 Å resolution (Figure 1). The final model shows good statistics with R_{work} and R_{free} values of 0.159 and 0.198, respectively, and 98.2% of the residues in the allowed regions of the Ramachandran plot (Table 1). The model comprises one heterodimer per asymmetric unit and contains α GC residues 471–662, β GC residues 411–608, 342 water molecules, and six ethylene glycol molecules. Residues at the N- and C-termini of α GC (466–470 and 663–690, respectively) and β GC (407–410 and 609–626, respectively) were not visible in the electron density and were not included in the final model. To rule out the possibility that these proteins may be proteolyzed or degraded and to unambiguously determine the masses of the purified proteins, we performed nano-ESI/MS analysis on the α GC and β GC proteins purified individually or copurified (Table 2, Supporting Information, and Figure S1 of the Supporting Information). We confirmed that we crystallized heterodimeric $\alpha\beta$ GC with a truncated α GC(466–662) subunit.

The structure of heterodimeric $\alpha\beta$ GC resembles the Chinese yin-yang symbol with the two subunits arranged in a head-to-tail conformation (Figures 1A,C). The catalytic domains were crystallized without metal or substrate. Accordingly, the structure reveals an inactive heterodimer conformation compared to that of active adenylate cyclase.³⁴ This is evidenced both by the relative orientation of the two subunits leading to an open active site and by the extended loop

Table 1. X-ray Data Collection and Refinement Statistics

	Data Collection ^d
space group	P212121
wavelength (Å)	1.116
resolution (Å)	69.7–1.9 (2.0–1.9)
unit cell parameters (Å)	$a = 49.5, b = 55.8, c = 139.4$
no. of measurements	240390 (31623)
no. of unique reflections	31270 (4438)
redundancy	7.7 (7.1)
completeness (%)	99.8 (98.7)
$\langle I/\sigma(I) \rangle$	14.2 (2.6)
$R_{\text{merge}} (\%)^a$	8.6 (68)
	Refinement ^d
resolution range (Å)	69.7–1.9 (1.96–1.90)
no. of protein atoms	2964
no. of water atoms	342
no. of heteroatoms	30
rmsd of bond lengths (Å)	0.012
rmsd of bond angles (deg)	1.3
$R_{\text{work}} (\%)^b$	15.9 (23.8)
$R_{\text{free}} (\%)^c$	19.8 (26.8)
Ramachandran plot (%)	
favored	98.2
allowed	0.8
generous	0.0
disallowed	0.0

^a $R_{\text{merge}} = \sum_h \sum_i |I(h,i) - \langle I(h) \rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the i th observation of reflection h and $\langle I(h) \rangle$ is the average intensity of redundant measurements of reflection h . ^b $R_{\text{work}} = \sum_h ||F_{\text{obs}} - |F_{\text{calc}}|| / \sum_h |F_{\text{obs}}|$. ^c $R_{\text{free}} = \sum_h ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_h |F_{\text{obs}}|$ for 5% of the reserved reflections. ^dValues in parentheses apply to the highest-resolution shell.

conformation of the core regions containing the catalytic residues (Figure 1A).

We superimposed our structure on that of the mutant heterodimeric human $\alpha\beta$ GC containing an engineered disulfide bridge (α GC C595–C476 β GC) at the dimer interface.²⁰ Aside from the mutations (G476C and C541A), the two structures are very similar, with an rmsd of 0.52 Å for 397 amino acids, but present subtle differences at the dimer interface (Movie S1 of the Supporting Information) and in surface-exposed regions (a detailed description of similarities and differences is presented in the Supporting Information). As a result, the α GC subunit and the β GC subunit present a slightly different orientation relative to each other in our structure. Overall, the α GC and β GC subunits are rotated by ~3° in the structure of the wild-type catalytic domains compared to the mutant structure. While these differences may seem subtle, similar conformational changes are observed in related adenylate cyclase between the inactive and active structures, in which one of the subunit rotates by 7° and secondary structure elements shift by 1–2 Å.³⁴ This example illustrates how small structural changes at the dimer interface in this protein family have profound effects on catalytic activity. In comparison to the structure of the mutant catalytic domains of sGC, our structure reveals an α GC– β GC interface with high plasticity that is necessary for sGC activity. Regardless, our structure is the closest to that of the catalytic domains in full-length sGC, as it is heterodimeric and devoid of mutations. As such, it represents an excellent starting model for the docking of small molecules that either inhibit or activate sGC.

Table 2. Comparison of Calculated and Experimental Molecular Masses for the Different sGC Constructs Determined by Nano-ESI/MS and Nano-ESI/MS/MS

construct	calculated mass (Da) ^a	calculated mass (Da) ^b	experimental mass (Da)
α GC(466–690) (monomer)	24671.2	24687.5	24740.8 ± 17.9 (10 μM) ^e
α GC(466–661) (monomer) ^c	21621.7	21636.1	21642.9 ± 18.3 (10 μM) ^e
α GC(466–690) (dimer)	49342.4	49375	49489 ± 20.3 (10 μM) ^e
α GC(466–662) (dimer) ^d	43499.6	43528.6	43500.1 ± 55.4 (10 μM)
α GC(466–661) (dimer)	43243.4	43272.2	43403.1 ± 5.5 (10 μM)
β GC(407–626) (monomer)	24769.2	24785.2	24820.5 ± 47.8 (10 μM) ^e
β GC(407–626) (dimer)	49538.4	49570.4	49636.7 ± 36.6 (10 μM)
β GC(407–626) (tetramer)	99076.8	99140.8	99929.9 ± 68.7 (10 μM)
$\alpha\beta$ GC (heterodimer)	49440.4	49472.7	49660.2 ± 38.7 (10 μM)
α GC661 β GC ^c (heterodimer)	46390.9	46421.3	46449.5 ± 20.2 (10 μM)

^aMonoisotopic masses were calculated from amino acid sequences with PeptideMass⁶⁶ from the ExPasy Web site. ^bAverage masses were calculated from amino acid sequences with PeptideMass⁶⁶ from the ExPasy Web site. ^cSpecies obtained by introducing a stop codon at position 662 in the α GC(466–690) construct. ^dSpecies obtained by cleavage of α GC(466–690) at the N- and C-terminal TEV cleavage sites. ^eMeasured mass obtained from nano-ESI/MS/MS at a SID voltage of 100 V.

Amino Acid Sequence Conservation Suggests Docking Sites for Other sGC Domains and Key Interfaces for Activation of sGC. To identify conserved regions in the catalytic heterodimer, we performed multiple-sequence alignments for both the α GC and β GC subunits (Supporting Information) and mapped the sequence identity percentage of each residue onto the $\alpha\beta$ GC structure (Figure 2). The regions with the highest degrees of sequence conservation are located in three regions of the heterodimer: (i) the substrate channel on the ventral face of the $\alpha\beta$ GC heterodimer (94% identical sequence), (ii) the C-terminal subdomain of α GC present on the ventral face of the heterodimer (78% identical sequence), and (iii) the dorsal face of the heterodimer (94% identical sequence). While the substrate channel is expected to be mainly invariant, the high level of sequence conservation of the α GC C-terminal domain (residues 616–662) is surprising and suggests that this domain may play an important role in the assembly and/or regulation of sGC. This hypothesis is supported by recent studies showing that the β HNOX–HNOXA domains directly interact with the C-terminal domain of α GC^{23,35} and could modulate the conformation of the active site. Interestingly, mutation of solvent-exposed residue α GC Arg624 in that domain to Ala leads to a dramatic increase in full-length sGC activity.³⁶ It is tempting to speculate that this residue plays a key role in domain–domain interaction with the N-terminal regulatory domains. Importantly, this region of the catalytic subunit could be targeted for the rational design of small molecules or protein therapeutics disrupting the inhibitory interactions between the regulatory and catalytic domains.

Second, the highly conserved region of the dorsal face of $\alpha\beta$ GC serves as the point of contact with the preceding $\alpha\beta$ CC

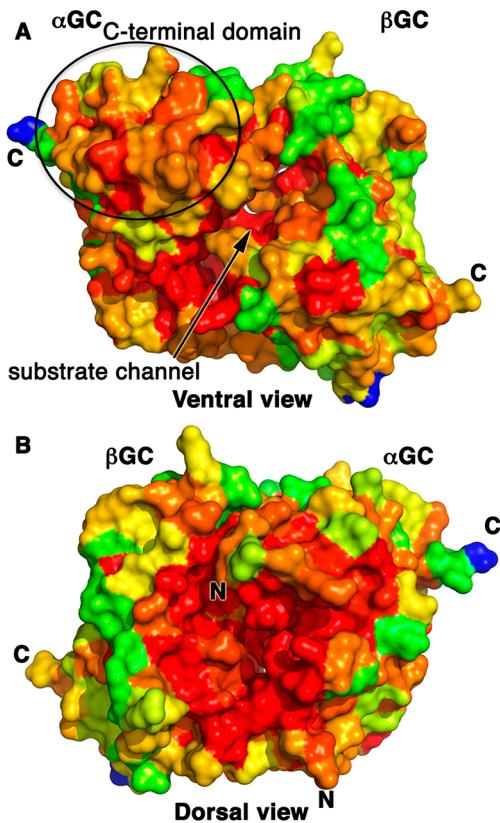


Figure 2. Map of the high levels of sequence conservation on the ventral face substrate channel and the α GC C-terminal domain and the heterodimer cleft on the dorsal face of the $\alpha\beta$ GC structure. We aligned >20 sequences of eukaryotic α GC and β GC domains (Supporting Information) and mapped the level of amino acid sequence conservation onto the $\alpha\beta$ GC crystal structure, colored from blue (0% identical) to red (100% identical). C-Termini of both subunits are marked with C, and N-termini are marked with N. (A) The highly conserved substrate channel on the ventral face is marked with an arrow. The C-terminal subdomain of α GC is moderately to strongly conserved (orange to red) as indicated by an oval. (B) The dimer interface region close to the N-termini of both subunits on the dorsal face is largely invariant.

dimerization domain, where it could modulate the interface between the α GC and β GC subunits. There is no crystal structure for heterodimeric $\alpha\beta$ CC, but recent studies unambiguously demonstrated a parallel orientation of the CC domains in sGC.^{21,26} In this arrangement, both C-termini of $\alpha\beta$ CC would be able to connect with the N-termini of the $\alpha\beta$ GC heterodimer separated by ~28 Å on the dorsal face of the heterodimer and located in the region with the highest level of sequence conservation. This position of $\alpha\beta$ CC is consistent with recent mass spectrometry²³ and FRET data.²⁵ This model is also coherent with the CC domain acting as a platform on which all the sGC domains assemble in full-length sGC.²⁶ The fact that the entire region surrounding the N-termini of the $\alpha\beta$ GC heterodimer is highly conserved in the sGC family points to more than simply an anchoring area for the preceding CC domain. Instead, we propose that the interface between the cyclase and the CC domains is essential for catalysis and that the CC promotes an optimal conformation of the catalytic subunits for activity.

Structural Determinants for Heterodimerization of the Catalytic Subunits. Our crystallization trials with

copurified wild-type $\alpha\beta$ GC protein yielded mostly homodimeric $\beta_1\beta_2$ GC crystals (98%) and some heterodimeric $\alpha\beta$ GC crystals (2%). Others also noted difficulties in obtaining heterodimer crystals of the wild-type catalytic domains and engineered a non-natural disulfide bridge at the interface to favor heterodimerization.²⁰ This suggests that $\beta_1\beta_2$ GC has a stronger propensity to crystallize and/or that the $\beta_1\beta_2$ GC interface is stronger. To address the first point, we performed native mass spectrometry and crystal packing analysis (Supporting Information) and showed that, in our case, TEV cleavage at the N-terminal and C-terminal sites in α GC yielded a minor population of α GC662 β GC heterodimers that were subsequently crystallized, thus explaining our low success in crystallizing $\alpha\beta$ GC heterodimers (Supporting Information). Second, to identify the structural determinants promoting heterodimerization of the catalytic subunits, we compared the heterodimeric $\alpha\beta$ GC structure with the homodimeric $\beta_1\beta_2$ GC structure (PDB entry 2WZ1) and analyzed both dimeric interfaces (Supporting Information). The structural superposition shows that the β_2 GC subunit of the homodimer and the β GC subunit of the heterodimer overlay closely, while the β_1 GC subunit of the homodimer and the α GC subunit of the heterodimer present distinct orientations (Figure 3A). In the heterodimer, α GC is rotated ~13° compared to β_1 GC in the homodimer, resulting in a different dimer interface. Both the heterodimeric and homodimeric interfaces contain a large number of hydrophobic interactions stabilizing the dimer core formed by both subunits and lining the substrate-binding site (Table S1 of the Supporting Information and Figure 3C,D). In addition, both interfaces contain several polar interactions between surface flexible structural elements (Figure 3E,F).

Despite the resemblance between the two dimeric interfaces, one major difference between the two structures is the conformation of the β -hairpins of residues 532–539 in β_1 GC and 587–593 in α GC. We will herein refer to these hairpins as “flaps”. In the symmetrical homodimer, both flaps—one contributed by each β GC subunit—participate in the dimer interface by wrapping securely onto the α 2 helix on the partner subunit, in a “double flap-wrap” conformation (Figure 3B). Residues from both flaps contribute one salt bridge and ten of the thirteen polar interactions that stabilize the homodimer. In the heterodimer, residues from the β_1 GC flap contribute three of the nine hydrogen bonds that stabilize the dimer (Table S1 of the Supporting Information), while the α 1GC flap is swung away and makes no interaction with the β GC subunit (Figure 3B). This suggests a different role of the flaps in the homodimer and the heterodimer. We further performed a structural comparison of all guanylate cyclase and adenylate cyclase catalytic domain structures (Supporting Information) and proposed that these surface-exposed structural flaps not only are involved in regulating the relative orientations of the subunits in various dimers but also may be important in modulating interactions with other domains or proteins. For sGC, our results indicate a key role of the interfacial flaps in stabilizing different dimer interfaces and suggest that the flaps may also be important for the proper orientation of the catalytic subunits in full-length sGC (see below).

Residues in the Interfacial Flap and Hydrogen Bond Network Play Key Roles in Modulating the Dimer Interface. To begin to understand the mechanisms involved in modulating sGC activity, we mapped sGC residues that have been shown to significantly impact activity and activation on our structure of the inactive heterodimer (Figures 4A,B) and

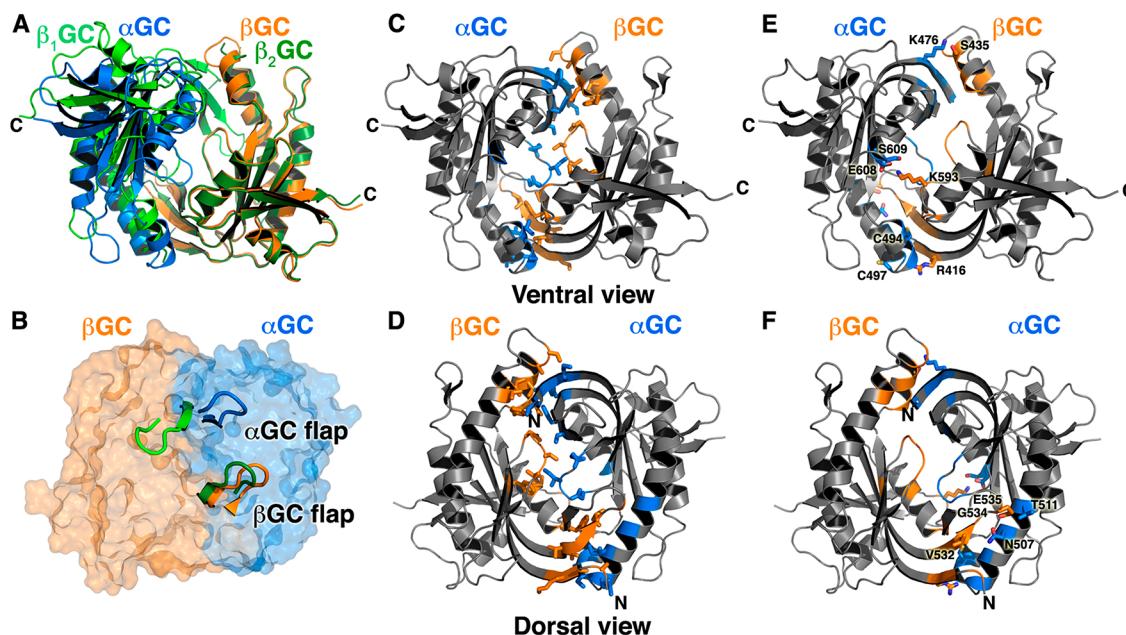


Figure 3. Structural determinants of catalytic domain dimerization. (A and B) Homodimeric $\beta_1\beta_2$ GC (PDB entry 2WZ1) is colored light green (β_1 GC) and dark green (β_2 GC). The $\alpha\beta$ GC heterodimer is colored blue (α GC) and orange (β GC). The ventral view (A) shows that β GC and β_2 GC subunits superimpose well, while α GC adopts a conformation different from that of β_1 GC in the heterodimer and homodimer, respectively. The dorsal view (B) shows the double flap-wrap conformation of the homodimer flaps (light and dark green cartoon). In the heterodimer depicted as a semitransparent solvent accessible surface, only the β GC flap (orange cartoon) wraps on the α GC subunit while the α GC flap (blue cartoon) is flipped out. (C and D) The core of the heterodimeric interface is formed by numerous hydrophobic interactions between residues from the β GC subunit (orange) and the α GC subunit (blue), depicted as sticks. (E and F) Polar interactions (hydrogen bonds and salt bridges) also contribute to the heterodimeric interface. Residues from β GC (orange) and α GC (blue) participating in these interactions are shown as sticks.

our model for the active heterodimer (Figures 4C,D). These residues fall in two areas of the dimer interface. (a) The first includes residues located in the flap or in the region interacting with the flap on the partner subunit. In full-length sGC, the Met537Asn mutation in β GC leads to increased constitutive activity and a very strong response to NO and/or YC-1 activators.³⁷ Met537 is located on the β GC flap, and its position is not expected to change drastically between the inactive and active conformation. However, reorientation of α GC in the active heterodimer leads to movement of its α 2 helix and β 3 strand closer to the β GC flap and places Met537 closer to hydrogen-bonding residues α GC Lys524 and Thr527 located on the β 3 strand (Figures 4B,D). Thus, the Met537Asn mutation would enhance interactions between the β GC flap and α GC by providing extra hydrogen bond contacts between the two subunits. This hypothesis is strongly supported by mutagenesis studies showing that two mutations abolishing hydrogen bonds between α GC and the β GC flap (α GC Lys524Ala and α GC Asp514Ala) in full-length sGC impair dimerization and activity.³⁶ Similar mutations introduced into adenylate cyclase yielded similar phenotypes. The C2a Lys1014Asn mutation (analogous to the β GC Met537Asn mutation) increased activity and affinity between the two subunits in the absence of forskolin or $G_s\alpha$.³⁸ In addition, the C1a Asp424Ala mutation (analogous to the α GC Asp514Ala mutation) severely impaired activity and activation.³⁶ (b) Other residues modulating sGC activity are located in the core of the heterodimer at the interface between both subunits. The α GC residues Glu526 and Cys595 are located in interfacial regions that would undergo major conformational changes in the active form (Figure 4B). On the basis of the structure of active AC,³⁴ we hypothesize that α GC Glu526, α GC Cys595, and β GC

Thr474 form an interfacial hydrogen bond network (equivalent to the C1a Lys436—C1a Asp505—C2a Thr939 adenylate cyclase triad), as these residues are also in the proximity of each other in the modeled catalytic domain active structure (within 3.5 Å of each other). This is strongly supported by studies showing that mutations that abrogate the ability to form hydrogen bonds at this interface severely impair sGC activity (α GC Cys595Tyr, α GC Cys595Asp, α GC Glu526Ala, and α GC Cys595Asp/Glu526Lys)^{36,37} and adenylate cyclase activity (C1a Lys436Ala and C1a Asp505Ala).^{38,39} In contrast, the α GC Cys595Ser mutation dramatically increases sGC basal activity.⁴⁰ The Cys to Ser substitution is often described as “silent”. However, the two amino acids have distinct properties because of differences in size and polarity between oxygen and sulfur. Thus, it is possible that the Cys595Ser mutation would enhance the ability of this residue to form hydrogen bonds.⁴¹ While we cannot rule out the possibility that the Cys595Ser mutation may prevent Cys595 oxidation and subsequent enzyme inhibition, it is more likely that Cys595 plays a key role in the communication between the two subunits. Previous studies proposed that Cys595 may participate in binding of the sGC stimulator YC-1 and modulate YC-1/NO activation.³⁷ However, several groups have unambiguously ruled out the possibility of binding of YC-1 to the catalytic domains.^{27,42–49} Instead, we propose that both the interfacial flap and the hydrogen bond network enhance interactions between the two catalytic subunits and guide an optimal conformation of the active center necessary for activity.

Copurified $\alpha\beta$ GC Catalytic Domains Assemble as a Mixture of Monomers, Homodimers, and Heterodimers. To confirm our structural prediction that the homodimeric $\beta_1\beta_2$ GC interface is stronger than the heterodimeric $\alpha\beta$ GC

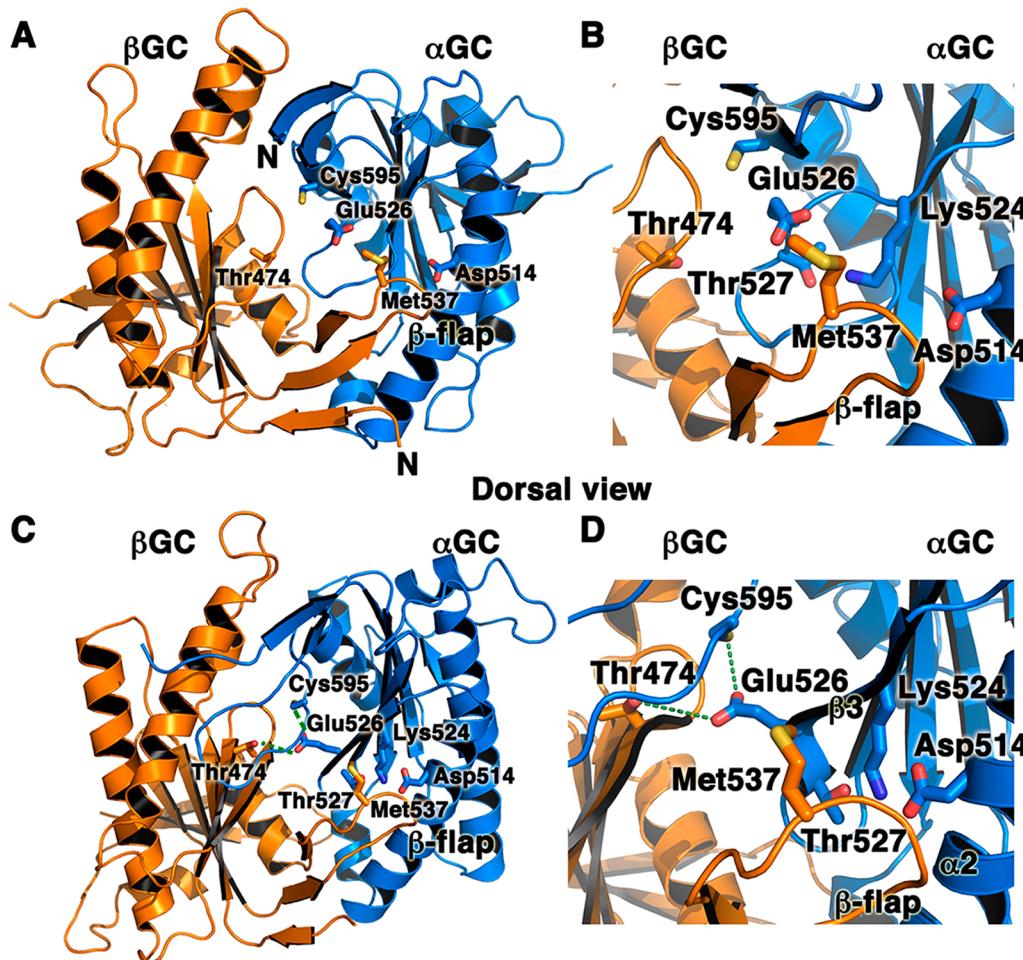


Figure 4. Interfacial mutations in adenylate cyclase and sGC that modulate enzyme activity underscore the key role of the heterodimeric interface for catalysis. We mapped AC and sGC mutations on the structures of the inactive $\alpha\beta$ GC heterodimer (A and B; PDB entry 4NI2) and the modeled active structure of $\alpha\beta$ GC (C and D). The model for the active conformation was generated with SWISSMODEL⁶⁷ by using active adenylate cyclase (PDB entry 1CJU) as a template. Residues that affect sGC or AC activity map to two regions of the $\alpha\beta$ GC heterodimer: (i) the interfacial hydrogen bond network among sGC residues α GC Cys595, α GC Glu526, and β GC Thr 474 (indicated by dashed green lines) and (ii) the sGC flap region (β GC Met537) or the region interacting with the flap in the partner subunit (α GC Asp514). Panels B and D are close-up views of panels A and C, respectively.

interface and the $\alpha\alpha$ GC interface, we used size-exclusion chromatography coupled to multiangle light scattering (SEC-MALS) and nano-ESI/MS (Figure S1 of the Supporting Information). Both the SEC (Figure S2A of the Supporting Information) and SEC-MALS profiles (Figure S2C of the Supporting Information) showed subtle deviations from perfectly symmetric peaks at higher elution volumes, suggesting slight heterogeneity in the sample composition. The molecular weight determined by SEC-MALS ranged from 96 to 106% of the theoretical molecular weight for the $\alpha\beta$ GC heterodimer. The subtle tail of the elution peak at high elution volumes contained more α GC than β GC as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). This suggested that the $\alpha\beta$ GC sample is a mixture of different oligomeric species in solution. However, we were unable to distinguish the different species in solution by SEC, MALS, or even mass spectrometry. Our result is not surprising given our overwhelming success in crystallizing $\beta\beta$ GC homodimers over $\alpha\beta$ GC heterodimers and has important implications when working with truncated sGC domains (see below).

Design of the Shorter α GC661 β GC Protein Allows Precise Quantification of Heterodimers in Solution. We showed above that the copurified $\alpha\beta$ GC protein is heterogeneous both in the length of the α GC subunit and in oligomer composition and that we were unable to quantify the different species in solution. This result is problematic for two reasons. First, structural mechanistic studies of the $\alpha\beta$ GC catalytic domains in the presence of metals, GTP, or GTP analogues require a reproducible homogeneous heterodimeric sample, and second, specific activity calculations require precise quantification of the $\alpha\beta$ GC heterodimers in solution, as only heterodimers are catalytically active.

Mass spectrometry and crystal packing analysis suggested that we had crystallized the heterodimeric $\alpha\beta$ GC catalytic domains with a truncated α GC(466–662) subunit (Supporting Information). To increase the likelihood of obtaining high-quality crystals of heterodimeric $\alpha\beta$ GC, we designed a shorter α GC construct. The XtalPred and DISOPRED2 web servers⁵⁰ predicted the region of residues 662–690 of α GC to be disordered. Therefore, we designed the shorter α GC661 construct that encompasses residues 466–661 after TEV cleavage of the N-terminal tag. The size-exclusion profile of

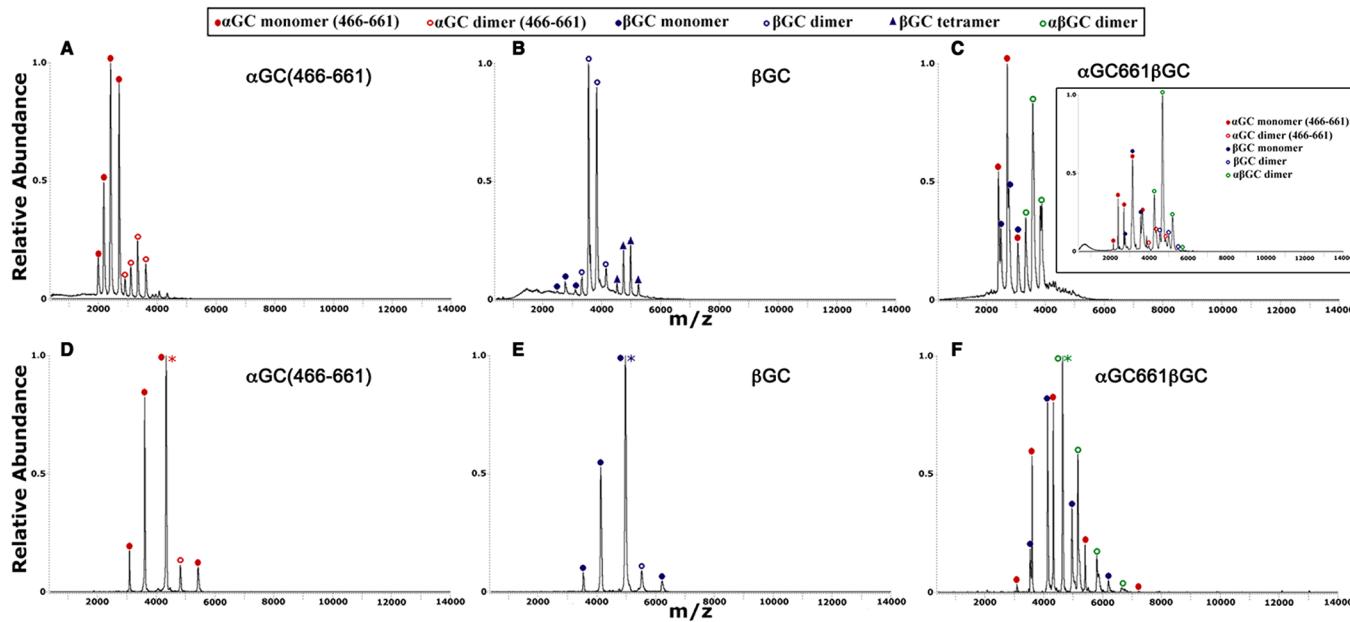


Figure 5. Nano-ESI/MS and nano-ESI/MS/MS (SID) reveal different oligomeric species present in α GC661, β GC, and α GC661/ β GC samples. (A–C) Nano-ESI/MS spectra were obtained by spraying a 10 μ M protein sample in 0.1 M NH_4OAc (pH 7.4). (A) α GC661 exists mostly as a monomer. (B) β GC exists predominantly as a dimer, with a small proportion of monomers and tetramers also present. (C) α GC661/ β GC is present in approximately equal amounts of monomer and dimer. The inset represents the spectrum for α GC661/ β GC (10 μ M) in 80 mM NH_4OAc and 20 mM TEAA (pH 7.4). The major species present was the α GC661/ β GC heterodimer. α GC661 and β GC monomers and $\alpha\alpha$ GC661 and $\beta\beta$ GC homodimers were also present. (D–F) Samples (10 μ M) in 80 mM NH_4OAc and 20 mM TEAA were sprayed, and the +11 precursor ion was chosen for MS/MS analysis at an SID voltage of 100 V. (D) α GC661 and (E) β GC dimers dissociate to give α GC661 and β GC monomers, respectively. (F) Dissociation of α GC661/ β GC results in an equal population of α GC661 and β GC monomers.

copurified α GC661/ β GC showed two overlapping peaks (Figure S2B of the Supporting Information) identified as homodimers and heterodimers by SDS-PAGE.

The calculated molecular masses for α GC661 and β GC differ by \sim 3 kDa (Table 2), allowing us to discriminate all species present in the α GC661/ β GC sample using mass spectrometry. First, we performed nano-ESI/MS for each subunit purified independently. Like that of α GC (Figure S1A of the Supporting Information), the spectrum for α GC661 showed that the protein was predominantly monomeric (mass of 21642.9 ± 18.3 Da) with a small amount of homodimers (mass of 43403.1 ± 5.5 Da) in a ratio of 79:21, as determined by AUC analysis (Figure 5A). As seen previously, the spectrum for β GC and AUC analysis showed that the monomer:dimer:tetramer ratio was 8:75:17 (Figure 5B). These results confirmed our prediction based on the crystal structure and modeling, and our size-exclusion experiments, and showed that the K_D for homodimerization of α GC661 was higher than that of β GC.

Second, we performed nano-ESI/MS for copurified α GC661/ β GC (Figure 5C). The complex spectrum showed a mixture of α GC661 monomers, β GC monomers, and α GC661/ β GC heterodimers, with an experimental mass for the heterodimer of 46449.5 ± 20.2 Da, which is close to the calculated value. After the addition of TEAA to reduce the overall charge of the complex and better separate the different charged species (Figure 5C, inset), the spectrum showed a mixture of α GC661 monomers, β GC monomers, $\alpha\alpha$ GC661 homodimers, $\beta\beta$ GC homodimers, and α GC661/ β GC heterodimers. We further used SID to confirm the identity of the different peaks (Figure 5D–F). These results unambiguously confirmed that the copurified α GC661/ β GC protein is a complex mixture of different oligomeric species.

To estimate the relative abundance of each species, we measured nano-ESI/MS spectra for α GC661, β GC, and α GC661/ β GC proteins at various protein concentrations. For α GC661, we observed only monomers and dimers. Thus, the K_D for α GC661 homodimerization was defined as the concentration at which the concentrations of the $\alpha\alpha$ GC661 dimer and the α GC661 monomer are equal and was determined to be 30 μ M (Figure S3A of the Supporting Information). For β GC, we observed several species in solution, including monomers, dimers, and tetramers, complicating the determination of K_D . Furthermore, the ionization efficiency of the β GC protein was significantly reduced at low concentrations. As a result, we can only estimate that the K_D for the $\beta\beta$ GC dimer is <2 μ M (Figure S3B of the Supporting Information). We repeated this analysis for various concentrations of α GC661/ β GC (Figure 6). First, we noted that at high concentrations, the relative abundance of all α GC species is almost equal to that of all β GC species. However, at low concentrations, the fraction of α GC is much higher than that of β GC. This may be due to the decreased ionization efficiency of β GC at low concentrations, as mentioned above. Therefore, we postulated that the K_D for the α GC661/ β GC heterodimer can be determined from the point at which the relative abundances of α GC and α GC661/ β GC are equal, which corresponds to a K_D of ~ 6.9 μ M. Finally, mass spectrometry analysis showed that mixing independently purified α GC661 and β GC subunits yielded less α GC661/ β GC heterodimer than purifying them together (data not shown).

Overall, the use of the truncated α GC661 construct allowed us to determine the K_D for the α GC661/ β GC heterodimer and quantify the amount of heterodimers present in solution at any given concentration to calculate the true specific activity normalized per mole of heterodimer. We firmly established that

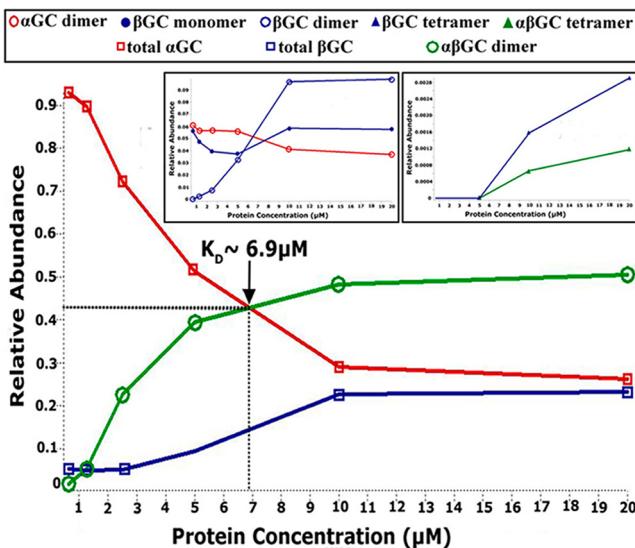


Figure 6. Determination of K_D for α GC661 β GC heterodimers by nano-ESI/MS. Spectra for α GC661 β GC were obtained by spraying the protein sample in 80 mM NH₄OAc and 20 mM TEAA (pH 7.4). The concentration of α GC661 β GC was varied by performing serial dilutions from an initial 50 μ M stock solution over a range of 0.6–20 μ M. The main panel shows the changes in the relative abundance of α GC661 β GC, total α GC661, and total β GC as a function of protein concentration. The insets show the relative abundance of the minor species in the α GC661 β GC sample as the concentration changes. For all experiments, the abundance of a particular species was determined by extracting its intensity from the ion mobility mobilogram that is produced with the mass spectrum, and the area under the curve (AUC) was determined using Origin.

the monomer–dimer dissociation constants for the catalytic domains increase as follows: $\beta\beta$ GC < $\alpha\beta$ GC ≪ $\alpha\alpha$ GC. This is in contrast to earlier studies suggesting that the K_D of $\beta\beta$ GC or $\alpha\alpha$ GC homodimers was much higher than the K_D of the $\alpha\beta$ GC heterodimer.²² These conflicting results may be due to distinct techniques used to characterize oligomeric assemblies. As opposed to low-resolution size-exclusion chromatography used previously,²² we used native mass spectrometry coupled to SID, which affords high accuracy in the stoichiometry of noncovalent complexes while preserving a nativelike quaternary structure.⁵¹ Importantly, our results support and extend a recent study showing that homodimers and heterodimers of the catalytic domains are both present in solution.²⁰

Our results have important implications for activity assay measurements with truncated sGC constructs commonly used by various laboratories. (i) A protein concentration well above the K_D should be chosen to ensure adequate heterodimer formation in the reaction mixture, and (ii) the true specific activity should be calculated on the basis of the heterodimer concentration only, as monomers and homodimers display no activity but contribute to the total protein concentration. While these requirements may not apply to wild-type full-length sGC, which mostly forms heterodimers *in vitro*, these results are critical for accurate activity assay measurements with mutant full-length sGC that show impaired dimerization.^{24,36,52–54}

Finally, our mass spectrometry approach to determine dimerization dissociation constants can be applied to truncated sGC constructs, encompassing various domains to precisely determine the contribution of each domain to heterodimerization of full-length sGC. Dimerization determinants were shown

to be predominantly contained in the HNOXA and coiled-coil (CC) domains,^{24,42,53–56} with some contribution from the catalytic domains.^{22,57} However, X-ray structures of homodimers were determined for HNOXA and CC domains,^{16,57} suggesting that, under these conditions, both domains can also homodimerize with K_D values that were estimated to be in the micromolar range.^{16,57} While a major role for the CC domain was proposed,⁵⁷ the precise determinants favoring sGC heterodimerization remain ambiguous and warrant further studies.

Isolated Catalytic Domains Display Low Activity

Despite Forming Heterodimers. Because we crystallized the apo $\alpha\beta$ GC heterodimer, we wanted to confirm that the isolated catalytic domains were nonetheless active. Others have shown that the regulatory N-terminal β HNOX–HNOXA domains inhibit the activity of the catalytic domains.^{22,23} Thus, in the absence of the regulatory domains, the isolated $\alpha\beta$ GC domains should be as active as activated full-length sGC. We measured cGMP formation with our purified $\alpha\beta$ GC and α GC661 β GC proteins, and human full-length sGC that was overexpressed in baculovirus (kind gift from E. Martin, The University of Texas Health Science Center at Houston, Houston, TX). We used α GC protein purified independently as a negative control.

The results presented above show that the copurified $\alpha\beta$ GC protein is a heterogeneous mixture of monomers, homodimers, and heterodimers. Because heterodimeric $\alpha\beta$ GC is the only catalytically active species, the true specific activity should be calculated by normalizing to the heterodimer concentration. This has proven to be difficult with wild-type $\alpha\beta$ GC protein (see above). The design of the truncated α GC661 β GC construct has allowed us to calculate the ratio of heterodimers at different total protein concentrations. First, we showed that α GC661 β GC [1.2 + 0.4 or – 0.3 fmol of cGMP min⁻¹ (pmol of enzyme)⁻¹] and untruncated $\alpha\beta$ GC catalytic domains [1.8 + 0.7 or – 0.5 fmol of cGMP min⁻¹ (pmol of enzyme)⁻¹] display comparable activity. Thus, the remainder of the analysis is based on α GC661 β GC for which we can calculate the true specific activity by normalizing to heterodimer concentration based on mass spectrometry data. For full-length sGC, mass spectrometry analysis showed that the protein is 100% heterodimeric at the concentration used for the cGMP reaction (data not shown). Importantly, copurified α GC661 β GC displayed only a fraction (0.01%) of the specific activity of basal full-length sGC in the presence of Mg²⁺ (Table 3). In the presence of Mn²⁺, the activities of α GC661 β GC and full-length sGC increased (779- and 2-fold, respectively), but α GC661 β GC still displayed <6% of the activity of full-length sGC. These results suggest that the isolated catalytic domains are not catalytically competent compared to basal full-length sGC, despite their ability to heterodimerize. Our structural studies of $\alpha\beta$ GC catalytic domains corroborate this result, as we crystallized the heterodimer in an inactive conformation. In the related adenylate cyclase, binding of Gs ω , forskolin, and ATP induces conformational changes leading to a closed and active catalytic center.³⁴ A similar mechanism is likely for sGC, whereby the catalytic center alternates between inactive and active conformations via structural rearrangements.

We propose that other sGC domains (including the coiled coil) will guide conformational changes leading to an optimal alignment of the active site residues. This model is supported by recent data showing that an $\alpha\beta$ HNOXA-CC-GC construct displays activity comparable to that of full-length sGC,⁵⁸ and by

Table 3. Guanylate Cyclase Activity^a

	$\alpha\text{GC}661\beta\text{GC}$		basal full-length sGC		αGC
	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺	Mg ²⁺
specific activity [fmol of cGMP min ⁻¹ (pmol of enzyme) ⁻¹]	1.2	899.9	10075.5	20578.5	0.5
upper bound error ^b	0.4	259.8	3163.7	5819.0	0.3
lower bound error ^b	0.3	200.7	2300.2	4433.3	0.2
relative abundance of the heterodimer (%) ^c	82.5	82.5	100	100	NA ^d
adjusted specific activity [fmol of cGMP min ⁻¹ (pmol of heterodimer) ⁻¹]	1.4	1090.8	10075.5	20578.5	NA ^d
upper bound error ^b	0.5	314.9	3163.7	5819.0	NA ^d
lower bound error ^b	0.4	243.2	2300.2	4433.3	NA ^d
activity normalized to full-length sGC (%)	0.01	5.3	100	100	NA ^d
<i>x</i> -fold increase in specific activity with Mn ²⁺	1	779	1	2	NA ^d

^aThe reaction was performed as described in Experimental Procedures. ^bError boundaries describe the 95% confidence interval.

^cDetermined by mass spectrometry. ^dNot applicable.

our results showing that substitution of Mg²⁺ with Mn²⁺ increases the basal activity of full-length sGC and $\alpha\text{GC}661\beta\text{GC}$ (Table 3). We propose that Mn²⁺ ions allow catalysis in both proteins without the need for the catalytic domains to undergo the transition to an optimal conformation. While Mg²⁺ requires a very specific coordination geometry,⁵⁹ Mn²⁺ has been shown to be less stringent.⁶⁰ In DNA polymerases, Mn²⁺ easily replaces Mg²⁺ but it also allows reactions to occur with mutated catalytic residues and decreased substrate specificity because of its higher tolerance for suboptimal metal coordination.⁶¹ Similarly, for full-length sGC and isolated $\alpha\beta\text{GC}$ catalytic domains, Mn²⁺ may favor a suboptimal conformation of the catalytic domains. This hypothesis is supported by studies showing that NO and/or YC-1 fails to fully activate sGC in the presence of Mn²⁺,^{40,55,62,63} suggesting that the enzyme is locked in an intermediate catalytic state. In conclusion, our activity measurements strongly support a role for other sGC domains in promoting a competent conformation of the active center.

■ DISCUSSION

The combined structural, biophysical, and mass spectrometry analyses presented here clarify widely accepted ideas regarding heterodimerization and activity of the isolated catalytic subunits of sGC and further provide the basis for understanding the regulation of sGC catalytic activity. We propose a novel role for interfacial structural elements in modulating the conformation of the active site for optimal activity. Finally, our results allow us to propose a comprehensive regulatory model in which distinct domain–domain interactions in sGC prevent or guide an optimal conformation of the sGC catalytic center associated with high activity.

Overall Structure of $\alpha\beta\text{GC}$ Catalytic Domains and Comparison with the Mutant Structure. To determine whether conformational changes are required for catalytic activity, we determined the X-ray structure of the $\alpha\beta\text{GC}$ catalytic domains from human sGC. We successfully obtained crystals of the wild-type catalytic domains, without the need for an engineered disulfide.²⁰ In our case, cleavage of the 28 C-terminal amino acids of αGC yielded a small amount of

truncated $\alpha\text{GC}662\beta\text{GC}$ catalytic domains that formed favorable crystal contacts, unlike the untruncated catalytic domains. The structural comparison of the mutant and wild-type $\alpha\beta\text{GC}$ catalytic domains reveals that the two structures are very similar but exhibit subtle differences at the dimer interface. Both our structure and that of the mutant $\alpha\beta\text{GC}$ catalytic domains were obtained in the absence of metal and nucleotide and show inactive conformations of the active center. This is evidenced by the extended loop conformation of the substrate binding regions at the core of the dimer interface, and the drastically different orientation of the αGC subunit relative to the βGC subunit, compared to the structure of active adenylate cyclase catalytic domains.³⁴ Potent sGC inhibitors have recently been described.⁶⁴ These nucleotide analogues show K_i values in the low nanomolar range and were predicted by docking to bind to an active conformation of the catalytic domains. We are currently exploring the use of these molecules to favor the crystallization of the active heterodimeric catalytic domains of sGC.

Residues at the Heterodimeric Interface Guide an Optimal Conformation of the Active Center. Our structure allowed us to propose a key role for structural elements of the catalytic domains in guiding an optimal conformation of the active center for activity. Our mass spectrometry studies, yielding a relatively weak heterodimerization affinity, and our structural comparisons strongly suggest that the dimer interface is flexible for achieving structural transitions from an inactive open form to an activated closed form, which has been observed for adenylate cyclase.³⁴ On the basis of our structural studies and mutagenesis data,^{36,37,40} we propose that both the interfacial flap and the hydrogen bond network enhance interactions between the two catalytic subunits and guide an optimal conformation of the active center necessary for activity. Studies are now underway to determine whether these activating mutations can facilitate structural studies of the sGC catalytic domains in the active state.

The Cyclase Domains Require Other sGC Domains for Optimal Activity. Our activity assay results demonstrate that even in the absence of the regulatory domains, additional sGC domain interactions are required to guide the appropriate conformation of the catalytic subunits associated with high activity. We propose that sGC activity is modulated by domain–domain interactions that allow the catalytic domains to undergo the transition from an inactive to an active conformation according to the following model: in the basal state, catalytic domains are constrained in a suboptimal conformation via inhibitory interactions with sGC domains, including the N-terminal βHNOX (and HNOXA) domain,^{22,23} which is itself maintained in an inhibited conformation by the αHNOX and αHNOXA domains.²⁷ Binding of NO and/or activators releases these inhibitory interactions and induces conformational changes transmitted to the catalytic domains via the coiled-coil domain²⁵ to yield the activated state (Figure 7). How the conformational transitions of these domains are orchestrated in sGC is still unknown. However, recent electron microscopy and hydrogen–deuterium exchange mass spectrometry suggest that full-length sGC presents multiple conformations with several interdomain pivot points allowing conformational changes to be transmitted from the regulatory domains to the catalytic domains.^{21,65} The observed “closing” of the catalytic interface upon NO binding⁶⁵ corroborates our proposal that other sGC domains, including the coiled coil, will

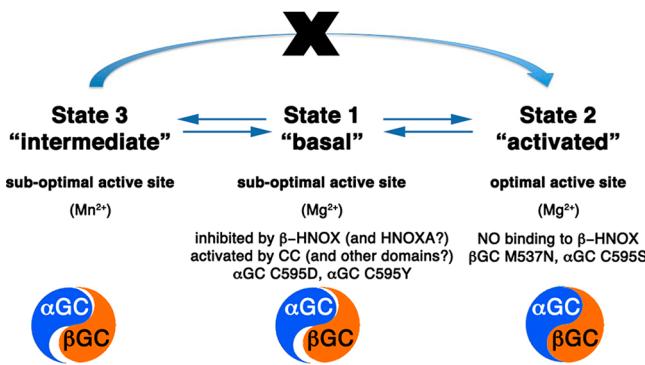


Figure 7. Model for domain–domain interactions that influence the conformation of the heterodimeric GC interface to modulate the activity of sGC. In state 1 (“basal”), competing domain–domain interactions yield sGC with low activity. While some sGC domains (including the coiled coil) promote conformational changes of the catalytic subunits, the regulatory β -HNOX domain (and possibly the HNOXA domain) inhibits activity via direct binding to α GC. Mutations in the α GC subunit (Cys595Asp and Cys595Tyr) also prevent conformational changes in the active site. In state 2 (“activated”), binding of NO to β -HNOX removes the inhibition and allows further conformational changes of the catalytic domains to yield fully active sGC. Mutations in catalytic subunits (β GC Met537Asn and α GC Cys595Ser) also yield an “activated” phenotype. In state 3 (“intermediate”), Mn^{2+} allows catalysis with a nonoptimal conformation of the catalytic domains. However, the enzyme is now locked in an intermediate state and cannot be further activated by NO and/or activators. The catalytic domains (represented as a blue and orange yin-yang) are in an inactive conformation (poor alignment) in states 1 and 3, and an active conformation in state 2 (perfect alignment of yin and yang).

guide conformational changes in the catalytic domains, leading to an optimal alignment of the active site residues for full sGC activity.

CONCLUSIONS

Our results allow us to propose that novel structural elements, the interfacial β -flap and hydrogen bond network, play a key role in sGC catalytic activity by enhancing interactions between the two catalytic subunits and guiding the active center to an optimal conformation.

This is the first study demonstrating that the catalytic $\alpha\beta$ GC domains require additional sGC domains for activity, despite their ability to heterodimerize. Overall, collective results provide evidence that other sGC domains modulate the relative orientation of the catalytic subunits and control the proper orientation of key residues in the catalytic domain for full enzyme activity. The fine balance between inhibitory and activating domain–domain interactions is modulated by NO and/or activators. As such, small molecules that influence the orientation of the catalytic subunits have a strong potential to shift this equilibrium. This work provides the basis for a novel model for sGC activation and opens additional drug discovery routes for targeting the NO–cGMP pathway.

ASSOCIATED CONTENT

Supporting Information

Movie showing the superimposition of the wild-type and mutant (PDB entry 3UVJ) heterodimeric structures of the sGC catalytic domains (Movie S1), additional results, Table S1,

sequence alignment, and Figures S1–S3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Department of Chemistry and Biochemistry, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250. E-mail: egarcin@umbc.edu. Telephone: (410) 455-2512. Fax: (410) 455-2608.

Funding

This work was supported in part by grants from the American Heart Association (10SDG2600345 to E.D.G. and 13PRE17000045 to F.S.), the National Institutes of Health (R01GM039345 to J.A.T.), and the National Science Foundation (DBI0923551 to V.H.W.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Susan E. Tsutakawa, Robert P. Rambo, Scott Classen, Thomas Miller, and James Fishbein for critical reading of the manuscript, technical support, and insightful discussions. Part of this work was conducted at the Advanced Light Source (ALS), a national user facility operated by Lawrence Berkeley National Laboratory on behalf of the Department of Energy (DOE), Office of Basic Energy Sciences, through the Integrated Diffraction Analysis Technologies (IDAT) program, supported by the DOE Office of Biological and Environmental Research. Additional support comes from the National Institute of Health project MINOS (R01GM105404).

ABBREVIATIONS

sGC, full-length soluble guanylyl cyclase or soluble guanylate cyclase; CC, coiled coil; H-NOX or HNOX, heme-nitric oxide oxygen binding; HNOXA, HNOX-associated; GC, guanylate cyclase catalytic domain; SEC-MALS, size-exclusion chromatography coupled to multiangle light scattering; SID, surface-induced dissociation; $\alpha\beta$ GC, construct containing only the α and β catalytic domains of sGC; rmsd, root-mean-square deviation.

REFERENCES

- (1) Voetsch, B., Jin, R. C., and Loscalzo, J. (2004) Nitric oxide insufficiency and atherosclerosis. *Histochem. Cell Biol.* 122, 353–367.
- (2) Ghofrani, H.-A., Galiè, N., Grimminger, F., Grünig, E., Humbert, M., Jing, Z.-C., Keogh, A. M., Langleben, D., Kilama, M. O., Fritsch, A., Neuser, D., and Rubin, L. J. (2013) Riociguat for the Treatment of Pulmonary Arterial Hypertension. *N. Engl. J. Med.* 369, 330–340.
- (3) Follmann, M., Griebenow, N., Hahn, M. G., Hartung, I., Mais, F.-J., Mittendorf, J., Schäfer, M., Schirok, H., Stasch, J.-P., Stoll, F., and Straub, A. (2013) The Chemistry and Biology of Soluble Guanylate Cyclase Stimulators and Activators. *Angew. Chem., Int. Ed.* 52, 9442–9462.
- (4) Conole, D., and Scott, L. (2013) Riociguat: First global approval. *Drugs* 73, 1967–1975.
- (5) Stasch, J.-P., and Evgenov, O. V. (2013) Soluble Guanylate Cyclase Stimulators in Pulmonary Hypertension. In *Pharmacotherapy of Pulmonary Hypertension* (Humbert, M., Evgenov, O. V., and Stasch, J.-P., Eds.) pp 279–313, Springer, Berlin.
- (6) Moncada, S., and Higgs, A. (1993) The L-Arginine-Nitric Oxide Pathway. *N. Engl. J. Med.* 329, 2002–2012.
- (7) Naseem, K. (2005) The role of nitric oxide in cardiovascular diseases. *Mol. Aspects Med.* 26, 33–65.

- (8) Thomas, D. D., Ridnour, L. A., Isenberg, J. S., Flores-Santana, W., Switzer, C. H., Donzelli, S., Hussain, P., Vecoli, C., Paolocci, N., Amba, S., Colton, C. A., Harris, C. C., Roberts, D. D., and Wink, D. A. (2008) The chemical biology of nitric oxide: Implications in cellular signaling. *Free Radical Biol. Med.* 45, 18–31.
- (9) Isenberg, J. S., Ridnour, L. A., Perruccio, E. M., Espey, M. G., Wink, D. A., and Roberts, D. D. (2005) Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13141–13146.
- (10) Miller, T. W., Isenberg, J. S., and Roberts, D. D. (2010) Thrombospondin-1 is an inhibitor of pharmacological activation of soluble guanylate cyclase: TSP-1 inhibits sGC activators. *Br. J. Pharmacol.* 159, 1542–1547.
- (11) Ramanathan, S., Mazzalupo, S., Boitano, S., and Montfort, W. R. (2011) Thrombospondin-1 and Angiotensin II Inhibit Soluble Guanylyl Cyclase through an Increase in Intracellular Calcium Concentration. *Biochemistry* 50, 7787–7799.
- (12) Kamisaki, Y., Saheki, S., Nakane, M., Palmieri, J. A., Kuno, T., Chang, B. Y., Waldman, S. A., and Murad, F. (1986) Soluble guanylate cyclase from rat lung exists as a heterodimer. *J. Biol. Chem.* 261, 7236–7241.
- (13) Pellicena, P., Karow, D. S., Boon, E. M., Marletta, M. A., and Kuriyan, J. (2004) Crystal structure of an oxygen-binding heme domain related to soluble guanylate cyclases. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12854–12859.
- (14) Nioche, P., Berka, V., Vipond, J., Minton, N., Tsai, A. L., and Raman, C. S. (2004) Femtomolar sensitivity of a NO sensor from *Clostridium botulinum*. *Science* 306, 1550–1553.
- (15) Ma, X., Sayed, N., Beuve, A., and van den Akker, F. (2007) NO and CO differentially activate soluble guanylyl cyclase via a heme pivot-bend mechanism. *EMBO J.* 26, 578–588.
- (16) Ma, X., Sayed, N., Baskaran, P., Beuve, A., and van den Akker, F. (2008) PAS-mediated dimerization of soluble guanylyl cyclase revealed by signal transduction histidine kinase domain crystal structure. *J. Biol. Chem.* 283, 1167–1178.
- (17) Purohit, R., Weichsel, A., and Montfort, W. R. (2013) Crystal structure of the α subunit PAS domain from soluble guanylyl cyclase. *Protein Sci.* 22, 1439–1444.
- (18) Rauch, A., Leipelt, M., Russwurm, M., and Steegborn, C. (2008) Crystal structure of the guanylyl cyclase Cya2. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15720–15725.
- (19) Winger, J. A., Derbyshire, E. R., Lamers, M. H., Marletta, M. A., and Kuriyan, J. (2008) The crystal structure of the catalytic domain of a eukaryotic guanylyl cyclase. *BMC Struct. Biol.* 8, 42.
- (20) Allerston, C. K., von Delft, F., and Gileadi, O. (2013) Crystal Structures of the Catalytic Domain of Human Soluble Guanylyl Cyclase. *PLoS One* 8, e57644.
- (21) Campbell, M. G., Underbakke, E. S., Potter, C. S., Carragher, B., and Marletta, M. A. (2014) Single-particle EM reveals the higher-order domain architecture of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2960–2965.
- (22) Winger, J. A., and Marletta, M. A. (2005) Expression and characterization of the catalytic domains of soluble guanylyl cyclase: Interaction with the heme domain. *Biochemistry* 44, 4083–4090.
- (23) Underbakke, E. S., Iavarone, A. T., and Marletta, M. A. (2013) Higher-order interactions bridge the nitric oxide receptor and catalytic domains of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6777–6782.
- (24) Rothkegel, C., Schmidt, P. M., Atkins, D. J., Hoffmann, L. S., Schmidt, H. H., Schroder, H., and Stasch, J. P. (2007) Dimerization region of soluble guanylyl cyclase characterized by bimolecular fluorescence complementation in vivo. *Mol. Pharmacol.* 72, 1181–1190.
- (25) Busker, M., Neidhardt, I., and Behrends, S. (2013) Nitric Oxide Activation of Guanylyl Cyclase Pushes the α_1 Signaling Helix and the β_1 Heme-binding Domain closer to the Substrate-binding Site. *J. Biol. Chem.* 289, 476–484.
- (26) Fritz, B. G., Roberts, S. A., Ahmed, A., Breci, L., Li, W., Weichsel, A., Brailey, J. L., Wysocki, V. H., Tama, F., and Montfort, W. R. (2013) Molecular Model of a Soluble Guanylyl Cyclase Fragment Determined by Small-Angle X-ray Scattering and Chemical Cross-Linking. *Biochemistry* 52, 1568–1582.
- (27) Purohit, R., Fritz, B. G., The, J., Issaian, A., Weichsel, A., David, C. L., Campbell, E. V., Hausrath, A. C., Rassouli-Taylor, L., Garcin, E. D., Gage, M. J., and Montfort, W. R. (2014) YC-1 binding to the β subunit of soluble guanylyl cyclase overcomes allosteric inhibition by the α subunit. *Biochemistry* 53, 101–114.
- (28) Leslie, A. G. W., and Powell, H. R. (2007) Processing diffraction data with mosflm. In *Evolving Methods for Macromolecular Crystallography* (Read, R. J., and Sussman, J. L., Eds.) pp 41–51, Springer, Dordrecht, The Netherlands.
- (29) McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* 40, 658–674.
- (30) Adams, P. D., Grosse-Kunstleve, R. W., Hung, L. W., Ioerger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K., and Terwilliger, T. C. (2002) PHENIX: Building new software for automated crystallographic structure determination. *Acta Crystallogr. D58*, 1948–1954.
- (31) Emsley, P., and Cowtan, K. (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr. D60*, 2126–2132.
- (32) Zhou, M., Dagan, S., and Wysocki, V. (2012) Protein subunits released by surface collisions of noncovalent complexes: Nativelike compact structures revealed by ion mobility mass spectrometry. *Angew. Chem., Int. Ed.* 51, 4336–4339.
- (33) Galhena, A., Dagan, S., Jones, C., Beardsley, R., and Wysocki, V. (2008) Surface-induced dissociation of peptides and protein complexes in a quadrupole/time-of-flight mass spectrometer. *Anal. Chem.* 80, 1425–1436.
- (34) Tesmer, J. J., Sunahara, R. K., Johnson, R. A., Gosselin, G., Gilman, A. G., and Sprang, S. R. (1999) Two-metal-Ion catalysis in adenylyl cyclase. *Science* 285, 756–760.
- (35) Haase, T., Haase, N., Kraehling, J. R., and Behrends, S. (2010) Fluorescent Fusion Proteins of Soluble Guanylyl Cyclase Indicate Proximity of the Heme Nitric Oxide Domain and Catalytic Domain. *PLoS One* 5, e11617.
- (36) Yuen, P. S., Doolittle, L. K., and Garbers, D. L. (1994) Dominant negative mutants of nitric oxide-sensitive guanylyl cyclase. *J. Biol. Chem.* 269, 791–793.
- (37) Lamothe, M., Chang, F. J., Balashova, N., Shirokov, R., and Beuve, A. (2004) Functional characterization of nitric oxide and YC-1 activation of soluble guanylyl cyclase: Structural implication for the YC-1 binding site? *Biochemistry* 43, 3039–3048.
- (38) Hatley, M. E. (2000) Isolation and Characterization of Constitutively Active Mutants of Mammalian Adenylyl Cyclase. *J. Biol. Chem.* 275, 38626–38632.
- (39) Tang, W.-J., Stanzel, M., and Gilman, A. G. (1995) Truncation and Alanine-Scanning Mutants of Type I Adenylyl Cyclase. *Biochemistry* 34, 14563–14572.
- (40) Friebel, A., Russwurm, M., Mergia, E., and Koesling, D. (1999) A Point-Mutated Guanylyl Cyclase with Features of the YC-1-Stimulated Enzyme: Implications for the YC-1 Binding Site? *Biochemistry* 38, 15253–15257.
- (41) Gregoret, L. M., Rader, S. D., Fletterick, R. J., and Cohen, F. E. (1991) Hydrogen bonds involving sulfur atoms in proteins. *Proteins: Struct., Funct., Genet.* 9, 99–107.
- (42) Koglin, M., and Behrends, S. (2004) Native human nitric oxide sensitive guanylyl cyclase: Purification and characterization. *Biochem. Pharmacol.* 67, 1579–1585.
- (43) Hu, X., Feng, C., Hazzard, J. T., Tollin, G., and Montfort, W. R. (2008) Binding of YC-1 or BAY 41-2272 to Soluble Guanylyl Cyclase Induces a Geminate Phase in CO Photolysis. *J. Am. Chem. Soc.* 130, 15748–15749.
- (44) Hu, X., Murata, L. B., Weichsel, A., Brailey, J. L., Roberts, S. A., Nighorn, A., and Montfort, W. R. (2008) Allostery in recombinant soluble guanylyl cyclase from *Manduca sexta*. *J. Biol. Chem.* 283, 20968–20977.

- (45) Denninger, J. W., Schelvis, J. P., Brandish, P. E., Zhao, Y., Babcock, G. T., and Marletta, M. A. (2000) Interaction of soluble guanylate cyclase with YC-1: Kinetic and resonance Raman studies. *Biochemistry* 39, 4191–4198.
- (46) Derbyshire, E. R., Fernhoff, N. B., Deng, S., and Marletta, M. A. (2009) Nucleotide Regulation of Soluble Guanylate Cyclase Substrate Specificity. *Biochemistry* 48, 7519–7524.
- (47) Sharina, I. G., Jelen, F., Bogatenkova, E. P., Thomas, A., Martin, E., and Murad, F. (2008) α_1 Soluble Guanylyl Cyclase (sGC) Splice Forms as Potential Regulators of Human sGC Activity. *J. Biol. Chem.* 283, 15104–15113.
- (48) Yoo, B.-K., Lamarre, I., Rappaport, F., Nioche, P., Raman, C. S., Martin, J.-L., and Negrerie, M. (2012) Picosecond to Second Dynamics Reveals a Structural Transition in *Clostridium botulinum* NO-Sensor Triggered by the Activator BAY-41-2272. *ACS Chem. Biol.* 7, 2046–2054.
- (49) Li, Z., Pal, B., Takenaka, S., Tsuyama, S., and Kitagawa, T. (2005) Resonance Raman Evidence for the Presence of Two Heme Pocket Conformations with Varied Activities in CO-Bound Bovine Soluble Guanylate Cyclase and Their Conversion. *Biochemistry* 44, 939–946.
- (50) Slabinski, L., Jaroszewski, L., Rychlewski, L., Wilson, I. A., Lesley, S. A., and Godzik, A. (2007) XtalPred: A web server for prediction of protein crystallizability. *Bioinformatics* 23, 3403–3405.
- (51) Zhou, M., Jones, C. M., and Wysocki, V. H. (2013) Dissecting the Large Noncovalent Protein Complex GroEL with Surface-Induced Dissociation and Ion Mobility–Mass Spectrometry. *Anal. Chem.* 85, 8262–8267.
- (52) Zabel, U., Hausler, C., Weeger, M., and Schmidt, H. H. (1999) Homodimerization of soluble guanylyl cyclase subunits. Dimerization analysis using a glutathione S-transferase affinity tag. *J. Biol. Chem.* 274, 18149–18152.
- (53) Wagner, C., Russwurm, M., Jager, R., Fribe, A., and Koesling, D. (2005) Dimerization of nitric oxide-sensitive guanylyl cyclase requires the $\alpha 1$ N terminus. *J. Biol. Chem.* 280, 17687–17693.
- (54) Shiga, T., and Suzuki, N. (2005) Amphipathic α -helix mediates the heterodimerization of soluble guanylyl cyclase. *Zool. Sci.* 22, 735–742.
- (55) Wedel, B., Harteneck, C., Foerster, J., Fribe, A., Schultz, G., and Koesling, D. (1995) Functional domains of soluble guanylyl cyclase. *J. Biol. Chem.* 270, 24871–24875.
- (56) Zhou, Z., Gross, S., Roussos, C., Meurer, S., Muller-Esterl, W., and Papapetropoulos, A. (2004) Structural and functional characterization of the dimerization region of soluble guanylyl cyclase. *J. Biol. Chem.* 279, 24935–24943.
- (57) Ma, X., Beuve, A., and van den Akker, F. (2010) Crystal structure of the signaling helix coiled-coil domain of the $\beta 1$ subunit of the soluble guanylyl cyclase. *BMC Struct. Biol.* 10, 2.
- (58) Sharina, I., Sobolevsky, M., Doursout, M.-F., Gryko, D., and Martin, E. (2011) Cobinamides Are Novel Coactivators of Nitric Oxide Receptor That Target Soluble Guanylyl Cyclase Catalytic Domain. *J. Pharmacol. Exp. Ther.* 340, 723–732.
- (59) Harding, M. M. (1999) The geometry of metal-ligand interactions relevant to proteins. *Acta Crystallogr. D55*, 1432–1443.
- (60) Yang, W., Lee, J. Y., and Nowotny, M. (2006) Making and Breaking Nucleic Acids: Two-Mg²⁺-Ion Catalysis and Substrate Specificity. *Mol. Cell* 22, 5–13.
- (61) Tabor, S., and Richardson, C. C. (1989) Effect of manganese ions on the incorporation of dideoxynucleotides by bacteriophage T7 DNA polymerase and *Escherichia coli* DNA polymerase I. *Proc. Natl. Acad. Sci. U.S.A.* 86, 4076–4080.
- (62) Hoenicka, M., Becker, M., Apeler, H., Sirichoke, T., Schroder, H., Gerzer, R., and Stasch, J. P. (1999) Purified soluble guanylyl cyclase expressed in a baculovirus/Sf9 system: Stimulation by YC-1, nitric oxide, and carbon monoxide. *J. Mol. Med.* 77, 14–23.
- (63) Beste, K. Y., Burhenne, H., Kaever, V., Stasch, J.-P., and Seifert, R. (2012) Nucleotidyl Cyclase Activity of Soluble Guanylyl Cyclase $\alpha_1\beta_1$. *Biochemistry* 51, 194–204.
- (64) Dove, S., Danker, K. Y., Stasch, J.-P., Kaever, V., and Seifert, R. (2014) Structure/Activity Relationships of (M)ANT- and TNP-Nucleotides for Inhibition of Rat Soluble Guanylyl Cyclase $\alpha_1\beta_1$. *Mol. Pharmacol.* 85, 598–607.
- (65) Underbakke, E. S., Iavarone, A. T., Chalmers, M. J., Pascal, B. D., Novick, S., Griffin, P. R., and Marletta, M. A. (2014) Nitric Oxide-Induced Conformational Changes in Soluble Guanylyl Cyclase. *Structure*, DOI: 10.1016/j.str.2014.01.008.
- (66) Wilkins, M. R., Lindskog, I., Gasteiger, E., Bairoch, A., Sanchez, J.-C., Hochstrasser, D. F., and Appel, R. D. (1997) Detailed peptide characterization using PEPTIDEMASS: A World-Wide-Web-accessible tool. *Electrophoresis* 18, 403–408.
- (67) Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* 22, 195–201.