Nitric oxide signaling controls collective contractions in a colonial choanoflagellate

Highlights

- The choanoflagellate *C. flexa* encodes a complete nitric oxide signaling pathway
- *C. flexa* responds to NO by contractions resulting in a feeding-to-swimming switch
- NO binds *C. flexa* soluble guanylate cyclase 1 (*Cf*sGC1) and induces cGMP synthesis
- sGC activity maintains NO-induced contractions

Authors

Josean Reyes-Rivera, Yang Wu, Benjamin G.H. Guthrie, Michael A. Marletta, Nicole King, Thibaut Brunet

Correspondence

marletta@berkeley.edu (M.A.M.), nking@berkeley.edu (N.K.), thibaut.brunet@pasteur.fr (T.B.)

In brief

Although nitric oxide signaling regulates key physiological processes in animals, its premetazoan origin is unclear. Reyes-Rivera et al. report a full animal-like NO signaling pathway in a close outgroup to animals, the multicellular choanoflagellate *Choanoeca flexa*, in which NO induces contractions, resulting in a switch from feeding to swimming.
Nitric oxide signaling controls collective contractions in a colonial choanoflagellate

Josean Reyes-Rivera,1 Yang Wu,2 Benjamin G.H. Guthrie,2 Michael A. Marletta,2,* Nicole King,1,* and Thibaut Brunet3,4,*

1Howard Hughes Medical Institute and the Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, USA
2Department of Chemistry and the Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, USA
3Institut Pasteur, Université Paris-Cité, Department of Cell Biology and Infection, and the Department of Developmental and Stem Cell Biology, 75015 Paris, France
*Lead contact
*Correspondence: marletta@berkeley.edu (M.A.M.), nking@berkeley.edu (N.K.), thibaut.brunet@pasteur.fr (T.B.)
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SUMMARY

Although signaling by the gaseous molecule nitric oxide (NO) regulates key physiological processes in animals, including contractility,1–3 immunity,4,5 development,6–9 and locomotion,10,11 the early evolution of animal NO signaling remains unclear. To reconstruct the role of NO in the animal stem lineage, we set out to study NO signaling in choanoflagellates, the closest living relatives of animals.12 In animals, NO produced by the nitric oxide synthase (NOS) canonically signals through cGMP by activating soluble guanylate cyclases (sGCs).13,14 We surveyed the distribution of the NO signaling pathway components across the diversity of choanoflagellates and found three species that express NOS (of either bacterial or eukaryotic origin), sGCs, and downstream genes previously shown to be involved in the NO/cGMP pathway. One of the species coexpressing sGCs and a bacterial-type NOS, Choanoeca flexa, forms multicellular sheets that undergo collective contractions controlled by cGMP.15 We found that treatment with NO induces cGMP synthesis and contraction in C. flexa. Biochemical assays show that NO directly binds C. flexa sGC1 and stimulates its cyclase activity. The NO/cGMP pathway acts independently from other inducers of C. flexa contraction, including mechanical stimuli and heat, but sGC activity is required for contractions induced by light-to-dark transitions. The output of NO signaling in C. flexa—contractions resulting in a switch from feeding to swimming—resembles the effect of NO in sponges1–3 and cnidarians,11,16,17 where it interrupts feeding and activates contractility. These data provide insights into the biology of the first animals and the evolution of NO signaling.

RESULTS

C. flexa encodes both NOS and sGC

C. flexa is a colonial choanoflagellate that forms concave sheets capable of global inversion of their curvature through collective contractility.15 C. flexa inversion mediates a trade-off between feeding and swimming: relaxed colonies (with their flagella pointing inside) are slow swimmers and efficient feeders, whereas contracted colonies (with their flagella pointing outside) are inefficient feeders but fast swimmers15 (Figure 1A). Inversion has been shown to be induced by light-to-dark transitions through the inactivation of a rhodopsin phosphodiesterase (Rho-PDE) and accumulation of cyclic guanosine monophosphate (cGMP).15 (Figure 1B). The involvement of cGMP in collective contraction in C. flexa and the connection between nitric oxide (NO)/cGMP signaling and tissue contraction in nonbilaterian animals18 led us to investigate whether NO signaling might exist in a colonial choanoflagellate phylogeny.12,22 make it difficult to pinpoint exactly when this horizontal gene transfer event occurred and whether it followed or preceded the loss of the ancestral eukaryotic NOS in most or all choanoflagellates. These choanoflagellate NO signaling pathways are therefore likely to have evolved convergently in multiple lineages.
genes, like those of cyanobacteria, differ from metazoan NOS, in which they encode an upstream globin domain with unknown function and lack the calmodulin-binding domain that mediates regulation of metazoan NOSs by Ca\(^{2+}\), suggesting that calcium signaling does not regulate NO synthesis in *C. flexa*. The *C. flexa* transcriptome also encodes complete biosynthetic pathways for the NOS cofactors tetrahydrobiopterin (BH\(_4\)), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and nicotinamide dinucleotide phosphate (NADPH) (Figure S2A) as well as downstream genes in the NO/cGMP signaling pathway: cGMP-dependent kinase (PKG), cGMP-gated ion channels (CNG), and cGMP-dependent PDE (PDEG) (Figure S2B), providing additional evidence that *C. flexa* employs NO signaling as part of its physiology.

Nearly all sGCs from choanoflagellates, including *C. flexa*, encode the canonical domains observed in animal sGCs: the heme NO-/O\(_2\)-binding domain (HNOB or H-NOX), the HNOB-associated domain (HNOBA), and the C-terminal catalytic domain (guanylate cyclase)\(^{22}\) (Figure 2C). Phylogenetic analysis revealed that all animal and most choanoflagellate sGCs (including those of *C. flexa*) evolved from a single ancestral sGC found in the last common ancestor of choanoflagellates and metazoans that diversified separately into multiple paralogs in these two lineages (Figure S1B). In contrast, the predicted sGC from one choanoflagellate species, *S. helianthica*, more closely resembles the sGCs of chlorophyte algae (which are the only protist group previously known to encode sGC proteins with a metazoan-like domain architecture\(^{24}\); Figure S1B).

Importantly, not all animal sGCs are regulated by NO: in *Drosophila melanogaster* and *Caenorhabditis elegans*, the so-called “atypical sGCs” preferentially bind soluble O\(_2\) instead of NO and are thought to be involved in the regulation of feeding by oxygen concentration.\(^{25–27}\) Discrimination between NO and O\(_2\) is mediated by the presence of a hydrogen-bonding network, where a distal pocket tyrosine residue is critical for stabilizing the heme-O\(_2\) complex.\(^{28}\) We generated preferential binding predictions based on this motif\(^{29,28}\) and found that both NO- and O\(_2\)-preferential binding sGCs are widely distributed among choanoflagellates with no obvious pattern (Figures 2A and S2D). Interestingly, predicted NO-selective
sGCs are present in choanoflagellate species in which NOS was not detected, suggesting that these species might detect NO from an exogenous source (i.e., environmental bacteria or other protists), might possess an alternative NO-producing mechanism, or might encode an NOS that was not detected in the transcriptome. In *C. flexa*, one of the four sGC transcripts was predicted to be selective for NO and was named *Cf*sGC1 (Figure S2D). The other two choanoflagellate species found to possess both an NOS and sGCs, *C. perplexa* and *S. infusionum*, were also predicted to encode at least one NO-sensitive sGC (Figure 2A).

**NO/cGMP signaling controls colony contraction in *C. flexa***

To test whether NO signaling regulates collective contractions in *C. flexa*, we treated *C. flexa* cultures with several NO donors, compounds capable of releasing NO in solution. We found that treatment of *C. flexa* with the NO donors prolinoNOSate and
DEANONOate led to an increase in intracellular NO as detected by the NO-sensitive fluorescent probe 4-Amino-5-Methylamino-2',7'-Difluorofluorescein (DAF-FM), demonstrating that they could be effective reagents for studying NO signaling in C. flexa in vivo (Figures 3A and S3A–S3C). Treatment of C. flexa with proliNONOate (Figures 3B and 3C) induced colony contraction within 1–2 min (although the inversion process itself only lasted a few seconds, as previously described for darkness-induced inversion\textsuperscript{15}; see Video S1). The inversion response of C. flexa to proliNONOate was concentration dependent (Figure 3D), reaching a plateau of nearly 100% inversion at a concentration of 0.1 m M. As a negative control, C. flexa did not invert in response to proline, the molecular backbone of proliNONOate and the end product of NO release (Figure 3D). Moreover,
In animals, NO-selective sGCs display three levels of activity: (1) in the absence of NO, the protein has a low basal guanylate cyclase activity; (2) when one NO molecule is bound at the heme moiety, the protein is partially activated (to several-fold the basal activity); and (3) in the presence of excess NO, the protein reaches maximal activation.34 To characterize the enzymatic activity of Cf sGC1, we measured cGMP production by purified Cf sGC1 under unliganded, equimolar NO, and excess NO conditions using an endpoint activity assay. We found that Cf sGC1 has an activity profile similar to that of animal sGCs, with a 2-fold increase in activity under equimolar NO concentration and ~6-fold increase under excess NO (Figure 3J). Overall, Cf sGC1 exhibits similar ligand binding properties and NO-stimulated activity profile to animal NO-specific sGCs.35 These results further support the existence of NO/cGMP signaling in C. flexa and is consistent with it being mediated (at least in part) by Cf sGC1.

**NO/cGMP signaling acts independently from most other inducers of colony contraction**

In animals, NO signaling can be induced by a broad range of stimuli, which include chemical signals (for example, acetylcholine in mammalian blood vessels35), mechanical cues (for example, shear stress in blood vessels36), or heat shocks.37–40 Interestingly, collar contractions in choanoflagellates can often be induced by mechanical stimuli, such as flow and touch.31–44 We thus set out to test whether NO signaling in C. flexa responds to or intersects with environmental inducers of inversion.

We observed that C. flexa colonies invert in a matter of seconds in response to agitation of culture flasks (which presumably combines the effect of flow and shocks with other colonies or the walls of the flask) and to heat shocks (Figures 4A and 4B). To test whether mechanically or heat-induced contraction requires NO/cGMP signaling, we incubated the colony cultures with the pan-sGC inhibitor ODQ and exposed them to either of the two different stressors. We found that the inhibition of sGCs did not abolish mechanically induced or heat shock-induced contraction (Figures 4A and 4B). Taken together, these findings suggest that the mechanosensitive and thermosensitive pathways that induce inversion in C. flexa are independent of sGCs and hint at complex behavioral regulation in this choanoflagellate.

Previous work has shown that C. flexa colonies invert in response to light-to-dark transitions that they detect through a Rho-PDE pathway.15 In the presence of light, a Rho-PDE hydrolyzes cGMP into 5′-GMP, thus preventing cGMP signaling. In darkness, the Rho-PDE is inactivated, which allows cGMP to accumulate and trigger colony inversion (Figure 1B). Interestingly, this pathway requires the presence of cGMP, which is presumably synthesized by either particulate (i.e., membrane bound) or soluble (i.e., cytosolic) guanylate cyclases,45 both of which are predicted to be encoded by the C. flexa transcriptome (Figure S2C). A third family of guanylate cyclases—NIT-GCs, recently discovered in animals—could not be detected in choanoflagellates.46

We next set out to answer whether sGCs are necessary for synthesizing the cGMP required for phototransduction. To address this, we treated light-sensitive colonies with ODQ and found that this entirely abolished darkness-induced inversion (Figure 4C). These results suggest that sGCs (either NO dependent or O2 dependent) are responsible for synthesizing baseline
levels of cGMP that are then used during phototransduction. Even though ODQ-treated light-sensitive colonies did not invert in response to darkness, we confirmed that they could still respond to NO by undergoing brief contractions (which were sustained for a much shorter time than in controls; Figure 4D), consistently with earlier results (Figure 3E).

**DISCUSSION**

Here, we report the presence of NOS, sGCs, and downstream components of NO/cGMP signaling in three choanoflagellate species, at least two of which (C. flexa and C. perplexa) are capable of collective contractions. To our knowledge, this is the first observation of both NOS and sGCs in a nonanimal model. We found that NO causes sustained colony contraction in C. flexa and an increase in cGMP concentration in live cells, and that inhibition of sGCs (and thereby reduction in cGMP concentration) accelerated colony relaxation. Moreover, in vitro experiments confirmed that NO directly binds Cf sGC1, which it activates with a two-step profile in response to different NO concentration, as in animal sGCs.

The observation that colonies treated with the sGC inhibitor initially contracted in response to NO at levels matching untreated colonies, only to relax much more quickly, was unexpected. We hypothesize that NO-induced contractions are mediated through at least two different pathways: a slow pathway (described above) that maintains contraction and requires sGC/cGMP, and an (unidentified) fast pathway independent of sGC/cGMP. Moreover, treatment of light-sensitive colonies with the sGC inhibitor abolished darkness-induced contractions (which are known to be mediated by cGMP) but did not prevent NO-induced contractions, further supporting the existence of a second pathway. In other organisms, cGMP-independent NO signaling can involve S-nitrosation, the modification of proteins through the formation of an S–NO covalent bond, although the direct targets and functions of S-nitrosation in animals are less well understood than NO/cGMP signaling. It is possible that this mechanism explains the cGMP-independent pathway underlying NO-induced colony contraction in C. flexa.

The control of multicellular behavior by NO/cGMP signaling in C. flexa is reminiscent of its function in animals, most notably in sponges. In the demosponges Tethya wilhelma, Ephydatia muelleri, and Spongilla lacustris, NO induces global contractions and stops flagellar beating in choanocyte chambers, which interrupts feeding, allows expulsion of clumps of waste, and flushes the aquiferous canal system (a behavior sometimes called “sneezing”). Recently, single-cell RNA sequencing in Spongilla lacustris revealed that pinacocytes (epithelial cells that cover and shape the sponge body) coexpress NOS and sGC, the actomyosin contractility module, and the transcription factor serum response factor (Srf), a master regulator of contractility. Control of motor and feeding behavior by NO/cGMP signaling in C. flexa is also observed in cnidarians and some bilaterians. In the jellyfish Aglantha digitale, NO/cGMP signaling in neurons induces a switch from slow swimming (associated with feeding) to fast swimming (associated with escape) and inhibits tentacular ciliary beating. In the sea pansy (a type of colonial cnidarian) Renilla koellikeri, NO/cGMP
increases the amplitude of peristaltic contractions associated with the movement of body fluids through the gastrovascular cavity.\textsuperscript{17} Finally, in the nudibranch \textit{Clione limacina} and the snail \textit{Lymnaea stagnalis}, NO activates both feeding and locomotory neural circuits.\textsuperscript{56-58} Thus, as in \textit{C. flexa}, the ancient functions of NO/cGMP signaling in animals may include the regulation of feeding and contraction.\textsuperscript{16,18,56,59–61} Interestingly, NO signaling also controls metamorphosis in sponges,\textsuperscript{7,62} gastropods,\textsuperscript{63} annelids,\textsuperscript{64} echinoderms,\textsuperscript{65,66} and ascidians\textsuperscript{65,67,68} thus regulating a switch from swimming to feeding during irreversible developmental programs. The conservation of the NO-sensitive transduction pathway across choanozoans (including sGC, PKG, CNG, and PDEG; Figure S2C) is consistent with a possible homology of the behavioral response to NO between choanoflagellates and animals. However, the mosaic distribution of eukaryotic and bacterial NOS across choanozoans (Figures 2A and S1A) suggests that the source of NO itself might have switched an unknown number of times during evolution between the ancestral eukaryotic NOS, the horizontally transferred bacterial NOS, and exogenous sources.

In future, identifying the function of \textit{Cf} NOS and \textit{C. flexa} NO- or O\textsubscript{2}-selective sGCs will require gene knockout, which is not yet possible in \textit{C. flexa}. Moreover, studies on \textit{Trichoplax} (in which NO/cGMP signaling has been predicted to exist based on genomic data\textsuperscript{69}), additional animal phyla, and other choanoflagellates will help flesh out reconstitutions of the early evolution of animal NO signaling.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.04.017.
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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** |
| *Escherichia coli* BL21 Star (DE3) | UC Berkeley QB3 Macrolab | N/A |
| *Escherichia coli* DH10b-T1R | Prepared in-house | N/A |
| **Biological samples** |
| *Choanoeca flexa* | Our lab, isolated from Curacao splash pool | [https://doi.org/10.1126/science.aay2346](https://doi.org/10.1126/science.aay2346) |
| **Chemicals, peptides, and recombinant proteins** |
| ODQ | BioVision | 2051 |
| proliNONOate | Cayman Chemical Company | 82145 |
| DEANONOate | Cayman Chemical Company | 82100 |
| DAF-FM | Invitrogen | D-23844 |
| DL-Proline | Sigma Aldrich | 609-36-9 |
| poly-D-lysine | Sigma Aldrich | P6407 |
| 16% paraformaldehyde | Fisher Scientific | 50-980-487 |
| Guanosine-5’-triphosphate, sodium salt hydrate | Sigma Aldrich | 36051-31-7 |
| Sodium dithionite | Sigma Aldrich | 7775-14-6 |
| DL-dithio-1,4-threitol | BACHEM | 3483-12-3 |
| Magnesium chloride hexahydrate | MP Biomedicals | 7791-18-6 |
| Pyridine | Sigma Aldrich | 110-86-1 |
| Potassium ferricyanide | Mallinckrodt Pharmaceuticals | 13746-66-2 |
| Precision Plus Protein Standard, unstained | BioRad | 161-0363 |
| **Critical commercial assays** |
| ENZO Direct cGMP ELISA kit | Enzo Life Sciences | ADI-900-014 |
| ENZO cGMP ELISA kit, extracellular | Enzo Life Sciences | ADI-901-013 |
| **Deposited data** |
| *Cf NOS* | GeneBank | ON075806 |
| *Cf sGC1* | GeneBank | ON075810 |
| *Cf sGC2* | GeneBank | ON075809 |
| *Cf sGC3* | GeneBank | ON075808 |
| *Cf sGC4* | GeneBank | ON075807 |
| **Oligonucleotides** |
| pYW5 Forward: TAAGAAGGAGATATACCA TG TATGGCTTGGTGCACGAAGC | This Paper | N/A |
| pYW5 Reverse: TAAATGGGTATGATGGTGA TG AACTATAGTCTGCTTGCCAACG | This Paper | N/A |
| **Recombinant DNA** |
| pYW5 (Cf sGC1 in pET28b backbone) | This paper | N/A |
| pGro7 (GroEL/GroES) | Takara Bio | 3340 |
| **Software and algorithms** |
| ImageJ version 2.3.0 | ImageJ Software Analysis | [https://imagej.nih.gov/ij/index.html](https://imagej.nih.gov/ij/index.html) |
| PRISM version 9.0.0 | GraphPad | [https://www.graphpad.com/](https://www.graphpad.com/) |
| **Other** |
| Agilent Technologies Cary 300 UV-vis Spectrophotometer | Agilent Technologies | 10071600 |
| Nanodrop 2000 Microvolume Spectrophotometer | Thermo Scientific | ND-2000 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thibaut Brunet (thibaut.brunet@pasteur.fr).

Materials availability
This study did not generate new unique reagents.

Data and code availability
All NOS and sGC sequences from C. flexa were deposited onto GenBank (accession numbers below). All other data reported in this paper will be shared by lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Culture of Choanoeca flexa
Colonies were cultured in 1% to 15% Cereal Grass Medium (CGM3) in artificial seawater (ASW). Polyxenic cultures (continuously passaged from a previously described environmental isolate15) were maintained at 22°C under a light-dark cycle of 12:12 hours in a Caron low temperature incubator equipped with a lamp (Venoya Full Spectrum 150W Plant Growth LED) controlled by a programmable timer (Leviton VPT24 1PZ Vizia). Polyxenic cultures used in most experiments were not light-sensitive, possibly due to progressive loss of bacterial diversity during serial passaging (as bacterially provided retinal is known to be required for photosensation in C. flexa15). Light-sensitive sheets used in photosensation experiments (Figures 4C and 4D) were thawed from stocks that had been frozen immediately after clonal isolation from a Curacao isolate and cultured as described above.

METHOD DETAILS

Light microscopy—Imaging
Colonies were imaged in FluoroDishes (World Precision Instruments FD35-100) by differential interference contrast (DIC) microscopy using a 20x Zeiss objective mounted on a Zeiss Observer Z.1 with Hamamatsu Orca Flash 4.0 V2 CMOS camera (C1140-22CU).

Compound treatments and colony inversion assays
Small molecule inhibitor treatments and colony inversion assays were performed in 24-well plates (Fisher Scientific 09-761-146) containing 1 mL C. flexa culture per well. ODQ (pan-soluble guanylate cyclase inhibitor, BioVision 2051) was added 1 hour before behavioral assays. Addition of each small molecule compound was followed by a gentle swirl of the 24-well plate to ensure mixing. For each assay, all colonies visible within a well were counted (at least 30 colonies per biological replicate). All behavioral experiments were conducted under ambient light in the laboratory, unless indicated otherwise.

NO donor-induced inversion
The NO donors proliNONOate (Cayman Chemical Company 82145) and DEANONOate (Cayman Chemical Company 82100) were dissolved according to provider’s instructions and stored as single-use aliquots at -80°C. Addition of NO donor proliNONOate induced inversion within 1-2 minutes. Prior to counting, colonies were fixed by addition of 16% ice-cold PFA in a 1:3 volumetric ratio, resulting in a final concentration of 4% PFA. Contracted and relaxed colonies were then manually counted by observation under a Leica DMIL LED transmitted light microscope.

Light-induced sheet inversion
After treatment with small molecule compounds, light-to-dark transitions were performed by manually switching off the light source of the DMIL LED microscope. The “light off” condition lasted for one minute before sheets were fixed and scored as described above.

REAGENT or RESOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| AKTA Purifier UPC 100 FPLC System | GE Healthcare | 10475 |
| POROS HQ20 10x100mm Anion Exchange Column | Applied Biosystems | 1232907 |
| Superdex 200 increase 10/300 GL Size Exclusion Column | GE Healthcare | GE28-9909-44 |
| Zeba Spin Desalting Column | Thermo Scientific | 89882 |
| His60 Ni Superflow Resin | Takara Bio | 635677 |
| SpectraMax M3 plate reader | Molecular Devices | 89429-536 |
Mechanically induced sheet inversion

3 mL of C. flexa culture were transferred to T12.5 culture flasks (Fisher Scientific 353107) and mechanically stimulated by vortexing on a Vortex Genie 2 (Scientific industries) on “Slow” setting for 5 seconds. Sheets were immediately fixed and scored as above.

Heat shock-induced sheet inversion

Colonies in 24-well plates were treated with inhibitors as described above and placed at the surface of a 37°C warm bath for one minute. Sheets were immediately fixed and scored as above.

cGMP ELISA

For in vivo quantification of cGMP was performed with an ENZO Direct cGMP ELISA kit (ADI-900-014, 96 wells) as directed by the manufacturer. For each biological replicate, 90 mL of dense (>10^6 cells/mL) C. flexa culture was centrifuged for 5 minutes at 3000 x g and resuspended in 25 mL of ASW to wash the bacteria away. After the third wash, the cells were resuspended in 200 μL of ASW and split into one control (100 μL) and one treated sample (100 μL). The samples were lysed and quantified in parallel in each assay. Colonies from the “NO donor” group were treated with 0.25 μM proline NONOate 5 minutes before lysis. Values were read on a SpectraMax M3 plate reader (Molecular Devices).

NO labeling, imaging, and image analysis

C. flexa cultures were transferred into 15 mL Falcon tubes and vortexed in “fast” setting on a Vortex Genie 2 for one minute to dissociate colonies into single cells. Cells were washed 3 times with artificial seawater (ASW) by centrifuging them for 5 minutes at 3000 x g and resuspending them in 25 mL of ASW. After the last wash, cells were resuspended in 1.5 mL ASW and transferred into a 1.5 mL Eppendorf tube. Cells were incubated in 10 μM DAF-FM (Invitrogen, D-23844) for 1 hour and rinsed twice with ASW to wash away the unincorporated dye. Cells were then transferred into a FluoroDish charged with a Corona surface treater and coated with poly-D-lysine, following a previously published protocol.15 We let the cells adhere to the bottom of the dish for 30 minutes before imaging on a Z.1 Zeiss Imager with a Hamamatsu Orca Flash 4.0 V2 CMOS camera (C11440-22CU) and a 40x water immersion objective (C-Plan). Images were taken using ImageJ. Change in fluorescence intensity was calculated by subtracting the fluorescence intensity at minute 1 from fluorescence intensity at minute 30.

Phylogenetic analysis and protein domain identification

We screened a selection of fully sequenced genomes for homologs of sGC and NOS with the following strategy: the protein sequences of Homo sapiens sGC1 and brain nitric oxide synthase (NOS1) were used as BLASTp queries against the NCBI database restricted to the following list of species:

- Eukaryotes: Homo sapiens (Hsa), Branchiostoma floridae (Bfl), Drosophila melanogaster (Dme), Capitella teleta (Cte), Nematostella vectensis (Nve), Amphimedon queselandica (Amq), Mnemiopsis leidyi, Trichoplax adhaerens (Tadh), Salpingoeca rosetta (Sro), Capsasporsa owczarzaki, Sphaereoides arctica (Spac), Aequorea victoria, Creolimax fragrantissima, Pirum gemma, Aspergillus oryzae (Asor), Jigmerdemania flammicorona (Jfll), Rhizoctonia solani (Rhs), Pterula gracilis (Ptega), Schizosaccharomyces pombe, Tuber melanosporum, Cryptococcus neoformans, Ustilago maydis, Cryptococcus neoformans, Ustilago maydis, Rhizopus oryzae, Allomyces macrognos, Batrachochytrium dendrobatidis, Spizellomyces punctatus, Thecamonas trahens, Dictyostelium discoideum, Polysphondylium pallidum, Entamoeba histolytica, Arabidopsis thaliana, Selaginella moellendorffii, Phycomytila patens, Chlamydomonas reinhardtii, Volvox carteri (Vcar), Chlorella variabilis (Chl), Ostreococcus tauri (Ostau), Ectocarpus siliculosus, Phaeodactylum tricornutum, Thalassiosira pseudonana, Phytophthora infestans, Toxoplasma gondii, Tetrahymena thermophila, Perkinsus marinus, Guillardia theta, Naegleria gruberi (Ngru), Trypanosoma cruzi, Leishmania major, Trichomonas vaginalis, Giardia lamblia, Bigelowiella natans, Emiliana huxleyi
- Archaea: Nanoarchaeum equitans, Ignicoccus islandicus, Natronolimnibius baerhuensis, Haloradiobacter regularis, Halostagnibacter aridus, Halobacterium sp. strain NRC-1, Haloarcula marismortui, Halorubrum lacus NOI, Halocarboxydus azovensis, Halococcus lipolytica
- Bacteria: Actinobacteria, Kibdelosporangium aridum (Kibd), Crossiella equi (Cross), Lentzea xinjiangensis (Lentz), Nocardioides oriculaci (Noc), Saracharopolyspora sp. (Sspa), Synechococcus sp. PCC 7335 (Syn), Nostoc cycladis (Nocy), Anabaenopsis circularis (Ancir), Planktothrix paucivesiculata (Plank), Cricnalis epipsammum (Crinep), Spirosoma radiotolerans (Spir), Roseinatronobacter monicus (Rose)

Additional BLASTp searches were conducted against a published dataset of 19 choanoflagellate transcriptomes,21 the C. flexa transcriptome,16, the Mnemiopsis leidyi genome (https://research.nhgri.nih.gov/mnemiopsis/sequenceserver/) and the Ministeria vi-brans transcriptome22 (and courtesy of Daniel J. Richter). The C. flexa NOS and sGC predicted protein sequences were deposited onto NCBI with the following accession numbers: GenBank: ON075806 (for Cf NOS), GenBank: ON075810 (for CfsGC1), GenBank: ON075809 (for CfsGC2), GenBank: ON075808 (for CfsGC3), and GenBank: ON075807 (for CfsGC4).

Domain architectures were predicted using the CD-search tool from NCBI. For phylogenetic reconstructions, sequences were aligned using Clustal implemented in Geneious Prime (2021 version). The NOS sequence alignment was manually trimmed to be restricted to the

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Domain architectures were predicted using the CD-search tool from NCBI. For phylogenetic reconstructions, sequences were aligned using Clustal implemented in Geneious Prime (2021 version). The NOS sequence alignment was manually trimmed to be restricted to the
oxygenase domain and the sGC alignment was trimmed using Gblocks with minimally stringent parameters. Phylogenetic trees were reconstructed using PhyML and BMGE implemented on http://phylogeny.lirmm.fr/phylo_cgi/index.cgi. Trees were visualized using iTOL (https://itol.embl.de/) and further edited in Adobe Illustrator 2021. Species silhouettes were added from PhyloPic (http://phylopic.org/).

**Construction of expression plasmid**

First-strand C. flexa cDNA (extracted as in\(^{15}\)) was used as the template for cloning Cf sGC1. Forward and reverse primers were designed against 5' and 3' ends of the target transcript (transcript name: TRINITY_DN6618_c0_g1_i1 in the published transcriptome\(^{15}\)). Forward: TAAAGAACGATATACCATG TATGCGTGGTGCAAGAAGC; reverse: TAAAGGTGATGAGGGTGATG AACTATAGCTG CTTGCAACG. Underlined portions anneal to the sequence template. The PCR product was inserted into a pET28b vector using Gibson assembly, and the cloning product was verified by sequencing (UC Berkeley sequencing facility).

**Protein expression and purification**

pET_Cf sGC1 was transformed into E. coli BL21star (DE3) cells co-expressing the chaperone GroEL/ES from the pGro7 plasmid (Takara Biosciences). After overnight incubation at 37 °C in LB Miller media supplemented with 50 μg/mL kanamycin, 20 μg/mL chloramphenicol and 500 μM iron (III) chloride, cells were subcultured 1:200 into TB media supplemented with 50 μg/mL kanamycin, 20 μg/mL chloramphenicol, 500 μM iron (III) chloride, 0.5 mg/mL L-arabinose, and 2 mg/mL glucose, grown at 37 °C. Once cell density reached OD\(_{600}\) = 0.6, 1 mM S-aminolevulinic acid was added to the culture, and culturing temperature was lowered to 18 °C. After 15 minutes of incubation, protein production was induced by addition of 500 μM isopropyl β-D-1-thiogalactopyranoside and cultures were incubated for an additional 18 hours. Cell culture was harvested by centrifuging at 4200 g for 25 min. Cells were collected, flash frozen in liquid nitrogen, and stored at -80 °C until purification.

All protein purification steps were done at 4 °C unless otherwise noted. Cells were resuspended in equal volume of buffer A (50 mM sodium phosphate, 150 mM NaCl, 5 mM imidazole, 5% glycerol, pH 8.0) supplemented with 110 mM benzamidine, 0.4 mM AEBSF, and 0.3 mg/mL DNase. Cell resuspension was lysed using an Avestin EmulsiFlex-C5 homogenizer. Cell lysate was collected and clarified by centrifugation at 32,913 g for 55 min, and the supernatant was collected and loaded onto a His60 Ni Superflow gravity column (Takara Bio). The column was washed twice, first with 10 CV buffer A, and then with 10 CV of a 9:1 mixture of buffer A and buffer B (50 mM sodium phosphate, 150 mM NaCl, 400 mM imidazole, 5% glycerol, pH 8.0). Protein was eluted with 5 CV buffer B in 1 mL fractions. Fractions with high absorbance were pooled and concentrated using a 50 kDa cutoff spin concentrator and supplemented with 5 mM DTT and 1 mM EDTA for overnight storage. Subsequently, protein was passed over a POROS HQ2 anion exchange column (Applied Biosystems). After loading, the column was washed with 5 CV of buffer C (25 mM triethanolamine, 25 mM NaCl, 5 mM DTT, pH 7.4) and developed over a gradient of 100 mM ~ 300 mM NaCl over 17 CV. The protein absorption spectrum was measured using a Nanodrop 2000 microvolume spectrophotometer (ThermoFisher Scientific). Subsequently, protein was aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C for future use.

**Analytical size exclusion chromatography**

Purified Cf sGC1 and the protein standard mixture were injected onto a Superdex 200 Increase 10/300 GL column (GE healthcare) equilibrated with buffer F (50 mM triethanolamine, 150 mM NaCl, 5 mM DTT, 5% glycerol). Protein elution was monitored by UV absorbance at 280 nm.

**Gas ligand binding of bacterial-produced Cf sGC1**

Cf sGC1 was handled in an argon-filled glove bag. Protein-bound heme was reduced by adding sodium dithionite (~500-fold excess over the protein conc.). Excess dithionite was removed by gel filtration of the protein into Buffer E (50 mM HEPES, 150 mM NaCl, 5% glycerol, pH 7.4) using a pre-equilibrated Zeba spin desalting column. A ferrous, ligand-free UV-vis absorption spectrum was collected on a Cary 300 UV-vis Spectrophotometer. Fe(II)-NO bound Cf sGC1 was generated by adding the NO-releasing molecule proline NONOate (~10-fold excess) to the protein sample, and Fe(II)-CO bound Cf sGC1 was generated by adding CO-sparged Buffer E to the protein sample before collecting a spectrum.

**Extinction coefficient of Cf sGC1**

The extinction coefficient of the Soret maximum of reduced Cf sGC1 was measured using two assays performed in tandem: heme concentration in a sample of Cf sGC1 was determined using pyridine hemochromagen assay, and the heme Soret absorption of the protein sample was measured using UV-vis spectroscopy as described above. The pyridine hemochromagen assay was carried out following a reported protocol. Briefly, a reduced protein sample with a known Soret band absorbance was diluted 5-fold in Buffer E, and then further diluted 2-fold in Solution I (0.2 M NaOH, 40% pyridine, 500 μM potassium ferricyanide) to yield the oxidized pyridine hemochromagen. An aliquot (10 μL) of Solution III (0.5 M sodium dithionite, 0.5 mM NaOH) was then added to the oxidized pyridine hemochromagen sample to yield the reduced pyridine hemochromagen. The UV-vis absorption spectrum of the reduced pyridine hemochromagen was measured on a Cary 300 UV-vis spectrophotometer. Absorption at 557 nm(ε = 34,700 mM⁻¹ cm⁻¹) was used to calculate the heme concentration in the pre-dilution sample, and the extinction coefficient of the heme cofactor of Cf sGC1 was calculated by dividing the reduced heme absorption by the heme concentration.
Activity assays and quantification

Specific activity for Cf sGC1 was measured by quantifying the amount of cGMP produced in duplicate end-point activity assays, done in biological triplicate. Cf sGC1 from previously frozen aliquots was thawed and reduced in an anaerobic chamber as described above. The reduced protein was used without further treatment for the basal (unliganded) activity assay. A UV-vis spectrum for the unliganded sample was obtained using a Nanodrop 2000 microvolume spectrophotometer, and the Soret maximum was used to quantify heme-bound protein concentration ($\epsilon_{428} = 101,000 \text{ M}^{-1}\text{cm}^{-1}$). Only protein with a Soret:280 ratio > 1 was used for activity assays. To the remaining reduced protein, prolinONOate was added to a final concentration of 400 $\mu$M by the addition of 1 $\mu$L of a stock solution of 10 mM prolinONOate in 10 mM NaOH, and the protein sample was incubated at 4 °C for 5 min to yield the xsNO sample. The xsNO-bound UV-vis spectrum was then collected. The protein concentration of the xsNO sample was assumed to be the same as the unliganded sample. The xsNO sample was then buffer exchanged by gel filtration using a Zeba spin column into buffer E to yield the 1-NO sample, and the UV-vis spectrum was collected. The concentration of the 1-NO sample was calculated based on the NO-bound Soret absorbance at 399 nm compared to that of the xsNO sample. Activity assays were carried out at 25 °C in Buffer E supplemented with 5 mM DTT and 3 mM MgCl$_2$ with Cf sGC1 concentration at 40 nM. To obtain the xsNO state, 70 $\mu$M prolinONOate was added. The reaction was initiated by addition of 1.5 mM GTP, and timepoints were quenched by diluting the reaction mixture 1:4 to a solution of 125 mM zinc acetate, and pH adjusted by adding equal volume of 125 mM sodium carbonate. Assay samples were stored at -80 °C until analyzed. Quenched assay samples were thawed at room temperature, centrifuged at 21,130 x g at 4 °C to remove zinc precipitate. Supernatant was collected and diluted 250-fold for the analysis. cGMP content of each assay sample was quantified in duplicate using an enzyme-linked immunosorbent assay (Enzo Life Sciences) following the manufacturer’s protocol. Initial rate of the reaction was calculated using the linear phase of the time course, where <10% of substrate has been depleted.

QUANTIFICATION AND STATISTICAL ANALYSIS

Information about the quantification and statistical details of experiments can be found in the corresponding figure legends. Statistical tests and graphs were produced using Prism 9.0.0.