Localization of the Heme Binding Region in Soluble Guanylate Cyclase[†]

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Received July 28, 1997; Revised Manuscript Received September 23, 1997[⊗]

ABSTRACT: Soluble guanylate cyclase (sGC) is a heterodimeric hemoprotein composed of α1 and β1 subunits. sGC is activated by nitric oxide (NO) and therefore plays a central role in NO signal transduction. Activation of sGC by NO is believed to be mediated by the interaction between NO and the heme of sGC. Spectroscopic and kinetic studies have shown that the heme of sGC is in a unique environment. Characterization of the heme environment is critical to the understanding of the mechanism of NO activation. To approach this goal, the β1 N-terminal fragment consisting of residues 1−385 [β1(1−385)] of sGC was expressed in *E. coli*. β1(1−385) was then purified to homogeneity in two steps by DEAE ion exchange and gel filtration chromatography. Purified β1(1−385) was found to contain a stoichiometric amount of heme. The UV−visible spectrum of β1(1−385) is almost identical to that of the native heterodimeric sGC purified from bovine lung. β1(1−385) binds both NO and CO, leading to a shift in the Soret maximum from 431 nm to 398 and 423 nm, respectively. These spectral shifts are identical to those observed with heterodimeric sGC purified from bovine lung. These results suggest that the heme in the β1(1−385) is similar to that in the heterodimeric sGC. Therefore, for the first time, the heme binding region of sGC has been unambiguously localized to the N-terminal region of the β1 subunit. Our data also suggest that the N-terminal region of the β1 subunit of sGC is itself sufficient for heme binding.

Nitric oxide (NO)[1] has been shown to play a significant role in several biological processes including neuronal signaling, vasodilation and the host response to infection (*1*−*6*). In responses that involve cell to cell signaling, the receptor for NO is the soluble isoform of guanylate cyclase (sGC) which catalyzes the conversion of GTP to cGMP (*7, 8*). NO has been shown to activate sGC up to 400-fold (*9*). When isolated from lung tissue, sGC is a heterodimeric protein composed of α1 and β1 subunits (*10*−*12*). Other types of sGC subunits (α2 and β2) have been cloned but not characterized at the protein level (*13, 14*). Both α1 and β1 subunits are necessary for sGC activity since transfection of COS cells with cDNAs encoding either α1 or β1 subunits of sGC alone did not generate active enzyme. Only coexpression of the α1 and β1 subunits of sGC generated catalytic activity (*15, 16*). sGC is a hemoprotein containing a protoporphyrin-IX type heme (*10, 17*). Spectroscopic studies have shown that the heme of sGC isolated from bovine lung is five-coordinate, ferrous high-spin (*10*). The ferrous heme of sGC can readily form complexes with CO and NO, and the UV−visible spectra of the complexes are distinct from that of ferrous sGC (*10, 18*). Binding of NO to the heme of sGC leads to the formation of a 5-coordinate complex by severing the axial amino acid ligand (*19*). NO activation of sGC is believed to be mediated by the interaction between NO and the heme of sGC, although the activation mechanism is not yet fully understood. Knowledge of the heme environment of sGC and the interaction between NO and the heme of sGC is critical for understanding the mechanism of NO activation.

The C-terminal region of sGC has been shown to be both necessary and sufficient for basal guanylate cyclase activity (*20, 21*). As mentioned above, expression of catalytically active sGC requires both α1 and β1 subunits; however, it is not clear if the enzyme contains two catalytic sites or if the active site is formed using residues from both subunits. The heme binding region of sGC has not been clearly localized. Since the C-terminal region of sGC is responsible for catalysis, it has been speculated that the N-terminal region is involved in heme binding and NO activation. Mutagenesis studies have shown that the N-terminal regions of both the α1 and β1 subunits are important for heme binding (*20*−*22*). However, it is still not clear whether the N-terminal region of sGC is sufficient for heme binding. As is the case with catalytic activity, it is not known whether interaction between the α1 and β1 subunits is necessary for heme binding. To address these questions, an N-terminal fragment of the β1 subunit of sGC consisting of residues 1−385 [β1-(1−385)] was expressed in *E. coli*. This recombinant β1-(1−385) was found to contain a stoichiometric amount of heme. Spectroscopic studies showed that the heme in β1-(1−385) is similar to that in heterodimeric sGC isolated from bovine lung. Therefore, for the first time, the heme binding region has been clearly localized to the β1 N-terminal region.

[†] These studies were supported by Searle chair endowment funds.

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[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1997.

[1] Abbreviations: BSA, bovine serum albumin; cGMP, guanosine 3′,5′-cyclic monophosphate; CO, carbon monoxide; DTT, dithiothreitol; GTP, guanosine 5′-triphosphate; IPTG, isopropyl β-D-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; NFDM, non-fat dry milk; NO, nitric oxide; PBS, phosphate-buffered saline; pGC, particulate guanylate cyclase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; sGC, soluble guanylate cyclase.

S0006-2960(97)01825-4 CCC: $14.00

In addition, we conclude that the C-terminal region of the β1 subunit and the interaction between α1 and β1 subunits are not necessary for heme binding to the β1 subunit.

EXPERIMENTAL PROCEDURES

Materials. Plasmid-containing rat lung sGC cDNAs were provided by Dr. Masaki Nakane (Abbott Laboratories). The plasmid pET-20b and *E. coli* BL21(DE3)pLysS competent cells were obtained from Novagen. Restriction enzymes and T4 DNA ligase were purchased from GIBCO BRL. IPTG and X-gal were also from GIBCO BRL. The Expand High Fidelity PCR kit was from Boehringer Mannheim. The pGMT vector used for cloning PCR products was from Promega. TSK DEAE 650M ion exchange resin was obtained from Tosohaas. The plasmid purification kits and gel extraction kits for recovering DNA from agarose gel were from Qiagen. Precast polyacrylamide gels, Mark 12 protein standards and SeeBlue prestained protein standards were from Novex. Carbon monoxide (99.5%) and nitric oxide (99.0%) were from Matheson. The Superdex 200 HiLoad 26/60 gel filtration column was purchased from Pharmacia. SDS, Coomassie Blue R-250, and Bradford protein dye reagent were purchased from Bio-Rad. PCR primers were synthesized by the Biomedical Core Facility, University of Michigan. All other chemicals were purchased from Sigma Chemical Co. unless otherwise stated.

Construction of Expression Plasmid. PCR was used to amplify the rat lung sGC β1 N-terminal fragment cDNA (encoding residues 1−385). The upstream primer and downstream primer were 5′-CATATGTACGGTTTTGT-GAACCAT-3′ and 5′-GGATCCCTAATCCTCCAAAGC-CCTCAG-3′, respectively. Plasmid pcDNAI-Beta that contains the entire rat lung sGC β1 cDNA was used as the PCR template. The β1(1−385) cDNA was amplified at 94 °C, 30 s; 55 °C 30 s; and 72 °C, 30 s for 15 cycles. The PCR product was purified from 1% agarose gel and then was cloned into the pGMT vector (Promega). The fidelity of the PCR reaction was checked by sequencing the entire PCR fragment. To subclone the N-terminal fragment cDNA from the pGMT vector into the pET-20b expression vector (Novagen), a *Nde*I/*Bam*HI fragment containing the entire β1 N-terminal fragment cDNA (residues 1−385) was removed from the pGMT vector, purified from agarose gel, and then cloned into pET-20b using the same set of restriction enzymes. The correct expression plasmid pET-20b-HBD was identified by restriction mapping and DNA sequencing.

Expression of Rat Lung sGC β1(1−385) in E. coli. The expression construct pET-20b-HBD was transformed into *E. coli* BL21 (pLysS) cells (Novagen). The recombinant *E. coli* were selected on LB plates containing 50 μg/mL ampicillin and 35 μg/mL chloramphenicol. The recombinant *E. coli* were grown in a 4 L glass flask containing 1 L of modified Terrific Broth (12 g of casein enzymatic hydrolysate, 24 g of yeast extracts, 4 mL of glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4) with 50 μg/mL ampicillin and 35 μg/mL chloramphenicol. Cultures were incubated at 37 °C, and shaken at 250 rpm in a New Brunswick incubator to $OD_{600} = 0.6$. The incubation temperature was then lowered to room temperature (approximately 25 °C). After a 30-min incubation at room temperature, IPTG was added to a final concentration of 0.5 mM. Cells were harvested by centrifugation 12−14 h after addition of IPTG. The cell pellets were stored at −80 °C.

Purification of β1(1−385). The frozen cell pellet from 3 L of culture was thawed on ice for about 2 h, resuspended in 120 mL of Buffer A (50 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM DTT, and 1 mM PMSF), and lysed by sonication for 5 min on ice. The resulting cell homogenate was centrifuged at 10000*g* for 1 h. The supernatant from this centrifugation was applied to a TSK DEAE (Tosohaas) anion exchange column (25 cm × 2.5 cm) that had been equilibrated with buffer A. The flow rate was 1.2 mL/min. The DEAE column was then washed with 300 mL of buffer A at a rate of 1.2 mL/min. β1(1−385) was eluted with a linear NaCl gradient from 100 to 500 mM in a total volume of 1 L. Fractions containing β1(1−385), identified by a characteristic red−brown color and UV−visible spectrum, were pooled and concentrated to 10 mL using an Amicon concentrator and a disk membrane (Filtron, 30 kDa MW cutoff). The concentrated sample was then loaded onto the Superdex 200 HiLoad 26/60 gel filtration column (Pharmacia) that was equilibrated with buffer B (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT). The flow rate was 1.7 mL/min. Fractions containing β1(1−385) were pooled and concentrated to 2 mL, and glycerol was added to 50% final volume. The purified β1(1−385) was aliquoted and stored at −80 °C under argon.

Protein Concentration Determination. Protein concentration was determined by the Bradford microassay using BSA as the standard. The Bradford assay was calibrated by quantitative amino acid analysis, and a correction factor was obtained. The quantitative amino acid analysis was performed by the University of Michigan Biomedical Core Facility. BSA standard solution from Sigma was used to verify the accuracy of the quantitative amino acid analysis.

Heme Content. Heme concentrations were determined by the pyridine−hemochromagen assay using horse heart myoglobin as the standard as previously described (*23*). The heme concentration in a myoglobin standard solution was calculated using the extinction coefficient of 121 mM^{-1} cm^{-1} at 434 nm for ferrous myoglobin heme (*23*). β1(1−385) samples used for the hemochromagen assay were desalted into 100 mM phosphate buffer, pH 7.5, to remove DTT.

Estimation of the Native Molecular Mass of β1(1−385). Gel filtration was used to estimate the native molecular mass of β1(1−385). Gel filtration protein standards used in this study were thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). The running buffer was 50 mM Hepes, pH 7.4, 150 mM NaCl, and 5 mM DTT. The Superdex 200 HiLoad 26/60 gel filtration column (Pharmacia) was run using a BioLogic Chromatography System (Bio-Rad). A standard curve was obtained by plotting the retention time against the logarithm of the molecular masses.

Optical Spectroscopy. UV−visible spectra were recorded on a Cary 3E spectrophotometer equipped with a Neslab RTE-100 temperature controller set at 10 °C. The ferrous heme−CO complex was formed by flushing the headspace of the cuvette containing purified β1(1−385) with CO for 10 min on ice. The ferrous heme−NO complex was obtained in the following way. Purified β1(1−385) was made anaerobic in a cuvette by argon replacement using an oxygen-scavenged gas train, and then NO was flushed

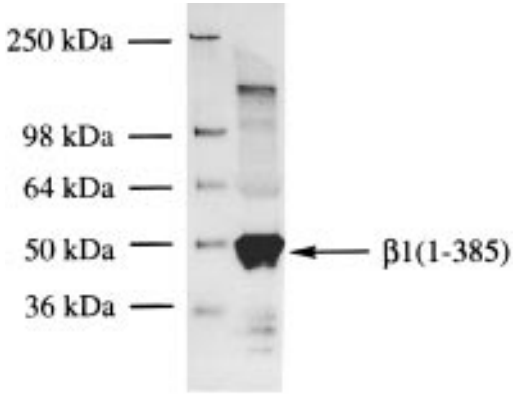

FIGURE 1: Western blot of β1(1−385) using antibody raised against bovine lung sGC. The sample was from a 10000*g* supernatant of cells harvested 14 h after IPTG induction.

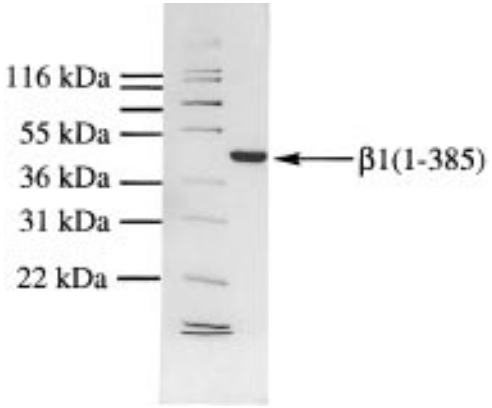

FIGURE 2: SDS−PAGE of β1(1−385) shown after the two-step purification procedure as described under Experimental Procedures.

through a saturated KOH solution and then into a reaction vial for 10 min. An aliquot of NO gas (5 μL) was removed from the reaction vial using gas-tight syringe and injected into the solution containing β1(1−385). Ferric β1(1−385) was made by ferricyanide oxidation of ferrous β1(1−385) as follows. β1(1−385) was first desalted into 50 mM Hepes, pH 7.4, 150 mM NaCl, and then ferricyanide (100 μM) was added gradually to β1(1−385) (9 μM) and the spectrum between 300 and 800 nm was followed until the Soret peak shifted completely from 431 nm to 392 nm. Ferricyanide was removed by gel filtration. KCN (20 mM) was added to the ferric form of the N-terminal fragment to form a cyano−heme complex.

SDS−PAGE and Western Blot Analysis.[2] SDS−PAGE was carried out with Novex 12% polyacrylamide precast gels following the instructions provided by the manufacturer. Protein gels were visualized by staining with Coomassie Blue R250. For the Western blot, proteins were transferred from the gel to a nitrocellulose membrane for 1 h at 250 mA in a Bio-Rad Mini Trans-Blot Cell. The membrane was blocked in 1% NFDM in PBS overnight with gentle shaking. The primary antibody was diluted 1:3000 in 5% NFDM and incubated with the membrane for 1 h. The membrane was then washed with 210 mL of 0.2% NFDM in PBS (three washes, 70 mL and 10 min for each wash). The membrane was then incubated with the secondary antibody in 5% NFDM for 1 h followed by three washes as described above. The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase. The membrane was developed using the chromogenic substrate 4-chloro-1-naphthol.

RESULTS

Expression and Purification of β1(1−385). The rat lung sGC β1(1−385) was expressed in *E. coli* under the control of a T7 promoter. About 1 h after induction with IPTG, β1(1−385) can be identified on both a Coomassie-stained protein gel and a Western blot (data not shown). The highest concentration of soluble β1(1−385) was obtained 12−16 h after induction, as detected by SDS−PAGE and Coomassie staining. Figure 1 shows a Western blot result of the *E. coli* supernatant after expression of β1(1−385) using antibody raised against bovine lung sGC. On the Western blot, the main band (47 kDa) was β1(1−385) (predicted molecular mass 44 kDa). Another indication of the expression of the heme binding β1(1−385) was that the cell pellet of the expression strain was red. We did not carry out a systematic approach to find out conditions giving maximal amounts of soluble β1(1−385) since there is no activity assay for β1(1−385) or any convenient measurement of the protein concentration that is specific for β1(1−385). However, a combination of lower incubation temperature and extremely rich media did produce satisfactory amounts of soluble protein. β1(1−385) was purified in two conventional steps: DEAE ion exchange and gel filtration chromatography. The calculated isoelectric point based on the amino acid sequence for β1(1−385) is 4.7; therefore, DEAE anion exchange was chosen as the first purification step. After the DEAE anion exchange column, there were 25 fractions (fraction size: 6.5 mL) that contained heme, based on the red/brown color and UV−visible spectrum. Only those fractions with a ratio of A_{280}/A_{Soret} lower than 2.0 were pooled, concentrated, and loaded onto the next column. The gel filtration column gave three well resolved peaks (data not shown). SDS−PAGE after these two steps purification reveals only a single band on a Coomassie-stained protein gel (Figure 2). Approximately 1 mg of purified β1(1−385) was obtained from 1 L of culture.

Heme Content of β1(1−385). Quantitative amino acid analysis was used to calibrate the Bradford assay in this study. A BSA solution (Sigma, catalog number P-0914) was used to test protein recovery in the quantitative amino acid analysis and found to be about 90%. The β1(1−385) concentration obtained from the Bradford microassay using BSA as the standard was 1.45 ± 0.05 (n = 3) fold higher than that obtained from quantitative amino acid analysis (uncorrected for recovery). Based on this protein concentration correction factor and the hemochromagen assay results, the heme content for the N-terminal fragment was found to be 0.90 ± 0.07 heme per monomer (n = 4).

Native Molecular Mass of β1(1−385). The native molecular mass of β1(1−385) was estimated using gel filtration. Proteins of known molecular mass were used to calibrate the Superdex 200 HiLoad 26/60 gel filtration column, and a standard curve was obtained by plotting the retention time against the logarithm of the molecular mass for the standards. Based on this standard curve, the native molecular mass of β1(1−385) was found to be 118 kDa. This result suggests that β1(1−385) is isolated as a homodimer.

[2] Antibody used for Western blot analysis in this study was raised in rabbits against purified sGC from bovine lung (Kim and Marletta, unpublished results).

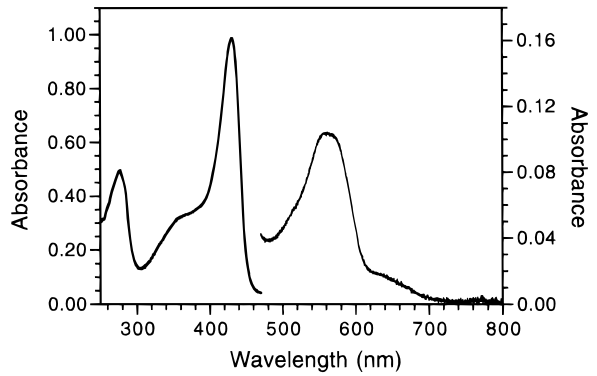

FIGURE 3: Electronic absorption spectrum of β1(1−385) (8 μM) in 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT. The left scale refers to the Soret peak and protein peak region, and the right scale refers to the α/β region.

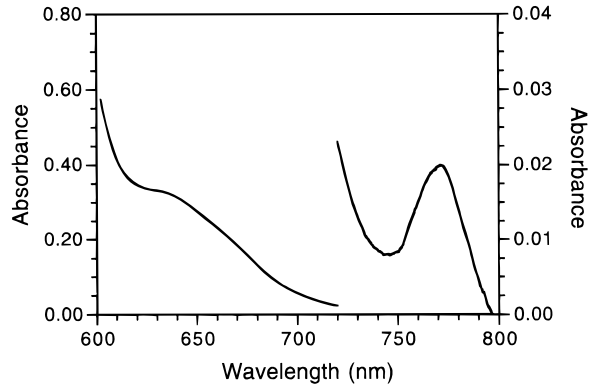

FIGURE 4: Electronic absorption spectrum of β1(1−385) (150 μM) in the near-IR region. The right scale refers to the 770 nm porphyrin to iron charge transfer band region, and the left scale refers to the 650 nm region.

Spectral Studies. The UV−visible spectrum of purified β1(1−385) as isolated is characterized by a sharp Soret band with a maximum at 431 nm and a single, broad α/β band with a maximum at 562 nm (Figure 3). The shape and the peak position of the Soret and the α/β band of β1(1−385) are almost identical to those of the heterodimeric native sGC isolated from bovine lung (*9, 10*). Therefore, the heme in β1(1−385) is 5-coordinate high-spin ferrous. A band near the infrared region with a maximum at 771 nm was also observed in the β1(1−385) spectrum (Figure 4). This near-IR band is believed to result from a porphyrin to Fe^{2+} charge transfer transition (*9*). Treatment of β1(1−385) with CO led to the formation of a CO−heme complex that has a very sharp Soret band with a maximum at 423 nm (Figure 5). The CO complex has a higher extinction coefficient than the ferrous heme in β1(1−385) (see Table 1) and a split α/β band (541 and 567 nm). β1(1−385) forms a complex with CO in the absence of a strong reducing agent such as dithionite, adding further support to the conclusion that the heme in β1(1−385) as isolated is ferrous. β1(1−385) also forms a complex with NO under anaerobic conditions. The nitrosyl complex of the heme in β1(1−385) has a Soret band at 399 nm and a split α/β region (Figure 6). A shoulder at 485 nm was also observed in the spectrum of the nitrosyl complex of β1(1−385) (Figure 6). The Soret position at 399 nm and the presence of the shoulder at 485 nm indicate that the nitrosyl complex is probably 5-coordinate (*19, 24*). Ferric heme in β1(1−385) was obtained by oxidation with ferricyanide. The ferric heme in β1(1−385) has a Soret peak at 392 nm (Figure 7). The ferric heme in β1(1−385) forms a complex with cyanide, and the Soret shifts from 392 to 420 nm (Figure 8).

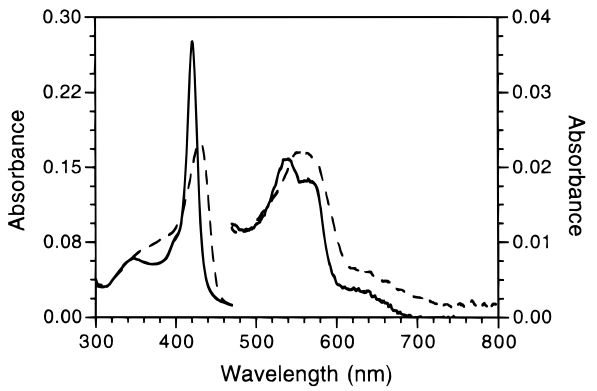

FIGURE 5: Electronic absorption spectra of the β1 CO complex (1.5 μM) in 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT: under air (- - -), and under CO (—). The left scale refers to the Soret peak region, and the right scale refers to the α/β region.

Table 1: UV−Visible Spectra Peak Positions (nm) and Millimolar Extinction Coefficients for the β1(1−385) and Bovine Lung sGC[a]

samples	Soret	α	β	Por−Fe
ferrous				
sGC	431 (111)	562 (12)		770 (0.2)
β1(1−385)	431 (124)	562 (13)		770 (0.13)
CO complex				
sGC	423 (145)	541 (12)	567 (12)	
β1(1−385)	423 (196)	541 (15)	568 (13)	
NO complex				
sGC	399 (85)	537 (11)	572 (11)	
β1(1−385)	399 (108)	537 (14)	572 (15)	
ferric				
sGC	391 (110)	511 (14)		642 (4)
β1(1−385)	392 (113)	520 (15)		645 (4)
cyanide complex				
sGC	418 (93)		538 (11)	
β1(1−385)	419 (110)		538 (11)	

[a] The sGC data were taken from ref (*10, 18*). Por−Fe refers to the putative porphyrin to iron charge transfer band.

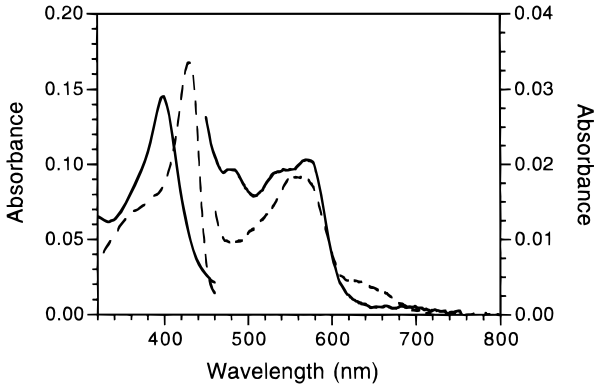

FIGURE 6: Electronic absorption spectra of the β1(1−385) nitrosyl complex (1.5 μM) in 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT: under air (- - -), and under NO (—). The left scale refers to the Soret peak region, and the right scale refers to the α/β region.

DISCUSSION

In this report, we present the first direct evidence showing that the β1 subunit of sGC alone is competent to bind heme. The heme binding region of sGC also has been located unambiguously in the N-terminal region of sGC. Our results

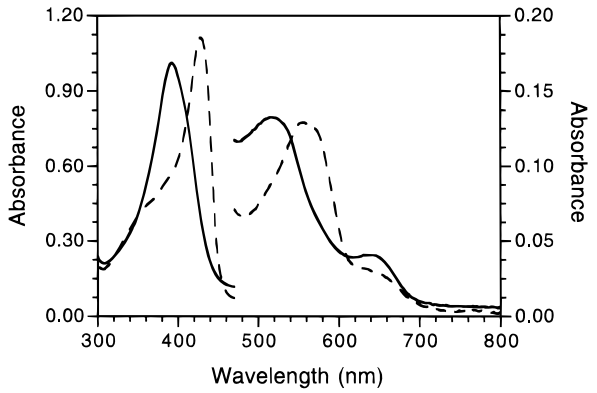

FIGURE 7: Electronic absorption spectra of the β1(1−385) ferric complex (9 μM) in 50 mM Hepes, pH 7.4, 150 mM NaCl: ferrous (- - -), ferric (—). The left scale refers to the Soret region, and the right scale refers to the α/β region.

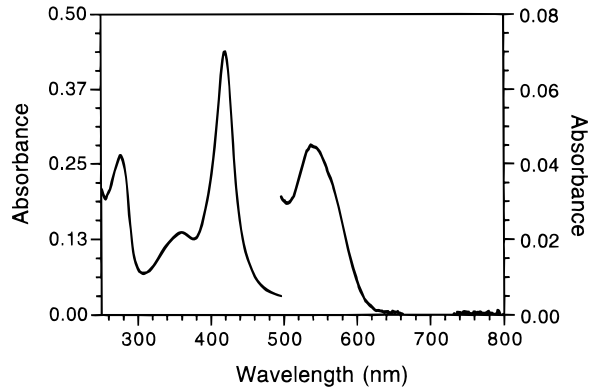

FIGURE 8: Electronic absorption spectrum of the β1(1−385) cyanide complex (4 μM) in 50 mM Hepes, pH 7.4, 150 mM NaCl. The left scale refers to the Soret region, and the right scale refers to the α/β region.

show that the heme in β1(1−385) is similar to the heme in the heterodimeric sGC purified from bovine lung. The heme in both of these proteins is 5-coordinate, ferrous high-spin and consistent with histidine as the axial ligand. The availability of this heme binding β1(1−385) provides an excellent tool to further characterize the sGC heme binding pocket and will eventually lead to studies on the NO activation/deactivation mechanism. UV−visible absorption spectroscopy was used to characterize the heme environment in β1(1−385). The spectroscopic results suggest that the heme environment in β1(1−385) is largely identical to that in the heterodimeric sGC purified from bovine lung. Table 1 lists the UV−visible spectrum peaks for heterodimeric sGC purified from bovine lung, β1(1−385), and their respective complexes with different ligands. The UV−visible spectrum of β1(1−385) as isolated is largely identical to that of bovine lung sGC which has a sharp Soret band (431 nm), a broad α/β region (562 nm), and a porphyrin to Fe^{2+} charge transfer band (770 nm) (Figures 3 and 4). Therefore, the heme in β1(1−385) is 5-coordinate, ferrous high-spin with histidine as the axial ligand. Similar to the bovine lung sGC, the heme in β1(1−385) can form a 6-coordinate complex with CO and shift the Soret from 431 to 423 nm and split the α/β region into two peaks (541 and 567 nm) (Figure 5). The spectrum of the nitrosyl complex of the heme in β1(1−385) is similar to that of the bovine lung sGC, which has a Soret peak at 398 nm, a split α/β region, and a shoulder at 485 nm (Figure 6). Like the heterodimeric sGC, the heme in β1(1−385) can be oxidized using ferricyanide, yielding a ferric heme which has a Soret maximum at 392 nm (Figure 7). Cyanide can also form a 6-coordinate low-spin complex with the ferric heme in β1(1−385) and shift the Soret peak to 420 nm (Figure 8), as is found in the spectrum of the cyanide−heme complex in the heterodimeric sGC. All these spectral studies support the conclusion that the heme environment in β1(1−385) is similar to that in bovine sGC.

The C-terminal portion of sGC is believed to be the catalytic site(s) because this portion is homologous to the catalytic domain of pGC and adenylate cyclase (*7*, *25*). The fact that coexpression of sGC α1 subunit residues 367−691 and β1 subunit residues 306−619 is sufficient for basal guanylate cyclase activity but not for the NO-stimulated activity further supports the hypothesis that the active site(s) is (are) at the C-terminus of sGC (*20*, *26*). The heme binding region of sGC, however, has not been localized. It is not clear whether the heme binding region lies in a separate region from the catalytic site(s) or whether the C-terminal portion of sGC is required for heme binding. Since the C-terminal portion of sGC is involved in catalysis, it has been speculated that the N-terminal portion of sGC is involved in heme binding. There are some reports that suggest that the N-terminal region is important for heme binding. The single point mutations, H105F and C78S, in the β1 subunit disrupted heme binding and NO-stimulated activity in heterodimeric sGC (*20*−*22*). Deletion of the N-terminal 64 amino acid residues in the β1 subunit or deletion of the N-terminal 131 amino acid residues in the α1 subunit also resulted in a loss of NO-stimulated activity (*20*, *21*, *26*). These results suggest that the N-terminal region of sGC may be necessary for heme binding; however, whether the N-terminal portion is sufficient for heme binding or whether the C-terminal region is directly involved is not clear. The data we have presented in this paper clearly demonstrate that β1(1−385) itself is sufficient for heme binding and that the C-terminal region of sGC is not necessary for heme binding to sGC. Therefore, the heme binding region and the catalytic sites are separable and located in different regions of the protein.

Recently, it was suggested that heme binding to sGC requires the presence of both subunits (α1 and β1) based on a study of N-terminal deletion mutants (*20*). However, since expressed and purified β1(1−385) alone has been found to contain a stoichiometric amount of heme, the interaction between the α and β subunits is not necessary for heme binding to the β1 subunit. These results do not rule out an *in vivo* heme binding site involving residues contributed by both the α and β subunits. Given the degree of homology in the N-terminus of the subunits, it is possible that the β1(1−385) homodimer is an *in vitro* outcome of expression in the absence of the α1 subunit. Expression of β1(1−385) and the α1 counterpart will help to definitively answer questions regarding heme stoichiometry and environment in the heterodimeric sGC.

Both bovine and rat lung sGC are known to be heterodimeric proteins ($\alpha1\beta1$); however, the dimerization mechanism for sGC is not yet understood. Formation of a heterodimeric sGC was reported to be necessary for both basal and NO-stimulated guanylate cyclase activity (*15*, *16*). All nucleotide cyclases (adenylate cyclase, pGC, and sGC) appear to require at least two catalytic domains to be active (*7*). It is not clear whether α1 or β1 subunits of sGC fail to

form homodimers or whether the homodimers, once formed, are inactive. Interestingly, β1 (1−385) appears to be a homodimer. A stretch of amino acid residues (residues 340−385) in the β1 subunit that is homologous to the dimerization stretch of pGC (*27*) was included in the expressed β1(1−385). It is not known if the full-length β1 subunit can form a homodimer. It is also not clear whether the C-terminal region of the β1 subunit interferes with the dimerization stretch and prevents β1 from forming a β1 homodimer *in vivo* or whether the interaction between α1 and β1 subunits is simply more favorable. Although the dimerization mechanism for sGC is not understood, the short stretch of amino acid residues (residues 340−385) in the β1 N-terminal fragment that is homologous to the dimerization stretch in pGC may also be responsible for the dimerization of sGC. The fact that the point mutation H346A of β1(1−385) leads β1(1−385) to be exclusively monomeric suggests that the stretch is indeed important for dimerization (Zhao and Marletta, unpublished data). Although the mutant is monomeric, it still binds a stoichiometric amount of heme (Zhao and Marletta, unpublished data). Interestingly, a similar case was found for MoFe protein of nitrogenase from *Azotobacter vinelandii*. The native MoFe protein is a heterotetramer ($\alpha_2\beta_2$). When the β subunit was expressed alone, it formed a homotetramer (β_4) (*28*).

In summary, the N-terminal region of the β1 subunit of sGC is not only necessary but also sufficient for heme binding. The heme in the heme binding N-terminal fragment of the β1 subunit of sGC is similar to the heme in heterodimeric sGC based on a series of UV−visible spectroscopic studies. Successful expression and purification of this heme binding N-terminal fragment will facilitate answering questions ranging from the heme environment of sGC to the mechanism of dimerization.

ACKNOWLEDGMENT

We thank Dr. Masaki Nakane (Abbott Laboratories) for kindly providing the rat sGC β1 cDNA and Jason Perry for helpful discussions. We also thank the members of the Marletta lab for their critical comments on the manuscript and Rae Herrst for editorial assistance.

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BI971825X