Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information

Human Soluble Guanylate Cyclase as a Nitric Oxide Sensor for NO-Signalling Reveals a Novel Function of Nitrite Reductase

Jie Pan[a], Qiming Xu[a], Ying-Wu Lin[b], Fangfang Zhong[a] and Xiangshi Tan[a]*

Materials and methods

Materials

Plasmid FL 14116 containing the entire human sGC β1 cDNA was purchased from Fulengen Company, China. The vector pET 22b(+) and the *E.coli* strains XL 10-Gold and Rosetta(DE3) pLysS was obtained from Novagen. *Pfu* DNA polymerase, T4 DNA ligase, dNTP and restriction enzymes (NdeI and XhoI) were purched from New England Biolabs. Oligonucleotide PCR primer pair were synthesized and DNA sequencing reactions were performed by Shanghai Invitrogen Biotech Co. Ltd. The plasmid purification kits, gel extraction kits, Nickel nitrilotriacetic acid (Ni-NTA) resin and Sephadex G-25 resin were purchased from QIAGEN (Chatsworth, CA, USA). DEAE Sepharose™ Fast Flow was from GE Healthcare Bio-Science. The Superdex™ 200 10/300 gel filtration column was from Pharmacia.Diethylammonium (Z)-1-(N,N-diethlamino)-diazen-1-ium-1,2-diolate (DEA/NO) was from Cayman Chemical Company. All other reagents were of analytic grade.

Subcloning of hsGC β1 (1-195) and hsGC β1 (1-385)

hsGC β1(1-195) encoding human sGC β1 N-terminal fragment cDNA residues 1-195 was amplified using FL 14116 containing the entire human sGC β1 cDNA as template. The purified PCR product was digested with the corresponding restriction enzmes and then ligated with T4 DNA ligase into the pET 22b(+) vector. The final construct has a $(His)_6$-tag fused onto the C-terminus of the hsGC β1(1-195) and no other extra residues in the N-terminus, which is different from the one we constructed before.[1] hsGC β1(1-385) was subcloned using the same procedure as for hsGC β1(1-195).

Expression and purification of hsGC β1(1-195) and hsGC β1(1-385)

Expression of the protein hsGC β1(1-195) was performed as described previously[1] with the following modifications. Cultures were grown to an OD_{600} of 0.6 and cooled to 20°C. IPTG was added to 0.1 mM, and aminolevulinic acid was added to 0.5 mM. Cultures were grown overnight for 12-16 h and harvested by centrifugation. The cell pellets were frozen and stored at -80°C.

All purification steps were performed under anaerobic conditions in a glove box with High purity nitrogen gas. Cell pellets were suspended in lysis buffer A (50 mM Na-Pi, 500 mM NaCl, 10 mM dithionite, 5 mM β-mecaptoethanol (β-Me) 20% glycerol, pH 8.0) with 2 mg/ml lysozyme and 1 mM phenyl methyl sulfonyl fluoride (PMSF), and lysed by sonication for 5min on ice. The resulting cell homogenate was centrifuged. The supernatant from this centrifugation was applied to Ni-NTA column that had been pre-equilibrated with buffer A. The column was washed with 20 bed volumes of 25 mM imidazole in buffer A, and the protein was eluted with 200 mM imidazole in buffer A or 100 mM EDTA. Brown-colored fractions were pooled, diluted 5 times with buffer B (50 mM Na-Pi, 150 mM NaCl, 5 mM DTT, 5% glycerol, pH 7.4) and then loaded onto DEAE Sepharose™ anion-exchange column. The column was washed with 5 bed volumes of buffer B and eluted with a linear gradient of NaCl from 150 to 500 mM. Brown-colored fractions were pooled and concentrated, and then subjected to the Superdex™ 200 10/300 gel filtration column equilibrated with buffer B (50 mM Na-Pi, 150 mM NaCl, 5 Mm DTT, 5% glycerol, pH 7.4). Fractions were selected on the basis of the intensity of the red/brown color, with a ratio of $A_{424\ nm}/A_{398nm}$ about 1.5 and stored at -80°C. The protein concentration was estimated by the Soret peak using the pyridine-hemochromagen assay with an extinction coefficient of $\varepsilon_{R\text{-}O,557\text{-}540}$ = 22.1 $mM^{-1}cm^{-1}$ as reported previously.[1] hsGC β1(1-385) was expressed and purified using the same procedure as for hsGC β1(1-195).

Spectrophotometric studies

hsGC β1(1-195) or hsGC β1(1-385) was fully oxidized with 1 mM potassium ferricyanide or fully reduced with 50 mM sodium dithionite at 4 °C and excess reagents were removed by passing a Sephadex G-25 column under anaerobic conditions in a glove box filled with High purity nitrogen gas. The NO-bound form was formed by addition of NO donator, DEA/NO. Reaction kinetics of

known amounts of ferrous hsGC β1(1-195) or hsGC β1(1-385) with different concentration nitrite were monitored by absorbance spectroscopy in an anaerobic cuvette in the presence of 2 mM sodium dithionite on a HP8453 UV-visible spectrophotometer. All reactions were conducted at 25 °C in phosphate buffer (50 mM K-Pi, 100 mM KCl), pH 6.5, pH 7.0, or pH 7.5 .

EPR Spectroscopy

Determination of NO-bound form of hsGC β1(1-195) (100 μM) was performed using electron paramagnetic resonance (EPR) spectroscopy using a Bruker EMX X-band spectrometer equipped with an Oxford-910 cryostat cooled to 110 K using liquid helium. The spectra were recorded under the following conditions: microwave frequency 9.44 GHz, microwave power 2.0 mW, modulation frequency 100 kHz, modulation amplitude 4.00 G, and time constant 164.84 ms.

Molecular modeling of nitrite or nitric oxide binding to hsGC β1(1-195)

Nitrite (NO_2^-) or nitric oxide (NO) binding to ferrous hsGC β1(1-195) was simulated by using a procedure as reported recently.[2] The initial structure of for hsGC β1(1-195) was obtained from previous homology modeling study.[1] The protein was then solvated in a cubic box of TIP3 water, which extended 10 Å away from any given protein atom. A NO_2^- anion or NO was added in the heme pocket, and an atomic charge of O1 (−0.43 e and −0.46 e for O- and N-binding modes, respectively) was used for the nitrite anion, according to the previous calculation.[3] Counter ions (Na^+ and Cl^-) were further added to obtain the physiological ionic strength of 0.15 M by using the autoionize plug-in of VMD 1.9.[4] The resulting system was minimized with NAMD (Nanoscale Molecular Dynamics),[5] using 1,000 minimization steps with conjugate gradient method at 0 K, and equilibrated for 5,000 molecular dynamics steps (1 fs per step) via an NVT ensemble (where the number of particles N, the volume V, and the temperature T of the system are kept constant) at 310 K, then further minimized for 1,000 steps at 0 K. Visualization and data analysis were done with VMD 1.9.

Reference:

1. Zhong, F.; Wang, H.; Ying, T.; Huang, Z. X.; Tan, X. *Amino Acids*, **2010**, *39* (2), 399-408.
2. Lin, Y. W.; Nie, C. M.; Liao, L. F. *J. Mol. Model.* **2012**, *18*(9):4409-15.
3. Perissinotti, L. L.; Marti, M. A. ; Doctorovich, F. ; Luque, F. ; Estrin, D. A. *Biochemistry*

2008, 47: 9793–9802.

4. Humphrey, W.; Dalke, A.; Schulten K. *J. Mol. Graph.* **1996**, 14: 33-38.
5. Kalé, L.; Skeel, R.; Bhandarkar, M.; Brunner, R.; Gursoy, A.; Krawetz, N.; Phillips, J.; Shinozaki, A.; Varadarajan, K.; Schulten, K. *J. Comp. Phys.* **1999**,151: 283-312.

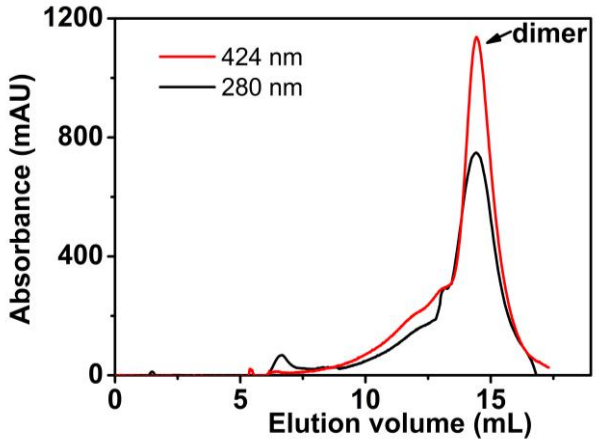

Figure S1. Elution curves of FPLC purification of hsGC β1(1-195) by Superdex^{TM} 200 10/300 column.

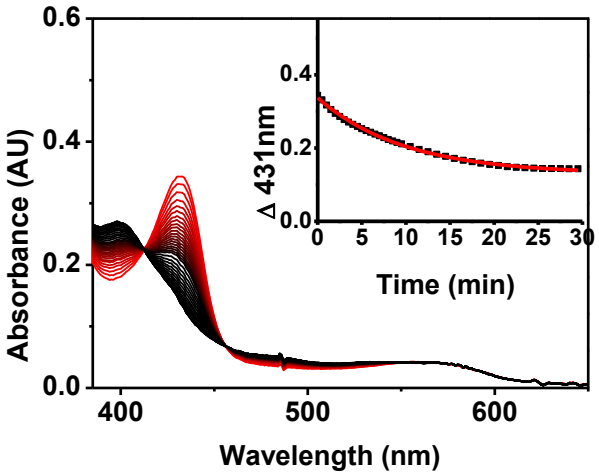

Figure S2. Spectral changes for reduction of nitrite (5 mM) by ferrous hsGC β1(1-385) (4 μM) at 15 sec intervals in the presence of 2 mM sodium dithionite (pH 7.0, 25°C). Kinetic trace at 431 nm was shown as an inset. (k_{obs} = 0.11 ± 0.003 min^{-1}).

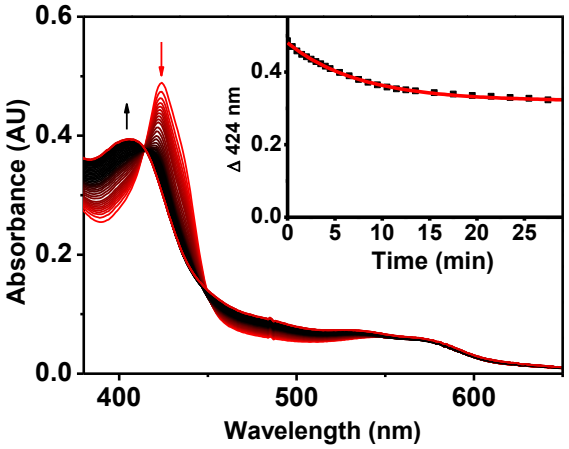

Figure S3. Spectral changes for reduction of nitrite (5 mM) by ferrous hsGC β1(1-195) (5 μM) at 15 sec intervals in the absence of sodium dithionite (pH 7.0, 25°C). Kinetic trace at 424 nm was shown as an inset (k_{obs} = 0.13 ± 0.004 min^{-1}).

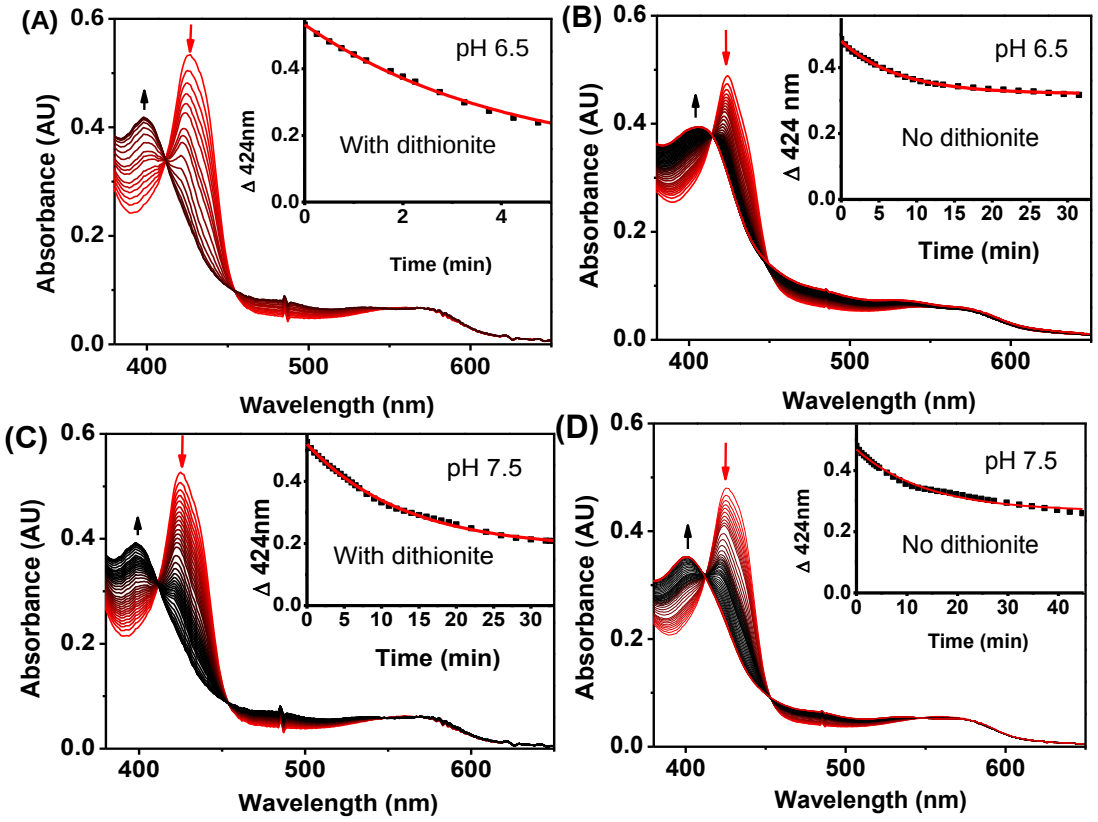

Figure S4. Spectral changes for reduction of nitrite (5 mM) by ferrous hsGC β1(1-195) (5 μM) at 15 sec intervals at pH 6.5 in the presence (A, k_{obs} = 0.24 ± 0.02 min^{-1}) and absence of sodium dithionite (2 mM) (B, k_{obs} = 0.19 ± 0.002 min^{-1}), and at pH 7.5 in the presence (C, k_{obs} = 0.08 ± 0.002 min^{-1}) and absence of sodium dithionite (2 mM) (D, k_{obs} = 0.07 ± 0.004 min^{-1}) (25°C). Kinetic traces at 424 nm was shown as insets.

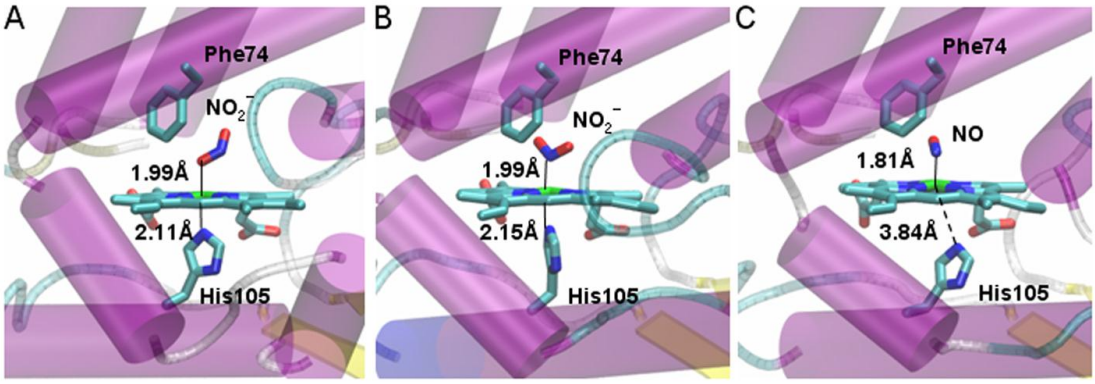

Figure S5. Heme pocket view of nitrite (O-binding model, A and N-binding model, B) and nitric oxide (C) binding to ferrous hsGC β1(1-195). Heme group, axial ligand His105, distal Phe74, and ligand NO_2^- or NO are highlighted.