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The process of S-nitrosation in sGC β1(1–194) revealed by infrared spectroscopy

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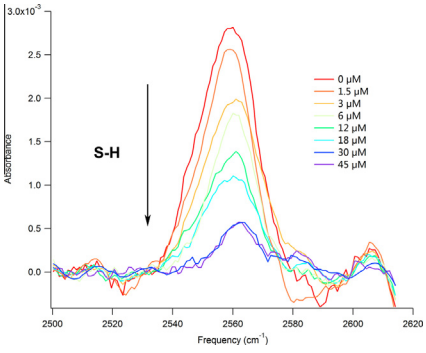
HIGHLIGHTS

- Infrared spectroscopy and site-directed mutagenesis were used in this study.
- The thiol signal was attenuated in the IR spectra of GSNO-treated protein.
- The process of S-nitrosation was GSNO concentration-dependent.

GRAPHICAL ABSTRACT



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ABSTRACT

Soluble guanylate cyclase (sGC) is the most important receptor for the signaling molecule NO. NO activates sGC by binding to its heme cofactor and reacts with free thiols in the protein itself. The S-nitrosation of cysteine thiols affects the activity of soluble guanylate cyclase (sGC). In this study, infrared (IR) spectroscopy and site-directed mutagenesis were used to investigate S–H vibration and the process of S-nitrosation in the β1 subunit (amino acids 1–194) of sGC. Fourier transform IR spectroscopy revealed that wild-type and mutants (C78S and C122S) of sGC β1(1–194) exhibited S–H peaks around 2560 cm^{-1}. The signals were attenuated in the IR spectra of S-nitrosoglutathione-treated mutants, demonstrating that S-nitrosation in sGC β1(1–194) occured at residues C78 and C122, and the process of the reaction was GSNO concentration-dependent.

Introduction

Soluble guanylate cyclase (sGC), a heme-binding heterodimeric protein consisting of subunits α1 and β1, catalyzes the conversion of GTP to cGMP [1,2]. NO binds to the heme at histidine 105 of the β1 subunit and leads to an increase in sGC activity by several hundred fold. Binding of NO to the heme group is not paralleled by activation of the enzyme indicating that heme is not the only binding site of NO [3]. Experiment reveals that NO activates sGC by binding to its heme cofactor and modifies the protein by cysteine S-nitrosation [4,5]. S-nitrosation, the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine, is a NO-dependent post-translational modification that may directly alter protein structure and function [6–8]. Several experiments pointed out S-nitrosation led to NO-desensitization in sGC [4,5].

The full-length sGC contains 34 cysteine residues [9] and only three of these (C78, C122 and C174) residues are in sGC β1(1–194) which has been characterized as the minimum functional ligand-binding heme domain of sGC and used to study

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cysteine S-nitrosation [10]. Using a biotin switch method and activity assays, the Marletta group demonstrated that C78 and C122 on the β1 subunit are key residues for NO-desensitization in heme-oxidized sGC by S-nitrosation [4]. Biotin switch technique is a qualitative and relative quantitative method. However, it is a multi-step operation and each step contains potential sources error [11,12]. Effective blocking of free thiols is required and the incompletely blocking of the free thiols causes high levels of SNO-independent biotinylation. The proper control is necessary to avoid "false-positive" signal. Infrared (IR) spectroscopy, in which the vibrations of individual chemical groups are recorded, has been used to study the S–H vibration and the change of thiol state [13–17]. Vibrational spectroscopy is a potentially useful method for directly detecting cysteine nitrosation, because the stretching frequency of cysteine S–H is in the 2580–2525 cm^{-1} region, which does not contain any other vibrations [13]. Infrared spectroscopy is a high-sensitive technique to detect the cysteine nitrosation and it is also capable of quantitation.

The aim of this study was to investigate the S-nitrosation of the three cysteine residues and the process of the reaction in sGC β1(1–194) by IR spectroscopy. For this purpose, wild-type and mutant sGC β1(1–194) proteins were expressed and purified, the cysteine residues were nitrosated by mixing with S-nitrosoglutathione (GSNO), and the attenuation of the thiol signal was probed spectrometrically. The resulting IR spectrum provided direct evidence that sGC β1(1–194) was S-nitrosated at residues C78 and C122 and the process of the reaction was GSNO concentration-dependent.

Experimental

Materials

Plasmids containing rat lung sGC cDNAs were kindly provided by Dr. Michael A. Marletta (University of California). The plasmid pET-20b and *Escherichia coli* BL21(DE3)pLysS competent cells were obtained from Novagen. All restriction enzymes, T4 DNA ligase and DNA polymerase were obtained from TAKARA (China) and New England Biolabs (USA). Nickel-nitrilotriacetic acid (Ni–NTA) resin and Sephadex G-25 resin were purchased from GE Healthcare. All other reagents were of analytical grade and were commercially available.

Construction of expression plasmids

The N-terminal fragment of the rat lung sGC β1 cDNA (encoding residues 1–194) was amplified by PCR using the upstream primer 5′-GGAATTCCATATGTACGGTTTTGTGAACCATGCC-3′ and the downstream primer 5′-CGGGATCCCTAATGATGATGATGATGATGGCTGCCGCGCGGCACCAGG-3′. The plasmid containing the rat sGC β1(1–385) cDNA was used as the PCR template. The amplified PCR products were cloned into pET-20b and sequenced. The coding sequences of the mutants C78S, C122S and C78/122S were amplified by using primers containing a single mutation. PCR primer synthesis and DNA sequencing were performed by Sangon Biotech (Shanghai) Co., Ltd (China).

Expression and purification of wild-type and mutants sGC β1(1–194)

The plasmids were transformed into BL21(DE3)pLysS cells and the recombinants were grown in a 5-L glass flask containing 1.5-L of LB medium containing 50 μg/mL ampicillin and 35 μg/mL chloramphenicol. The cultures were grown to an OD_{600} of 0.6–0.7 and then incubated at 27 °C for 12–18 h after the addition of 10 μM IPTG and 1 mM aminolevulinic acid. sGC β1(1–194) was purified as follows: cells collected from the 1.5-L culture were resuspended in 80 mL of buffer A (50 mM potassium phosphate pH 7.4, 300 mM NaCl, 5 mM imidazole, 1 mM PMSF and 5 mM DTT), and then lysed by sonication and centrifuged at 50,000*g* for 40 min. The supernatant was then collected and loaded onto a 1-mL Ni–NTA column equilibrated in buffer A at a rate of 2–3 mL/min. Once loaded, the column was washed once with buffer A and then washed with buffer B (50 mM potassium phosphate pH 7.4, 300 mM NaCl and 25 mM imidazole) until the OD_{280} returned to background levels (0.02–0.05). Subsequently, the protein was eluted from the column with buffer C (50 mM potassium phosphate pH 7.4, 500 mM NaCl and 500 mM imidazole). The recombinant sGC β1(1–194) mutant proteins were obtained and purified using the same procedures. A PD-10 column (GE Healthcare) was used for desalting and buffer exchange. All protein manipulations were carried out at 4 °C. The purity and concentration of the protein were analyzed by SDS–PAGE and BCA method.

Synthesis of GSNO

GSNO was prepared by mixing glutathione (reduced form) with sodium nitrite in the dark for 30 min and then neutralizing with NaOH [18]. The concentration of the GSNO solution was approximately 500 mM.

UV–visible spectroscopy

UV–visible spectra were recorded on a UV-2550 detection system (Shimadzu, Japan) equipped with a temperature controller set at 10 °C. The spectra were collected over 240–700 nm at 600 nm/min with a data point interval of 0.5 nm.

S-nitrosation of sGC β1(1–194)

S-nitrosation was determined by the biotin-switch method as described in [4]. sGC β1(1–194) was incubated with GSNO 30 min at room temperature in 50 mM Hepes, pH 7.4, 50 mM NaCl. Reactions were then mixed with an equal volume of 50 mM Hepes pH 7.4, 1 mM EDTA, 0.1 mM neocuproine, 10% SDS, 250 mM MMTS. After that, reactions were incubated at 50 °C for 60 min and vortexed every 10 min. Samples were acetone precipitated and the pellets were dissolved in 50 μL of 50 mM Hepes pH 7.4, 0.25 mM EDTA, 0.025 mM neocuproine, 5% SDS, 1 mM maleimide-PEO2-biotin (Pierce), 10 mM sodium ascorbate, and incubated for 1 h at 25 °C. Samples were acetone precipitated again and the protein pellets were dissolved in 30 μL of 50 mM Hepes pH 7.4, 0.25 mM EDTA, 0.025 mM neocuproine, 5% SDS. SDS–PAGE loading buffer containing 50 mM DTT was added to the protein samples. The samples were then split and analyzed by both Neutravidin-HRP Western blot and Coomassie blue staining.

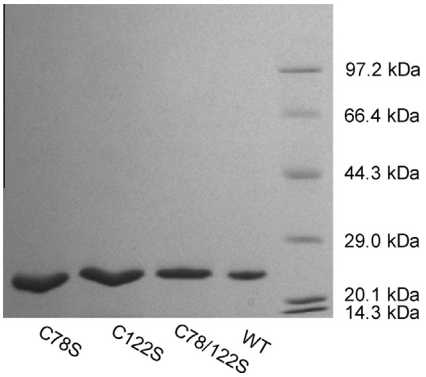

Fig. 1. SDS–PAGE of purified rat sGC β1(1–194) and mutants.