# Heme Stoichiometry of Heterodimeric Soluble Guanylate Cyclase[†]

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ABSTRACT: The soluble form of guanylate cyclase (sGC) is to date the only definitive receptor for the novel signaling agent nitric oxide (•NO). •NO increases the $V_{max}$ of sGC by 100−200-fold, and it has been proposed that this activation occurs subsequent to the binding of •NO to a heme moiety on the enzyme. It has previously been demonstrated that the enzyme can be purified in a state containing as much as 1 heme per heterodimer. However, since the two subunits of the heterodimer display considerable homology, and the enzyme routinely loses heme upon purification, it has been unclear whether the native heme stoichiometry is 1 per heterodimer or 2 per heterodimer. Using a novel procedure, the enzyme has been purified to homogeneity from bovine lung in a state containing 1.52 ± 0.10 equiv of heme per heterodimer, indicating that the native heme stoichiometry is 2 per heterodimer. The •NO-activated specific activity of this enzyme is increased by 50% over that of enzyme containing 1 heme per heterodimer and is the highest specific activity ever observed for sGC. Spectrally only one type of heme is observed, indicating that both hemes in the heterodimer are in similar environments. It is concluded that each subunit of the heterodimer binds 1 equiv of heme at a site conserved between the two subunits. Alignment of the nine published cDNA sequences for sGC indicates that the heme binding domain is the central portion of each subunit, corresponding to residues 213−370 in the bovine $\beta_1$ sequence.

The guanylate cyclases are a family of enzymes that catalyze the cyclization of guanosine 5′-triphosphate (GTP)[1] to guanosine 3′,5′-cyclic monophosphate (cGMP) (Garbers & Lowe, 1994; Waldman & Murad, 1987). There are two general classes of the cyclase, particulate guanylate cyclase (pGC) and soluble guanylate cyclase (sGC). Isoforms of pGC are activated by peptide ligands such as ANF, which bind to an extracellular receptor domain (Garbers & Lowe, 1994), while sGC serves as the predominant, if not the only, receptor for the novel signaling agent nitric oxide (•NO) [for recent reviews see Bredt and Snyder, (1994), Marletta (1993, 1994), and Nathan (1992)]. In the presence of •NO, the $V_{max}$ of sGC is increased by 100−200-fold. sGC is a heterodimer possessing an α subunit with a mass ranging from 73 to 88 kDa and a β subunit with a mass ranging from 70 to 76 kDa. Previously, the enzyme has been purified in a state containing one protoporphyrin-IX type heme per heterodimer (Gerzer et al., 1981; Ignarro et al., 1986; Stone & Marletta, 1994). The heme is five-coordinate high spin in both the ferrous and the ferric states with histidine as the sole axial ligand (Stone & Marletta, 1994). The heme readily forms a complex with •NO, and electronic absorption and electron paramagnetic resonance spectral studies have indicated that this nitrosyl complex is five-coordinate (Stone et al., 1995; Stone & Marletta, 1994). It was originally proposed that activation of sGC by •NO was a direct result of the formation of a five-coordinate nitrosyl-heme complex (Ignarro et al., 1984). The severing of the bond to the *trans*-axial ligand and the resulting changes in porphyrin structure were postulated to mediate a conformational change to the protein as a whole, which results in activation of the enzyme. This mechanism of activation is consistent with all published observations (Stone et al., 1995).

Since the highest heme content previously reported for purified sGC was approximately 1 per heterodimer, it has generally been assumed in the literature that the native heme stoichiometry is, in fact, 1 per heterodimer. Before the DNA sequences of two subunits were available, it was proposed that one subunit was regulatory and bound the heme and that the other subunit was catalytic (Kamisaki et al., 1986). However, analysis of the cDNA sequences revealed that the two subunits show a high degree of homology (Koesling et al., 1990; Nakane et al., 1990). With this result, the location of the binding site for the single heme in the heterodimer was not clear. There are currently no examples of hemoproteins in which the heme is sandwiched between two different subunits, and there is no direct evidence that only one subunit of sGC can bind heme. However, since the two subunits of the heterodimer are homologous, and the enzyme routinely loses heme upon purification, it seemed plausible that the native heme stoichiometry is actually 2 per heterodimer, with each subunit binding 1 equiv of heme.

Here we report the purification of protein that contains 1.5 equiv of heme per heterodimer, indicating a native stoichiometry of 2 per heterodimer. Also, for the first time

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[1] Abbreviations: sGC, soluble guanylate cyclase; pGC, particulate guanylate cyclase; •NO, nitric oxide; TEA, triethanolamine, EDTA, ethylenediaminetetraacetic acid; IBMX, isobutylmethylxanthine; DTT, dithiothreitol; GTP, guanosine 5′-triphosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; Hb, hemoglobin; Mb, myoglobin; SNP, sodium nitroprusside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride.

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the electronic absorption spectra are reported for the ferric form of the purified enzyme in the visible region and for the ferrous form of the enzyme in the near-IR region. On the basis of the heme stoichiometry, the electronic absorption spectral characteristics, and sequence alignments, the probable location of the heme binding site in sGC has been identified.

## MATERIALS AND METHODS

*Materials.* Research-grade argon (99.9999%) and •NO (99.0%) were obtained from Matheson. All other materials unless otherwise stated were obtained from Sigma.

*Purification of sGC from Bovine Lung.* sGC was purified from bovine lung using a novel procedure based on two previously published procedures (Mulsch & Gerzer, 1991; Stone & Marletta, 1994). All steps were performed at 4 °C. During the purification, assays for sGC were carried out in 50 mM TEA, pH 7.4, with 2 mM DTT, 5 mM $MnCl_2$, 100 $\mu$M GTP, 1 mM IBMX, 5 mM phosphocreatine, and 152 units of creatine kinase/L, with or without 100 $\mu$M SNP, in a total volume of 100 $\mu$L at 37 °C. The assays were quenched with 400 $\mu$L of 125 mM $Zn(CH_3COO)_2$ and 500 $\mu$L of 125 mM $Na_2CO_3$. The amount of cGMP generated was then quantitated via radioimmunoassay (Amersham).

Bovine lung (1100 g) was homogenized with a food processor in 1100 mL of homogenizing buffer (25 mM TEA, 50 mM NaCl, 5 mM DTT, 1mM PMSF, 1 mM EDTA, 2 mM benzamidine, 1 $\mu$M pepstatin A, 1 $\mu$M leupeptin, pH 7.4). The homogenate was then centrifuged at 100 000*g* for 60 min. The resulting supernatant was added to 450 mL of Q-Sepharose Fast Flow (Sigma) and stirred slowly with an overhead stirrer for 45 min. The buffer was removed by vacuum filtration, and the resin was washed twice by stirring slowly for 15 min with 1200 mL of homogenizing buffer. The resin was then poured into a 2.5 × 100 cm column and packed by washing the column with homogenizing buffer for 1 hour at 80 mL/h. sGC was then eluted at 80 mL/h with 1 L of a 0.0−0.4 M NaCl gradient followed by a 300 mL wash at 0.4 M NaCl. Active fractions were pooled (220 mL) and diluted by a factor of 3.1 with 25 mM TEA, 7.5 mM DTT, pH 7.4.

The sample was then applied at 200 mL/h to a 5 × 40 cm (800 mL) column of Blue agarose (Sigma, Type 300, 0.5 $\mu$mol of dye per mL of bed volume). The column was washed for 2.75 h at 110 mL/h with buffer A (25 mM TEA, 50 mM NaCl, 5 mM DTT, 0.1 mM PMSF, 0.2 mM benzamidine, pH 7.4). The column was then washed at 110 mL/h with an 11 h linear gradient running from 50 to 750 mM NaCl in buffer A, followed by a 2 h wash with buffer A containing 750 mM NaCl. The sGC activity eluted at ~0.3 M NaCl. Active fractions were pooled (280 mL), supplemented with additional DTT (5 mM) and NaCl (0.5 M), and concentrated by ultrafiltration to 8 mL on an Omega Series membrane (30 kDa MWCO, Filtron). The sample was then applied to a 2.5 × 95 cm column of Ultrogel AcA-34 (IBF Biotechnics, Villeneuve-la-Garenne, France) at 20 mL/h. The column was washed with 400 mL of 25 mM TEA, 50 mM NaCl, 5 mM DTT, 0.1 mM PMSF, 0.2 mM benzamidine, pH 7.4, at 20 mL/h. Active fractions were pooled (25 mL), provided with additional DTT (5 mM), and brought to 5 mM $MnCl_2$.

The sample was then applied at 50 mL/h to a 2.5 × 3 cm (15 mL) column of ATP−agarose (0.5 $\mu$mol of ATP per mL of bed volume, see below). The column was washed at 50 mL/h for 15 min with buffer B (25 mM TEA, 50 mM NaCl, 5 mM DTT, 0.1 mM PMSF, 0.2 mM benzamidine, 5 mM $MnCl_2$, pH 7.4). The column was then washed at 50 mL/h for 20 min with buffer C (25 mM TEA, 5 mM DTT, 0.1 mM PMSF, 0.2 mM benzamidine, 5 mM $MnCl_2$, 10 mM $NaNO_3$, 10 mM creatine, 0.2 mM ADP, pH 7.4), followed by buffer B at 50 mL/h until the enzyme eluted (~2 h), presumably as a result of enzymatic degradation of the column. The active fractions were pooled (70 mL), supplemented with additional DTT (5 mM), brought to 1.0 M NaCl, and concentrated by ultrafiltration to 0.5 mL on an Omega Series membrane. To remove $MnCl_2$ before freezing, the sample was then applied to a 0.7 × 20 cm Bio-Gel P-6 DG gel filtration column (Bio-Rad) equilibrated with 25 mM TEA, 5 mM DTT, 1 M NaCl, pH 7.4 (final volume = 1.0 mL). The sample was brought to 50% glycerol and stored as aliquots under nitrogen at −70 °C.

*Synthesis of ATP−Agarose.* The ATP−agarose used in the purification was synthesized by coupling the ligand [8-(6-aminohexyl)amino-ATP] (Sigma) to cyanogen bromide (CNBr)-activated Sepharose-4B (Pharmacia) following a previously published procedure (Barker et al., 1974). All steps were performed at 4 °C. Briefly, 15 mL of CNBr-activated resin was hydrated with 50 mL of cold water and then washed with an additional 50 mL of water in a 2.5 × 10 cm glass column. To this resin was added 15 mL of water containing 1.25 mM ligand and 10 mM NaCl. The resin was suspended, and the pH was adjusted to 10 with NaOH. The suspension was then agitated for 16 h in an end-over-end resin mixer. The resin was washed with 50 mL of 25 mM TEA, 2 M NaCl, pH 7.4, to remove any noncovalently bound ligand. The resin was stored in 25 mM TEA, pH 7.4, at 4 °C until use.

The ATP−agarose resin was analyzed for ATP content by total acid hydrolysis as previously described (Lowe, 1979). ATP−agarose (0.1 mL) was placed in a 1.5 mL microcentrifuge tube with water (100 $\mu$L) and 10 N sulfuric acid (1.0 mL) and incubated at 45 °C for 4 h. The sample was centrifuged at 10 000*g* for 2 min, and then the electronic absorption spectrum of the supernatant was recorded. The absorbances at 255 and 310 nm were determined, and the $\Delta$Abs(255−310) was calculated. For standards, 0.1 mL of unligated CNBr-activated resin was placed in a microcentrifuge tube with 1.0 mL of 10 N sulfuric acid and 100 $\mu$L of water or water containing a particular concentration of the ligand. The standards were then processed as described above for the sample. For the standards, plotting $\Delta$Abs(255−310) versus the concentration of ligand yields a straight line suitable for determining the ligand concentration of the sample. The $\Delta$Abs(255−310) was used rather than just the absorbance at 267 nm to avoid interference by furfuraldehyde ($\lambda_{max}$ = 280 nm) formed during the resin hydrolysis (Lowe, 1979). Typically, resin made as described above contained 0.5−0.6 $\mu$mol of ATP per mL of bed volume.

*Heme Content.* Heme concentrations were determined by the pyridine−hemochromagen assay using myoglobin as the standard as previously described (White & Marletta, 1992). Prior to analysis, all sGC samples were applied to a 0.7 × 20 cm Bio-Gel P-6 DG gel filtration column (Bio-Rad) equilibrated with 25 mM TEA, 50 mM NaCl, pH 7.4, to remove DTT.

Table 1: Purification Summary for sGC

| fraction | activity[a] (nmol/min) | mass protein[b] (mg) | specific activity (nmol/min mg) | purification factor |
|---|---|---|---|---|
| supernatant | 1460 | 28900 | 0.0506 | 1.0 |
| Q-Sepharose | 5230 | 2390 | 2.19 | 43.3 |
| Blue agarose | 2610 | 125 | 20.9 | 413 |
| Ultrogel AcA-34 | 2610 | 13.3 | 196 | 3870 |
| ATP−agarose | 763 | 1.89 | 404 | 7980 |

[a] Assay conditions were as described in the methods section with $Mn^{2+}$ as the required divalent metal. [b] Determined by Bradford microassay, not corrected by quantitative amino acid analysis.

*Activation of Purified sGC with •NO.* •NO was purified of other nitrogen oxides by bubbling the gas through saturated KOH. Buffer containing 50 mM TEA, pH 7.4, 2 mM DTT, 4.5 mM $MgCl_2$, and 1.5 mM GTP was placed in a gas-tight vial under 1 atm of either argon or argon containing 0.5% •NO and warmed to 37 °C. The reaction was initiated by the injection of 4 μL of sGC with a gas-tight syringe to give a final reaction volume of 1.0 mL with an enzyme concentration of 0.75 μg/mL. At time intervals, 100 μL aliquots were removed with a gas-tight syringe and then quenched and assayed for cGMP as described above.

*Protein Determination.* Protein concentrations were determined with the Bradford microassay (Bio-Rad) using BSA as the standard. The assay was calibrated for sGC using quantitative amino acid analysis as described previously (Stone & Marletta, 1994).

*Electronic Spectroscopy.* All electronic spectra were obtained on a Cary 3E UV−visible spectrophotometer at 10 °C. Buffer conditions were as described in the figure legends.

*Electrophoresis.* Reducing SDS polyacrylamide gel electrophoresis (SDS−PAGE) of purified sGC was performed using the Bio-Rad minigel system following the procedure provided by the manufacturer. The running gel contained 12% acrylamide, and the protein was visualized with silver stain (Bio-Rad).

*Sequence Alignments.* cDNA sequences were aligned using the Wisconsin Package, Version 8.0 (Genetics Computer Group, Madison, WI).

## RESULTS

*Purification/Heme Content.* The novel purification procedure described here results in the isolation of approximately 1.9 mg of sGC from 1100 g of bovine lung (Table 1). Reducing SDS−PAGE of the purified protein revealed two bands of approximately equal intensity with apparent masses of 69 and 78 kDa (data not shown), as observed previously (Stone & Marletta, 1994). Also as seen previously, the total activity increases upon placing supernatant over the ion-exchange column (Stone & Marletta, 1994; Wolin et al., 1982). The purified protein was found to contain 1.52 ± 0.10 equiv of heme per heterodimer ($n$ = 3 preparations) with an extinction coefficient of 105 ± 7 (mM heme)$^{-1}$ cm$^{-1}$ at 431 nm.

*Spectral Studies.* The electronic absorption spectra of the ferrous and ferric forms of the enzyme were obtained in 25 mM TEA, 50 mM NaCl, pH 7.4, under air (Figure 1). The spectrum of the ferrous enzyme has a sharp Soret at 431 nm and a single broad peak in the α/β region at 562 nm. Addition of 15 μM ferricyanide to thiol-free sGC shifts the

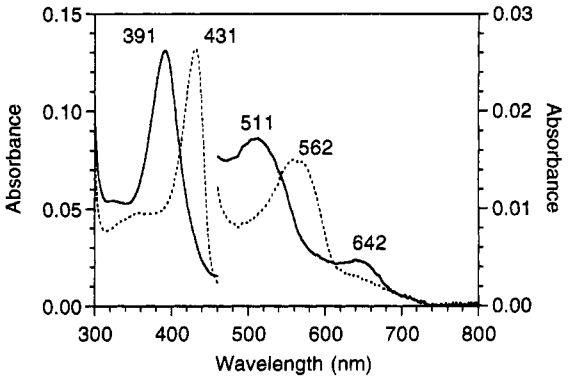


FIGURE 1: Electronic absorption spectra of sGC. sGC (1.2 μM heme) in 25 mM TEA, pH 7.4, 50 mM NaCl, under air, in the absence (···) and presence (—) of 15 μM potassium ferricyanide. The scale on the left refers to the Soret region, and the scale on the right refers to the α/β region. The sample used for this spectrum had been frozen in 50% glycerol, thawed, and then applied to a 0.7 × 20 cm Bio Gel P-G DG gel filtration column to remove the DTT and glycerol.

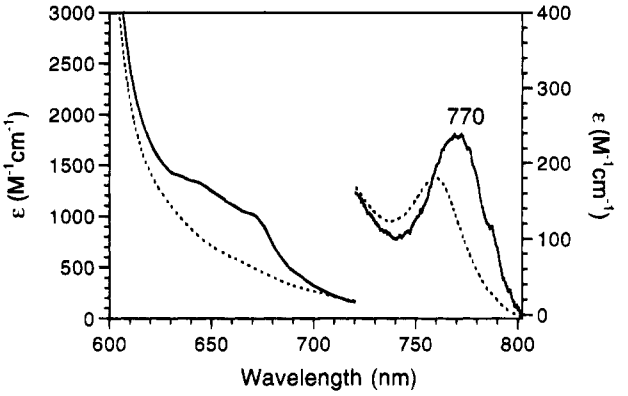


FIGURE 2: Visible/near-IR electronic absorption spectrum of ferrous sGC. Ferrous sGC (15 μM heme) in 150 mM TEA, 300 mM NaCl, pH 7.4, under air (—). Sperm whale myoglobin (100 μM) under argon in the same buffer containing 10 mM sodium dithionite (···). The scale on the left refers to the 650 nm region, and the scale on the right refers to the 770 nm region. The sample used for this spectrum had not been previously frozen and therefore had not been treated with glycerol.

Soret from 431 to 391 nm (Figure 1). Furthermore, the single broad peak at 562 nm is replaced by peaks at 511 and 642 nm. These spectra were obtained in the absence of glycerol, and the presence of 50% glycerol does not significantly alter either spectrum (data not shown). In fact, all of the spectra reported here were the same regardless if they were acquired before the addition of glycerol or after glycerol removal for samples that had had glycerol added or that were frozen in the presence of it for stability. Figure 2 depicts the electronic spectra of sGC and sperm whale deoxyMb in the visible and near-IR regions. Of particular note is the band at 760 nm in the spectrum of deoxyMb and the band at 770 nm in the spectrum of sGC. Also, in the spectrum of sGC there are bands with $\epsilon$ = 600 M$^{-1}$ cm$^{-1}$ around 650 nm. These bands are not present in the spectrum of deoxyMb.

*Activity Studies.* The activity of the enzyme containing 1.5 equiv of heme per heterodimer in the presence and absence of •NO was compared to that previously published for enzyme containing 1 equiv of heme per heterodimer (Stone & Marletta, 1994). The two different preparations of enzyme were assayed under identical conditions. For each

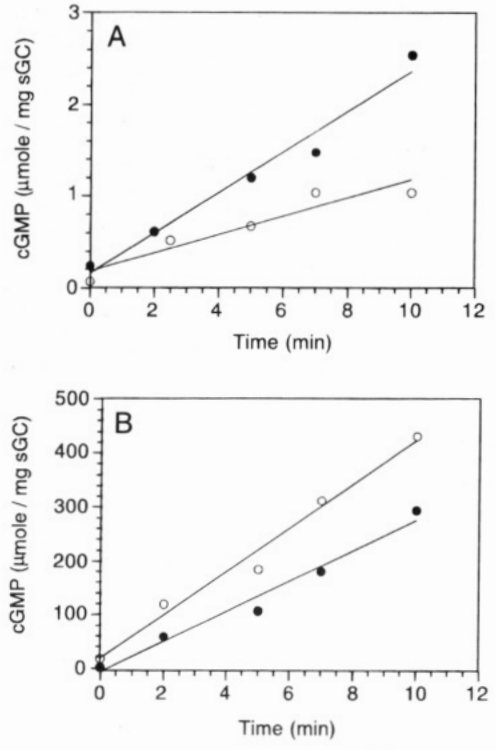


FIGURE 3: Activation of sGC by •NO. sGC was assayed as described in the Materials and Methods section. Panel A: in argon; current purification procedure (○), previous purification procedure (●). Panel B: 0.5% •NO in argon; current purification procedure (○), previous purification procedure (●). Note the difference in the ordinate scale between the two panels.

Table 2: Comparison of Specific Activities[a]

| hemes per heterodimer | basal sp act (nmol/min mg) | •NO sp act (nmol/min mg) | fold activation |
|---|---|---|---|
| 1.08 ± 0.17 | 221 ± 23 | 28 200 ± 2 800 | 128 ± 17 |
| 1.52 ± 0.10 | 100 ± 19 | 40 600 ± 3 200 | 406 ± 84 |

[a] The protein concentration was determined by the Bradford microassay, and the values have been corrected by values obtained from quantitative amino acid analysis. The assays conditions were as described in the Materials and Methods section with $Mg^{2+}$ as the required divalent metal.

sample, enzyme activity was linear for the 10 min assayed (Figure 3). There does not appear to be any lag for activation with •NO, nor do the activated rates diminish during the 10 min observed. The slopes of the lines in Figure 3 are the specific activities and are summarized in Table 2. The •NO-activated specific activity of enzyme containing 1.5 equiv of heme per heterodimer is 50% higher than that of enzyme containing only 1 heme per heterodimer. For both enzyme samples, the nonactivated (basal) specific activity is significantly lower when $Mg^{2+}$ is used as the required divalent metal (Table 2) rather than $Mn^{+2}$ (Table 1) (Waldman & Murad, 1987). The basal specific activity of enzyme containing 1.5 equiv of heme per heterodimer is lower than that of enzyme containing only 1 equiv of heme per heterodimer (Table 2). This lower basal rate combined with a higher •NO-activated rate results in a 400-fold activation for the enzyme containing 1.5 equiv of heme per heterodimer compared to a 130-fold activation for enzyme containing 1 equiv of heme per heterodimer.

*Sequence Alignments.* The nine published cDNA sequences for subunits of sGC (Giuli et al., 1992; Harteneck et al., 1991; Koesling et al., 1988, 1990; Nakane et al., 1988, 1990; Yoshikawa et al., 1993; Yuen et al., 1990) were aligned to determine the probable heme binding site in the protein. Figure 4 depicts the general domains of sGC and lists, for each of the three domains, the identity and homology between the subunits of the bovine lung enzyme as well as the number of invariant residues for all nine sGC sequences. A comparison of the nine cDNA sequences for the central domain of sGC is shown in Figure 5. Of the 25 invariant residues, two are histidines, $\beta$220 and $\beta$346. The alignment also shows that many of the other invariant residues are hydrophobic in nature (phenylalanine and leucine).


| Function | Regulatory | Regulatory | Catalytic |
|---|---|---|---|
| Residues (β1) | 1 - 212 | 213 - 370 | 371 - 619 |
| Identity | 20% | 45% | 43% |
| Homology | 47% | 65% | 60% |
| Invariant Residues | 1 | 25 | 51 |

FIGURE 4: Comparison of the cDNA sequences of the two subunits of sGC. The identities and homologies listed are for the two subunits of the bovine lung enzyme. The numbers of invariant residues listed are for all nine published sequences of sGC subunits.

## DISCUSSION

The new purification procedure detailed here provides for the purification of enzyme containing approximately 1.5 equiv of heme per heterodimer. The high heme content is the result of several modifications to our previously reported procedure (Stone & Marletta, 1994). One key modification that helped to increase the heme content was the inclusion of 50 mM NaCl in almost all buffers. The protein is more stable in the presence of NaCl, and all ultrafiltration concentration steps were performed in ~1 M NaCl. Another key factor was the observation that high ligand concentrations on the resins (≥1 μmol per mL of bed volume) could cause substantial heme loss regardless of the type of resin. This was found to be the case not only for dye columns but also for nucleotide columns. Since there was no commercially available ATP−agarose with a ligand concentration this low, this resin had to be synthesized.

In addition to a lower heme content, the previous method had several other problems. The procedure relied upon commercially prepared ATP−agarose and GTP−agarose resins. It was found over time that the manufacturers were unable to provide consistent preparations of these resins. Furthermore, these resins are quite expensive and not reusable. This was overcome by switching to the Blue agarose resin and by moving the ATP−agarose step to the end of the procedure. The Blue agarose can be easily regenerated by extraction with 1:1 chloroform:methanol, and the cost of the small (15 mL) ATP−agarose column is minimal compared to that of the nucleotide resins used in the previous procedure. Two other advantages of the current procedure are the time to complete the procedure and the yield of protein. The previous procedure required 6 days for completion, while the current one requires only 4 days. Also the current procedure yields three to four times more protein than does the previous one.

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                 **  ** *           *  *
β1-Bov    213  FCKAFPFHII FDRDLVVTQC GNAIYRVLPQ LQ.PGNCSLL  251
β1-Hum    213  FCKAFPFHII FDRDLVVTQC GNAIYRVLPQ LQ.PGNCSLL  251
β1-Rat    213  FCKAFPFHII FDRDLVVTQC GNAIYRVLPQ LQ.PGKCSLL  251
β2-Rat    209  FCDAFPFHIV FDEALRVKQA GVNIQKYVPG IL.TQKFALD  247
α1-Bov    283  FCKTFPFHFM LDRDMSILQL GHGIRRLMSR RDVQGKPHFD  322
α1-Rat    281  FCKTFPFHFM LDRDLAILQL GNGIRRLVNK RDFQGKPNFE  320
α1-Hum    280  FCKTFPFHFM FDKDMTILQF GNGIRRLMNR RDFQGKPNF.  318
α2-Hum    323  FCRAFPFHLM FDPSMSVLQL GEGLRKQL.R CDTHKVLKFE  361
Drosph    265  FCKMFPWHFI MNEQLELVQL GRGFSKLYKP YMADFGCQAT  304

                  *    *                   *
β1-Bov    252  SVFSLVRPH. IDISFHGILS HINTVFVLRS KEGLLDVEKS  290
β1-Hum    252  SVFSLVRPH. IDISFHGILS HINTVFVLRS KEGLLDVEKL  290
β1-Rat    252  SVFSLVRPH. IDISFHGILS HINTVFVLRS KEGLLDVEKL  290
β2-Rat    248  EYFSIIHPQ. VTFNISSICK FINSQFVLKT RKEMMPKAR.  285
α1-Bov    323  EYFEILTPK. ISQTFSGIMT MLNMQFLVRV RR........  353
α1-Rat    321  EFFEILTPK. INQTFSGIMT MLNMQFVIRV RR........  351
α1-Hum    319  EYFEILTPK. INQTFSGIMT MLNMQFVVRV RR........  349
α2-Hum    362  DCFEIVSPK. VNATFERVLL RLSTPFVIRT KP........  392
Drosph    305  TYFDFKRPKG LTMKFRDIVR RTYTPFLIGL NN........  336

                                  ***             *  **    *
β1-Bov    291  ECEDELTGTE ISCLRLKGQM IYLPEADSIL FLCSPSVMNL  330
β1-Hum    291  ECEDELTGTE ISCLRLKGQM IYLPEADSIL FLCSPSVMNL  330
β1-Rat    291  ECEDELTGAE ISCLRLKGQM IYLPEADSIL FLCSPSVMNL  330
β2-Rat    286  ........KS QPMLKLRGQM IWMESLRCMI FMCSPNVRSL  317
α1-Bov    354  ..WDNSMKKS SRVMDLKGQM IYMVESSSIL FLGSPCVDRL  391
α1-Rat    352  ..WDNLVKKS SRVMDLKGQM IYIVESSAIL FLGSPCVDRL  389
α1-Hum    350  ..WDNSVKKS SRVMDLKGQM IYIVESSAIL FLGSPCVDRL  387
α2-Hum    393  ..EASGSENK DKVMEVKGQM IHVPESNSIL FLGSPCVDKL  430
Drosph    337  ..PPGAVDFP AIGLEIKGQM VHCPESNSLL FIGSPFLDGL  374

                         ***  *   *    *   *       *
β1-Bov    331  DDLTRRGLYL SDIPLHDATR DLVLLGEQFR EEYKLTQELE  370
β1-Hum    331  DDLTRRGLYL SDIPLHDATR DLVLLGEQFR EEYKLTQELE  370
β1-Rat    331  DDLTRRGLYL SDIPLHDATR DLVLLGEQFR EEYKLTQELE  370
β2-Rat    318  QELEESKMHL SDIAPHDTTR DLILLNQQRL AEMELSCQLE  357
α1-Bov    392  EDFTGRGLYL SDIPIHNALR DVVLIGEQAR AQDGLKKRLG  431
α1-Rat    390  EDFTGRGLYL SDIPIHNALR DVVLIGEQAR AQDGLKKRLG  429
α1-Hum    388  EDFTGRGLYL SDIPIHNALR DVVLIGEQAR AQDGLKKRLG  427
α2-Hum    431  DELMGRGLHL SDIPIHDATR DVILVGEQAK AQDGLKKRMD  470
Drosph    375  DGLTCNGLFI SDIPLHDATR EVILVGEQAR AQDGLRRRMD  414
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FIGURE 5: Putative heme binding domain of sGC. The nine published cDNA sequences for the central domain of sGC have been aligned. Invariant residues are indicated by bold type and an asterisk.

As reported previously, the electronic absorption spectrum of the ferrous enzyme (Figure 1) is characteristic of a five-coordinate high-spin ferrous heme with imidazole as the axial ligand, such as in deoxyMb (Stone & Marletta, 1994). In the electronic spectrum of deoxyMb in the near-IR region, there is a band at 760 nm resulting from a porphyrin $\rightarrow Fe^{+2}$ charge transfer (Figure 2). In Hb and Mb this band is believed to be present only in the five-coordinate ferrous form of the protein and is often used as an indicator for that coordination/redox state (Eaton & Hofrichter, 1981). For sGC there is a band at 770 nm, which probably corresponds to the same transition (Figure 2). In Hb the position of this band is extremely sensitive to minor alternations in porphyrin structure. For example, during the first 10 ns after photolysis of CO from CO−Hb, this band is red-shifted 5 nm compared to that of deoxyHb, even though both species are five-coordinate ferrous (Sassaroli & Rousseau, 1987). Thus the presence of the charge transfer band at 770 nm in the spectrum of sGC indicates that the heme is five-coordinate ferrous, but there are probably minor structural differences between the heme of sGC and the hemes of deoxyHb and deoxyMb. Also for sGC, there are bands with $\epsilon = 600\ M^{-1}\ cm^{-1}$ around 650 nm. These bands are not present in the spectrum of deoxyMb, and the nature of the transition giving rise to them is unclear. The presence of these bands also indicates that there are structural differences between the heme in sGC and that in deoxyMb. The bands at 650 nm and 770 nm should prove useful in developing heme model compounds which structurally mimic the heme in sGC.

Previously, the ferric form of the heme in sGC was proposed to be five-coordinate high spin based solely on the position of the Soret (Stone & Marletta, 1994). Now that the electronic spectrum for the entire visible region has been obtained, a more complete analysis can be performed. In the electronic absorption spectrum of ferric sGC (Figure 1), the peak at 511 nm is primarily the $\beta$ band of the heme, and the peak at 642 nm results from a porphyrin $\rightarrow Fe^{3+}$ charge transfer (Adar, 1978). This pattern of peaks is characteristic

of a high-spin ferric heme, indicating a relatively weak axial ligand field (Antonini & Brunori, 1971). If the heme were low spin, peaks centered at ~550 nm would be observed. The position of the porphyrin → $Fe^{+3}$ charge transfer band at 642 nm indicates that ferric sGC is five-coordinate. These bands are usually in the range of 605−635 nm for six-coordinate high-spin complexes (Antonini & Brunori, 1971) and 640−650 nm for five-coordinate high-spin complexes (Gilles-Gonzalez et al., 1994; Yoshimura et al., 1985). Thus even in the ferric state, the heme of sGC is five-coordinate high spin, with the unusual property of not binding water. The most likely reason for the failure of ferric sGC to bind water is the presence of a hydrophobic distal pocket lacking a residue which can hydrogen bond to water and stabilize the coordination of the water by the iron (Quillin et al., 1993).

In two reports from one laboratory using a heme-reconstituted preparation of sGC, it has been suggested that the heme is low spin with bishistidyl coordination (Burstyn et al., 1995; Yu et al., 1994). The protein used in those studies was reported to be not more than 10% pure in sGC and required heme reconstitution. Also in those studies, the ferric form of the heme-reconstituted preparation was found to adopt a five-coordinate-like state in the presence of glycerol (Burstyn et al., 1995). They suggested that the difference between our conclusions, concerning the coordination state and their conclusions was that we performed our experiments in 50% glycerol. However, as described in the legend to Figure 4 in our previous work, the experiment in question was performed in the absence of glycerol (Stone & Marletta, 1994). Furthermore, all of the spectra in this current report were obtained in the absence of glycerol, and 50% glycerol does not alter the appearance of these spectra. The effect of glycerol on the spectrum of the heme-reconstituted preparation is reminiscent of the effect of glycerol on the EPR and electronic spectra of the D235E mutant of cytochrome *c* peroxidase (Goodin & McRee, 1993). In that report, spectra of the wild-type enzyme indicated the heme is five-coordinate and were unaffected by glycerol, as is the case for sGC purified with the heme intact. However, for the D235E mutant, the spectra indicated the heme is six-coordinate in the absence of glycerol and five-coordinate in the presence of glycerol, as is apparently the case for the heme-reconstituted partially-purified preparation of sGC. We have observed by electronic absorption spectroscopy that five-coordinate sGC can irreversibly denature to a six-coordinate low-spin form upon various treatments (pH > 10, pH < 5, or 1 mM SDS, data not shown) as has been observed with cytochrome *c'* (Horio & Kamen, 1961). Furthermore, when we have tried to reconstitute heme into purified protein containing 0.5 heme per heterodimer, the heme content only increased by ~0.1 heme per heterodimer, and the reconstituted heme appeared to be low spin (data not shown). Given all of the above observations, it is most likely that the six-coordinate low-spin state of sGC is a result of mild protein denaturation.

To determine if the extra 0.5 equiv of heme per heterodimer observed here is playing a functional role on the enzyme, the specific activity of this enzyme in the presence and absence of •NO was compared to that previously reported for enzyme containing only 1 heme per heterodimer (Stone & Marletta, 1994). It was observed that the •NO-activated specific activity increased by 50%, the same factor by which the heme content was increased (Table 2). Previously it had been suggested that the •NO-activated specific activity is directly proportional to the heme content (Ignarro et al., 1982). The increase in the •NO-activated specific activity observed here would then suggest that the extra 0.5 equiv of heme is in fact serving a functional role on the cyclase. The •NO-activated specific activity observed here of 41 000 nmol $min^{-1}$ $mg^{-1}$ is the highest specific activity ever reported for sGC.

There are currently nine published cDNA sequences for subunits of sGC (Giuili et al., 1992; Harteneck et al., 1991; Koesling et al., 1988, 1990; Nakane et al., 1988, 1990; Yoshikawa et al., 1993; Yuen et al., 1990). All of these sequences contain a domain at the C-terminus which is homologous to the catalytic domains of the particulate guanylate cyclases and adenylate cyclases and is assumed to be the cyclase catalytic site. The N-terminal half of each sequence displays considerable homology between the different sGC sequences but not to any other known protein sequence. This N-terminal half of each subunit is assumed to serve a regulatory role. The nine sGC sequences were analyzed to determine the probable site of heme binding in the protein. Since the heme stoichiometry is 2 per heterodimer, the subunits are homologous to each other, and only one type of heme has been identified spectrally, it is probable that each subunit binds 1 equiv of heme. The electronic spectra also indicate that the heme is coordinated by a single histidine from the protein. Thus at least one histidine should be conserved in all nine sGC cDNA sequences. Figure 4 depicts the general domains of sGC and lists the identity and homology between the subunits of the bovine lung enzyme as well as the number of invariant residues for all nine sGC sequences. On the basis of the sequence alignment, each subunit can be divided into three domains. There is an extreme N-terminal domain which displays little homology between the two subunits and contains only one invariant residue, a glycine. Therefore, it is unlikely that the heme binds in this portion of the protein. There is also a central domain to the protein which is still part of the "regulatory" region. This central domain displays much higher homology between the two subunits and contains 25 invariant residues. Thus, this domain is likely to be the site of heme binding. The catalytic domain at the carboxyl-terminus also displays considerable homology between the two subunits and contains several invariant residues, including one histidine. However, this domain is highly conserved with the catalytic domains of the particulate guanylate cyclases and the adenylate cyclases, and the conserved histidine is probably the catalytic base. None of these other proteins has been shown to bind heme, and it is unlikely that this conserved domain would then be able to bind heme in sGC. Thus the central domain is the most likely site for heme binding.

A comparison of the nine cDNA sequences for the central domain of sGC is shown in Figure 5. Of the 25 invariant residues two are histidines, $\beta$220 and $\beta$346, and it is quite probable that one of these two histidines is the axial ligand to the heme iron. It is also evident from the alignment that many of the other invariant residues are hydrophobic in nature (phenylalanine and leucine). These invariant hydrophobic residues may be critical in forming the heme-binding pocket. As discussed above, spectroscopic studies indicate that the ligand-binding (distal) side of the heme is in a hydrophobic environment.

A recent mutagenesis study implicated the histidine at position $\beta$105 as the heme ligand (Wedel et al., 1994). In that study, the histidine at position $\beta$105 was mutated to a phenylalanine. The protein was transiently expressed in COS cells, and the supernatant was assayed for activation by SNP. The H$\beta$105F mutant was found not to be activated by SNP in contrast to wild-type enzyme. However, a histidine is not present at this position in the $\alpha$ subunit. In light of the heme stoichiometry of 2 per heterodimer reported here, this histidine is unlikely to be the ligand to the heme. More likely, the nonconservative mutation resulted in the improper folding of the regulatory portions of the heterodimer. Also in that work, the single mutants H$\beta$220F, H$\beta$346F, and H$\alpha$407F were all found to be mildly activated by SNP. These histidines were thus excluded as possible heme ligands. However, since both subunits can presumably bind heme, and each of these histidines has a conserved counterpart in the other subunit, one would need to make the $\alpha/\beta$ double mutants for each position to rule out a particular histidine as the heme ligand. Thus at present it appears that the histidine that coordinates the heme iron is at position $\alpha$290/$\beta$220 or $\alpha$407/$\beta$346 in the bovine $\alpha_1\beta_1$ isoform of sGC.

In summary, sGC has been purified from bovine lung in a state containing 1.5 equiv of heme per heterodimer, indicating that the native heme stoichiometry is 2 per heterodimer. Spectrally only one type of heme is present in the enzyme, and this heme is five-coordinate high spin in both the ferrous and ferric states. It has been concluded that each subunit of the heterodimer binds 1 equiv of heme at a site conserved between the two subunits. Alignment of the nine published cDNA sequences for sGC indicates that the heme binding domain is the central portion of each subunit corresponding to residues 213−370 in the bovine $\beta_1$ sequence.

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