Studies of the Heme Coordination and Ligand Binding Properties of Soluble Guanylyl Cyclase (sGC): Characterization of Fe(II)sGC and Fe(II)sGC(CO) by Electronic Absorption and Magnetic Circular Dichroism Spectroscopies and Failure of CO To Activate the Enzyme

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ABSTRACT: The mechanism of activation of soluble guanylyl cyclase by NO is poorly understood although it is clear that NO interacts with a heme group in the protein via formation of a heme-nitrosyl adduct. The objective of this study is to investigate the coordination environment of the heme in the enzyme spectroscopically in the presence of known heme ligands and to correlate the spectral characteristics with other heme proteins of known structure. Comparison of the electronic and magnetic circular dichroism (MCD) spectra for ferrous bovine soluble guanylyl cyclase (Fe(II)sGC) in the absence and presence of the common heme ligand CO with those of other hemoproteins suggests that histidine is an axial ligand to the heme iron in Fe(II)sGC. Further analysis indicates that Fe(II)sGC is predominantly bis-histidine ligated; the ratio of MCD signal intensity in the visible region to that in the Soret region is most consistent with an admixture of pentacoordinate and hexacoordinate ferrous heme in Fe(II)sGC at pH 7.8. Spectral changes upon CO binding have been correlated with the activity of the enzyme to determine the relationship between coordination structure and activity. Although CO clearly binds to Fe(II)sGC to form a six-coordinate adduct, it fails to significantly activate the enzyme regardless of heme content or CO concentration. In contrast, the extent of activation of sGC by NO is dependent on the heme content in the enzyme and on the concentration of NO. These observations are consistent with a mechanism for activation of soluble guanylyl cyclase in which the bond between the heme iron and the proximal histidine must be broken for activation to take place.

The discovery of the role of nitric oxide (NO) in numerous important biological processes has greatly heightened interest in this unique biological effector (Moncada et al., 1991). Nitric oxide serves as a cellular regulatory agent in many physiological events including vascular smooth muscle relaxation (Ignarro & Kadowitz, 1985), platelet aggregation (Azuma et al., 1986; Furlong et al., 1987; Radomski et al., 1987a,b), and neuronal communication (Garthwaite et al., 1988; Bredt & Snyder, 1989). Nitric oxide exerts its effect through interaction with a heme moiety (Fe(II)PPIX)[1] in the soluble form of guanylyl cyclase (sGC) (Ignarro et al., 1982a; Craven & DeRubertis, 1983; Ignarro, 1989). Soluble guanylyl cyclase catalyzes the conversion of GTP to cGMP; the enzyme is a 150 kDa complex of two similar but not identical polypeptides (Garbers, 1979; Kamisaki et al., 1986) that contains one heme moiety per heterodimer (Gerzer et al., 1981; Ignarro et al., 1986; Stone & Marletta, 1994) that is not essential for activity. Catalytic activity requires the presence of either Mg^{2+} or Mn^{2+} in excess of the nucleoside triphosphate (Ignarro et al., 1982a; Wolin et al., 1982). Soluble guanylyl cyclase is markedly activated by NO and other substances capable of releasing or forming NO (Craven & DeRubertis, 1978; Gruetter et al., 1979). The presence of heme is absolutely required for the activation of sGC by NO (Ignarro et al., 1982a; Craven & DeRubertis, 1983), and activation has been proposed to result upon formation of NO-heme within the enzyme (Craven & DeRubertis, 1978; Craven et al., 1979; Ignarro et al., 1986). Interestingly, sGC is also activated by protoporphyrin IX (PPIX), the metal-free cofactor (Ignarro et al., 1982b).

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; ADP, adenosine 5′-diphosphate; DTT, D,L-dithiothreitol; SNP, $Na_2Fe(CN)_5NO{\cdot}2H_2O$; TEA, triethanolamine hydrochloride; IBMX, 1-methyl-3-isobutylxanthine; PEI-F, polyethylenimine cellulose with fluorescent indicator; TLC, thin layer chromatography; MCD, magnetic circular dichroism; sGC, soluble guanylyl cyclase; Mb, myoglobin; HRP, horseradish peroxidase; cyt b_5, cytochrome b_5; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton; PPIX, protoporphyrin IX; PEG, poly(ethylene glycol).

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The molecular mechanisms underlying the regulation of this enzyme are poorly understood, and only limited data regarding the heme coordination environment have been reported (Stone & Marletta, 1994; Yu et al., 1994). A number of laboratories have purified sGC to near homogeneity (Tsai et al., 1978; Garbers, 1979; Wolin et al., 1982; Humbert et al., 1991; Mülsch & Gerzer, 1991; Waldman et al., 1991), but only preliminary spectroscopic analysis has been carried out (Gerzer et al., 1981; Stone & Marletta, 1994; Yu et al., 1994). It is our goal to understand the interaction of NO with the heme moiety of its receptor, sGC, and to this end, we have undertaken a variety of spectroscopic studies of this unusual enzyme. We have recently reported resonance Raman spectra of Fe(II)sGC and its CO and NO adducts (Yu et al., 1994). It was suggested therein that Fe(II)sGC in its resting state contained two histidine ligands. Both CO and NO were observed to bind directly to the iron atom. The carbon monoxide adduct, Fe(II)sGC(CO), was apparently six coordinate, and histidine was the likely axial ligand, while the nitrosyl adduct was observed to be five coordinate. Based on these observations, displacement of the axial ligand by NO was proposed to trigger the activation of the enzyme.

In the present study, the nature of the heme coordination environment has been studied by electronic absorption and magnetic circular dichroism spectroscopy (MCD). MCD is a particularly useful method to probe the electronic structure of heme systems for all oxidation states of iron (Holmquist, 1978; Dawson & Dooley, 1989). The spectra are sensitive to the electronic state of the heme; the shape and intensity of the spectra can be correlated with the redox state, the spin state, and the axial ligation of the iron atom and can therefore be used to draw conclusions about the physical structure of the heme iron. The MCD results obtained in the present work with sGC have been compared with those previously reported for analogous complexes of the histidine-ligated heme proteins myoglobin (Mb), horseradish peroxidase (HRP), and cytochrome b_5 (cyt b_5), heme proteins known to have an endogenous histidine imidazole ligand (Vickery et al., 1976a,b; Sono & Dawson, 1984; Svastits & Dawson, 1986). The influence of the typical heme ligands, cyanide and carbon monoxide, on the activation of sGC by NO or nitroprusside and on the activity of sGC, respectively, has been examined. These observations are discussed in light of the proposed mechanism of activation of sGC.

EXPERIMENTAL PROCEDURES

Materials. NO (99+%) and CO (99.9%) were purchased from Liquid Carbonic. Hemin chloride, SNP, TEA, EDTA, DTT, IBMX, γ-globulin, GTP, cGMP, benzamidine, leupeptin, trypsin inhibitor, aprotinin, PEG, Q-Sepharose Fast Flow, Blue Sepharose, GTP-agarose (11-atom spacer at the ribose hydroxyl), and ADP were all purchased from Sigma. NO was purified prior to use by passage through solid KOH. The $Na_2S_2O_4$ used in this study was the highest purity available from Fluka Chemical; KCN, NaCN, $MgCl_2$, $MnCl_2$, and $CuSO_4$ were from Mallinckrodt, and NaOH, NaCl, and $NaNO_3$ were from Fisher. [α-^{32}P]GTP was from NEG-Dupont, and "Baker-flex" cellulose PEI-F TLC plates were from VWR Scientific. Unless stated otherwise, materials were used as purchased.

Isolation of Purified sGC from Bovine Lung. Fresh bovine lung (1 kg) was minced in a food processor. A protease cocktail containing 2 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL trypsin inhibitor, and 5 μg/mL aprotinin was added into a 40% w/v dilution of minced tissue in 25 mM TEA at pH 7.8, 1 mM EDTA, and 5 mM DTT. The mixture was homogenized in a Waring blender and then centrifuged at 10000*g* for 20 min. The supernatant was clarified at 100000*g* for 60 min, followed by precipitation with 1% PEG and centrifugation at 10000*g* for 30 min. The supernatant was brought to 15% PEG and centrifuged at 10000*g* for 30 min, and the pellets were redissolved in 25 mM TEA containing 5 mM DTT and 1 mM benzamidine (TEA-DTT buffer) at pH 7.8. Following batch adsorption of sGC onto 500 g of Q-Sepharose Fast Flow, the resin was poured into a column (2.5 cm diameter) and washed (240 mL/h) with pH 7.8 TEA-DTT buffer. Soluble guanylyl cyclase was eluted with a 1.5 L linear NaCl gradient (0−0.75 M) in pH 7.8 TEA-DTT buffer at 100 mL/h. Active fractions were concentrated with an Amicon ultrafiltration cell (PM-30 membrane) and dialyzed against TEA-DTT buffer at pH 6.5. The dialysate was loaded onto a Blue Sepharose CL-6B column, previously equilibrated with pH 6.5 TEA-DTT buffer, and the column was washed with pH 6.5 TEA-DTT buffer and then with pH 7.8 TEA-DTT buffer. The protein was eluted with a 0.4 L linear NaCl gradient (0−1.0 M) in pH 7.8 TEA-DTT buffer, and the active fractions were concentrated and dialyzed against the same buffer. A GTP-agarose column, previously equilibrated with pH 7.8 TEA-DTT buffer with 0.2 mM benzamidine and 5 mM $MnCl_2$ (buffer A), was loaded at 30 mL/h, and the column was washed to remove creatine kinase with 5 column volumes each of (1) buffer A, (2) buffer A containing 10 mM creatine, 10 mM $NaNO_3$, and 0.5 mM ADP, and (3) buffer A. Soluble guanylyl cyclase was eluted with 0.4 M NaCl in pH 7.8 TEA-DTT buffer, and active fractions were concentrated and stored at −80 °C. Protein purified through the Q-Sepharose column (partially-purified sGC) had a substantial amount of endogenous heme, whereas purified sGC contained a negligible amount. Protein concentrations were determined by Biuret protein assay according to a published protocol (Oser, 1965). The heme content of the isolated protein was determined by the pyridine−hemochromogen method (de Duve, 1948).

Soluble Guanylyl Cyclase Assay. The activity of sGC was determined by measuring the formation of [^{32}P]cGMP from [α-^{32}P]GTP as described previously (Garbers & Murad, 1979; Kim & Burstyn, 1994). Assays contained 40 mM TEA at pH 7.4, 1 mM GTP, 0.3 mM IBMX, 2 mM DTT, 3 mM Mn^{2+} or Mg^{2+}, 3 nmol of [α-^{32}P]GTP (800 Ci/mmol), and enzyme in a final volume of 0.2 mL and were incubated for 10 min at 37 °C. The natural cofactor, Mg^{2+}, was used in all activation and inhibition studies; Mn^{2+} was used only during purification when a higher basal activity was desired. Reactions were initiated by the addition of enzyme, unless noted otherwise, and terminated by the addition of 10 μL of quenching solution (0.5 M EDTA in 50 mM TEA at pH 8.4) at 0 °C. Reaction mixtures were chromatographed on "Baker-flex" cellulose PEI-F TLC plates to separate the cGMP from residual GTP (Kavipurapu et al., 1982). An aliquot of the assay mixture (10 μL) was applied to a pre-etched TLC plate, and then 5 μL of pre-spotting solution (5 mM GTP and 5 mM cGMP) was applied over the original spots. The TLC plate was developed with 0.15 M LiCl. Spots corresponding to cGMP were excised and shaken with

10 mL of Biosafe II scintillation cocktail, and the radioactivity was measured in a Beckman LS 6000E liquid scintillation counter.

Anaerobic Heme Reconstitution. As isolated, sGC was depleted in heme, but the heme-deficient protein could be readily reconstituted. A molar excess of hemin chloride was added to a solution of sGC in pH 7.8 TEA-DTT buffer containing 1 mM $Na_2S_2O_4$, and the mixture was stirred for 1 h under N_2 at 0 °C. The solution was then passed anaerobically through a Sephadex G-25 column in the same buffer to remove unbound heme. Heme-reconstituted sGC was characterized by electronic absorption spectroscopy and activity assay, and the heme content was determined by pyridine hemochromogen assay (de Duve, 1948).

Reaction of sGC with Carbon Monoxide, Nitric Oxide, or Cyanide. Saturated solutions of NO and CO were prepared by bubbling the appropriate gas through deoxygenated buffer for 10 min at room temperature. In enzyme assays, the gas solution was diluted to the appropriate concentration in an assay mixture containing sGC, and the reaction was initiated by the addition of substrate; assays containing CO were carried out in the dark. For spectroscopic studies, Fe(II)-sGC(CO) was prepared by addition of a CO-saturated buffer to anaerobic Fe(II)sGC. The reaction was monitored by following the Soret shift from 426 to 420 nm. For studies involving CN^-, solutions of KCN or NaCN were prepared and titrated with HCl to pH 7.4 in a fume hood. (**Caution:** *Addition of acid to solutions containing cyanide generates poisonous HCN gas.*)

Spectrophotometric Measurements. Optical absorption, MCD, and CD spectra of sGC were obtained in 25 mM TEA buffer, pH 7.8, at 4 °C with heme concentrations of 5−20 μM. Absorption spectra were recorded on a Varian-Cary 219 or a Hitachi U-3210 spectrophotometer. The heme contents of both as-isolated and heme-reconstituted sGC were determined based on the ϵ_{557} value of 33.4 mM^{-1} cm^{-1} for the reduced pyridine hemochromogen (de Duve, 1948), and the millimolar absorptivities were calculated based on the measured heme concentrations. CD and MCD spectra were obtained on a Jasco J-500 spectrometer with the 1.41 T magnetic field oriented parallel to the direction of light propagation, in the latter case. Optical absorption spectra were scanned before and after each MCD/CD measurement; MCD spectra of samples displaying <5% change were deemed acceptable. The calibration of the spectropolarimeter and the data handling system have been described previously (Huff et al., 1993). The MCD data, corrected for natural CD, are reported as a molar magnetic absorption coefficient, $\Delta\epsilon/H$, in M^{-1} cm^{-1} T^{-1}.

RESULTS AND DISCUSSION

Although previous studies of soluble guanylyl cyclase (sGC) have clearly established the necessity of heme for NO activation of the enzyme (Ignarro et al., 1982a; Craven & DeRubertis, 1983), relatively little is known about the axial ligands to the heme iron. To understand the mechanism of NO activation, it is imperative to characterize the heme coordination structure in the resting ferrous state as well as in ligand-bound derivatives of sGC, including the activated ferrous-NO state. In the present study, we have focused on the electronic absorption and magnetic circular dichroism (MCD) spectral properties of Fe(II)sGC and Fe(II)sGC(CO) and have examined the effect of CO and CN^- on the activity of the enzyme.

Properties of Heme-Reconstituted sGC. As noted by others (Craven & DeRubertis, 1978; Ignarro et al., 1982a; Craven & DeRubertis, 1983; Busygina & Severina, 1991), we have found the heme content of purified sGC to be variable. We observe 5−50-fold activation of the purified sGC and 12−150-fold activation of the partially-purified protein; this variability is indicative of partial heme loading in the protein (Ignarro et al., 1982a; Craven & DeRubertis, 1983). Ignarro and co-workers (1982a) determined that the heme-deficient protein was insensitive to activation by NO but NO activation could be restored when the protein was reconstituted with exogenous heme. We find that sGC is readily reconstituted with Fe(II)PPIX but not with Fe(III)-PPIX. A sample of partially-purified enzyme, initially activated 12-fold by 100 μM SNP, is activated 160-fold after reconstitution with Fe(II)PPIX as described in the Experimental Procedures. In contrast, reconstitution of the protein with Fe(III)PPIX in the open air does not result in any increase in the sensitivity of the enzyme to activation; the enzyme is activated 12-fold by 100 μM SNP both before and after aerobic reconstitution. Further evidence for discrimination between Fe(II)PPIX and Fe(III)PPIX is obtained when the protein is isolated anaerobically through the Q-Sepharose column. The anaerobically-isolated partially-purified protein is activated 120-fold by 100 μM SNP in contrast to the 12-fold activation of aerobically-isolated protein. Measurement of heme content by pyridine hemochromogen assay reveals substantially higher heme content in anaerobically isolated and anaerobically reconstituted protein than in the aerobically prepared protein. These data suggest that sGC binds Fe(II)PPIX more strongly than Fe-(III)PPIX; a differential affinity for ferrous and ferric heme has not been previously noted.

Typically, anaerobically-reconstituted purified sGC (HRsGC) is activated up to 150-fold by SNP compared to the as-isolated protein which is activated only 5−50-fold. HRsGC shows a decrease in basal activity relative to the as-isolated protein, consistent with Ignarro's observations that the heme itself is an inhibitor of sGC activity (Ignarro et al., 1984). The suppressed basal activity and high sensitivity to activation by NO observed for the reconstituted, purified enzyme are consistent with the presence of the heme in the appropriate location in the protein.

Because we were unable to obtain spectra on non-reconstituted, purified protein, we have studied partially-purified protein which contained substantial amounts of endogenously bound heme. The partially-purified protein contained not more than 10% sGC based on the specific activity. The partially-purified samples were highly sensitive to NO activation, but the sensitivity could be increased by heme reconstitution, suggesting that even partially-purified sGC was not saturated with heme. Neither purified nor partially-purified protein contained any bovine serum albumin (BSA), as determined by Western blotting with anti-BSA antibodies. Serum albumins are known to bind heme (Bunn & Jandl, 1968; Beaven et al., 1974), and BSA is a likely contaminant in preparations of sGC from bovine lung.

Electronic Absorption and MCD Spectral Characteristics of Fe(III)sGC, Fe(II)sGC, and Fe(II)sGC(CO). The spectral properties of sGC have been investigated using partially-purified sGC, in both the as-isolated and heme-reconstituted

Table 1: Electronic Absorption Data for Various Derivatives of Soluble Guanylyl Cyclase[a]

derivative	Soret (ϵ)	β (ϵ)	α (ϵ)
Fe(III)sGC	420 (160)	541 (23)	572 (18)
Fe(III)sGC + glycerol	394 (194)	490 (26)	604 (17)
Fe(II)sGC	426 (214)	528 (22)	558 (25)
Fe(II)sGC(CO)	420 (243)	536 (22)	561 (19)

[a] All peak positions are in nm; millimolar absorptivities are in mM^{-1} cm^{-1}. All values were determined in 25 mM TEA, pH 7.8.

forms, as well as purified HRsGC. As-isolated partially-purified sGC contains a mixture of Fe(II) and Fe(III) heme, characterized by a Soret absorption at 422 nm. Upon ferricyanide oxidation, the Soret peak shifts to 420 nm with decreased absorptivity (Table 1), and β and α bands are observed at 541 and 572 nm, respectively. The MCD spectrum of Fe(III)sGC at pH 7.8 (data not shown) was typical of a low-spin ferric heme adduct with derivative-shaped features centered at 420 and 572 nm. This spectrum was too noisy to warrant further interpretation as the ferric form of sGC is stable only marginally longer than the time scale of the MCD experiments. As was noted previously by Gerzer et al. (1981), the heme appears to dissociate from sGC in the ferric oxidation state. When partially-purified Fe(II)sGC was oxidized, either in the air or with ferricyanide, the Soret absorption shifted to 420 nm and then decreased in intensity with a half-life of 60 min. Oxidation of heme-reconstituted Fe(II)sGC with ferricyanide or dioxygen resulted in the formation of a dark purple precipitate over the course of several hours. The precipitate was isolated and identified as heme (FePPIX) by its characteristic pyridine hemochrome spectrum. Although it had been reported that CN^- also promoted the loss of heme from the enzyme (Gerzer et al., 1981), we did not observe significant changes in either the electronic absorption or MCD spectrum of Fe(III)sGC when CN^- was added to 250 mM. We therefore conclude that CN^- neither binds to nor promotes displacement of the heme in Fe(III)sGC at this concentration.

The spectral characteristics of Fe(III)sGC that we observe differ significantly from those recently reported by Stone and Marletta (1994) in which a Soret absorption peak at 393 nm for Fe(III)sGC was observed. Noting the spectral similarity of this peak position to that of procaryotic ferric cytochrome *c'* (Yoshimura et al., 1985), Stone and Marletta (1994) suggested that sGC contained a five-coordinate ferric heme center with a histidine imidazole as the sole axial ligand (Finzel et al., 1985). Because Stone and Marletta (1994) examined their enzyme in the presence of 50% glycerol, we have studied the effect of glycerol on the spectral properties of Fe(III)sGC. Making Fe(III)sGC 50% in glycerol results in a shift of the Soret absorption peak from 420 to 393 nm, and removal of the glycerol by gel filtration chromatography returns the Soret absorption to 420 nm, as illustrated in Figure 1. In addition, we observe that in 50% glycerol Fe(III)sGC binds CN^- readily at 10 mM CN^-, forming a species with a Soret absorption at 418 nm, consistent with the observations of Stone and Marletta (1994). On the basis of these observations, we conclude that the differences between the spectral properties of Fe(III)sGC reported here and those observed by Stone and Marletta (1994) could result from the presence of 50% glycerol in the buffers in the latter experiments.

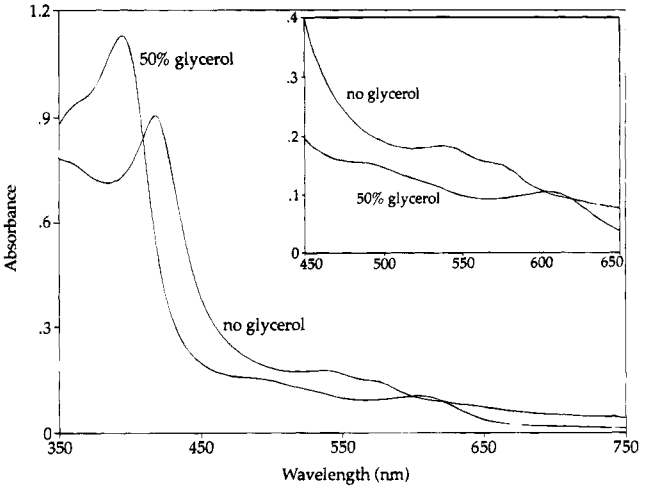


FIGURE 1: Electronic absorption spectra of Fe(II)sGC in the presence and absence of glycerol. The spectra of heme-reconstituted partially-purified sGC (6 μM heme) in 25 mM TEA, pH 7.8, oxidized with 2 mM $K_3[Fe(CN)_6]$. Spectra were recorded in the presence of 50% glycerol and after removal of the glycerol on Sephadex G25. Peak positions and molar absorptivities are listed in Table 1.

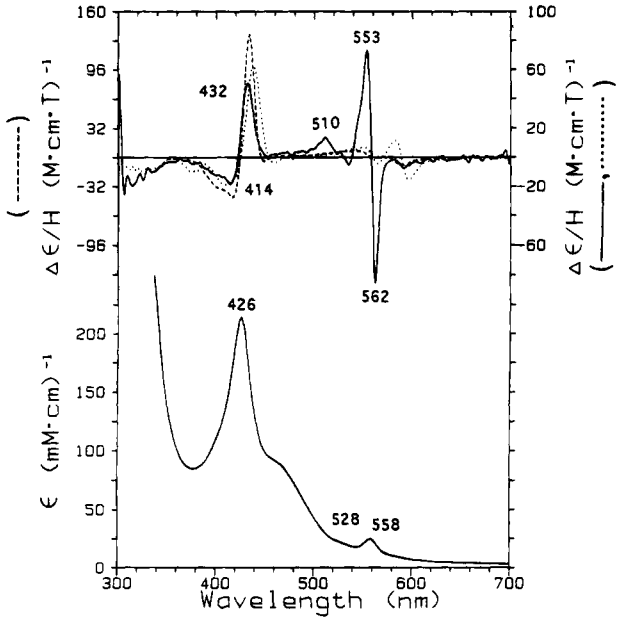


FIGURE 2: MCD (top) and electronic absorption (bottom) spectra of Fe(II)sGC. The spectra of as-isolated partially-purified sGC in 25 mM TEA, pH 7.8, after reduction with sodium dithionite. Spectral measurements were carried out as described in the text. Spectral peak positions and intensities are listed in Tables 1 and 2. The MCD spectrum of Fe(II)sGC (5.0 μM heme) (—) is overlaid with the spectra of Fe(II)Mb (48 μM heme) (- - -) in 0.1 M potassium phosphate buffer, pH 7.0, and Fe(II)HRP (61 μM heme) (• • •) in 0.1 M potassium phosphate buffer, pH 7.0, for comparison. The spectra of Fe(II)Mb and of Fe(II)HRP have been previously reported by Bracete et al. (1991) and by Dawson et al. (1992), respectively.

The ferrous form of partially-purified sGC, Fe(II)sGC, has been studied by visible and MCD spectroscopy, both as-isolated and in the heme-reconstituted form. Generation of the partially-purified Fe(II)sGC by anaerobic reduction of the ferric enzyme with $Na_2S_2O_4$ leads to a shift in the Soret absorption maximum from 420 to 426 nm with a concomitant increase in the molar absorptivity (Table 1 and Figure 2). The shoulder at 450 nm may reflect the impurity and low heme content of the protein; it is absent in the spectrum of

Table 2: MCD Spectral Data for Fe(II)sGC and Derivatives[a]

Fe(II)sGC, λ ($\Delta\epsilon/H$)	HRFe(II)sGC, λ ($\Delta\epsilon/H$)	Fe(II)sGC(CO), λ ($\Delta\epsilon/H$)
414 (−18)		413 (52)
422 (0)		419 (0)
432 (51)	427 (22)	424 (−58)
510 (14)	509 (14)	555 (21)
553 (74)	551 (70)	564 (10)
557 (0)	556 (0)	568 (0)
562 (−85)	561 (−78)	576 (−30)

[a] Spectra were recorded in 25 mM TEA, pH 7.8. All peak positions (λ) are in nm; molar magnetic absorption coefficients ($\Delta\epsilon/H$) are in M^{-1} cm^{-1} T^{-1}.

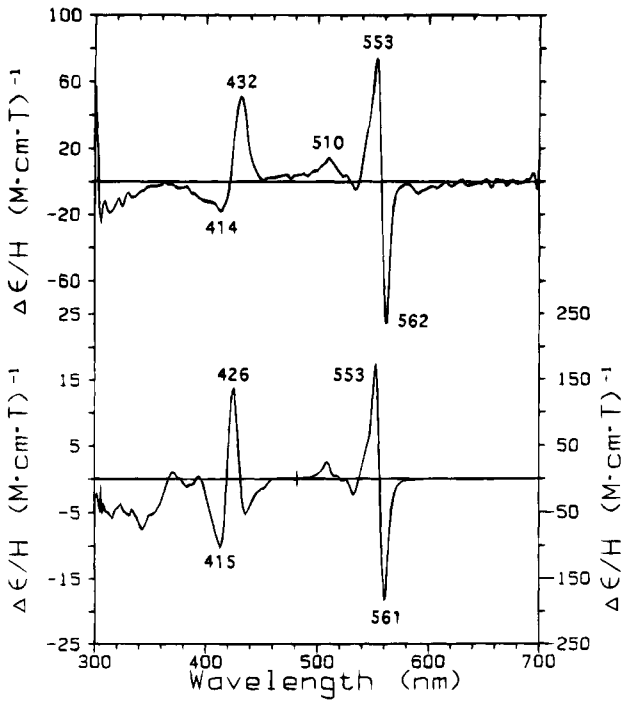


FIGURE 3: Comparison of MCD spectra of Fe(II)sGC (top) with Fe(II)cyt b_5 (bottom). Comparison of the MCD spectrum of Fe(II)sGC from Figure 2 with that of Fe(II)cyt b_5. Note the change in scale between the 300−480 and 480−700 nm regions of the spectrum of Fe(II)cyt b_5, which has been previously reported by Svastits and Dawson (1986).

heme-reconstituted sGC (see Figure 4). The electronic absorption spectrum of Fe(II)sGC is similar to that previously observed by Gerzer et al. (1981). The MCD spectrum of Fe(II)sGC features derivative-shaped curves in both the Soret and visible regions (Figure 2, Table 2) and most closely resembles the spectrum of Fe(II)cyt b_5 (Vickery et al., 1975, 1976a; Svastits & Dawson, 1986), a low-spin six-coordinate bis-histidine ligated hemoprotein (Figure 3). The MCD spectrum of Fe(II)sGC shows somewhat weaker intensities in the visible region and greater intensities in the Soret region than are observed for Fe(II)cyt b_5. The sharp, intense MCD feature in the visible region is typical of low-spin six-coordinate ferrous hemoproteins (Vickery et al., 1976a; Svastits & Dawson, 1986) and is distinctly different from the much weaker features observed for Fe(II)Mb or Fe(II)-HRP (Nozawa et al., 1976; Vickery et al., 1976b). In contrast, the spectral intensity in the Soret region of Fe(II)-sGC is more similar to that observed for Fe(II)Mb and Fe-(II)HRP than to that of Fe(II)cyt b_5. Overall, the MCD spectrum of Fe(II)sGC appears to be a linear combination

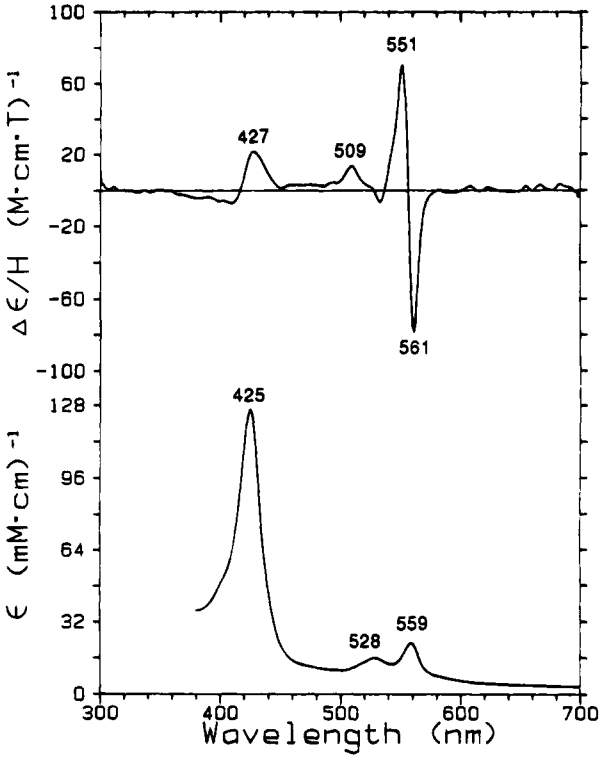


FIGURE 4: MCD (top) and electronic absorption (bottom) spectra of heme-reconstituted Fe(II)sGC. The spectra of partially-purified heme-reconstituted Fe(II)sGC (26 μM heme) in 25 mM TEA, pH 7.8, with 1 mM sodium dithionite and 10 mM DTT. Spectral peak positions and intensities are listed in Tables 1 and 2.

of the spectra of five-coordinate Fe(II)Mb and six-coordinate Fe(II)cyt b_5. As has been reported for secondary amine monooxygenase (Alberta et al., 1989), these data suggest that Fe(II)sGC contains a mixture of high-spin five- and low-spin six-coordinate heme, with one or two axial histidine ligands, respectively. The relative signal intensities indicate that the low-spin six-coordinate state predominates; in contrast, Stone and Marletta (1994) concluded that Fe(II)-sGC is exclusively five-coordinate in the presence of 50% glycerol.

The electronic absorption and MCD spectra of partially-purified HRFe(II)sGC (Figure 4) are similar to those of the as-isolated protein (Figure 2). Together with the increase in NO activation of the reconstituted enzyme, these data suggest that the added heme is bound at the native site. HRFe(II)sGC shows greater MCD spectral intensity in the visible region and weaker Soret intensity compared to as-isolated Fe(II)sGC, and the spectrum of HRFe(II)sGC is nearly superimposable on that of Fe(II)cyt b_5 (Vickery et al., 1975) (Figure 3). These data indicate that the heme in HRFe(II)sGC is present almost entirely in a six-coordinate, bis-histidine ligated form. The optical and MCD spectra of purified HRFe(II)sGC are identical to those observed for the partially-purified protein; however, noisier spectra were obtained due to the low protein concentration, and more of the heme was present in the five coordinate state.

Upon addition of CO to Fe(II)sGC, the Soret absorption maximum shifts from 426 to 420 nm with an accompanying increase in the molar absorptivity (Table 1, Figure 5). The positions of the Soret and visible bands in the electronic absorption spectrum are consistent with those previously reported by Gerzer et al. (1981). The MCD spectrum of

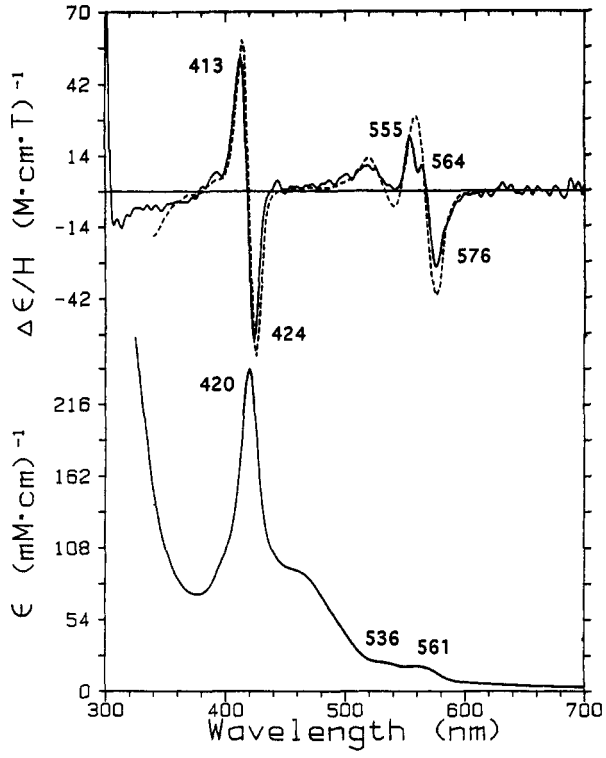


FIGURE 5: MCD (top) and electronic absorption (bottom) spectra of Fe(II)sGC(CO). The carbonmonoxy derivative of as-isolated partially-purified Fe(II)sGC was prepared as described in the text. Spectral peak positions and intensities are listed in Tables 1 and 2. The MCD spectrum of Fe(II)sGC(CO) (5.0 μM heme) (—) in 25 mM TEA buffer, pH 7.8, is overlaid with the spectrum of Fe(II)-Mb(CO) (46 μM) (- - -) in 0.1 M potassium phosphate buffer, pH 7.0, for comparison. The spectrum of Fe(II)Mb(CO) has been previously reported by Bracete et al. (1991).

Fe(II)sGC(CO) (Figure 5) is remarkably similar to those of Fe(II)Mb(CO) (Vickery et al., 1976b) and Fe(II)HRP(CO) (Nozawa et al., 1976). The MCD spectral comparison of Fe(II)sGC(CO) with the analogous complex of Mb does not lead to an unambiguous assignment for the proximal ligand of sGC because ferrous-CO heme model complexes with a variety of *trans* ligands display MCD spectra similar to that of Fe(II)Mb(CO) (Collman et al., 1976; Svastits, 1986). This spectral comparison does eliminate the possibility of either tyrosine phenolate or cysteine thiolate as the trans axial ligand in Fe(II)sGC(CO), as these ligands result in substantially different MCD spectral characteristics in ferrous-CO heme model complexes (Collman et al., 1976; Svastits, 1986). A distinct shoulder at 564 nm is present in the MCD spectrum of Fe(II)sGC(CO) even at higher CO concentrations (Figure 5). This peak appears at the position expected for the Fe-(II)sGC$(L)_2$ six-coordinate resting state, suggesting that a small amount of the protein is unreactive with CO.

Activation of sGC by Nitric Oxide and Other Small Molecules. We have examined the activity of sGC in the presence and absence of the potential heme ligands CO and CN^-. It has been reported that CO activates sGC (Brune et al., 1990; Utz & Ullrich, 1991), and a role for CO as a neurotransmitter has been postulated based on its ability to activate sGC (Verma et al., 1993). The spectroscopic evidence presented in this study demonstrates conclusively that CO binds to the heme iron; however, we see no significant activation of the enzyme by CO. We observed no more than a 2-fold increase in specific activity (typically

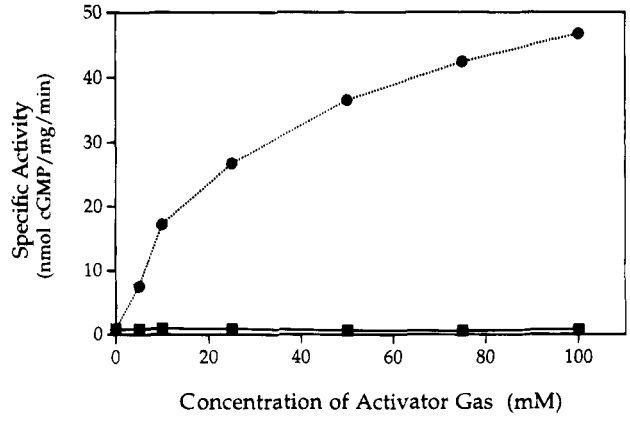


FIGURE 6: Concentration-dependent activation of sGC by NO and CO. Activity of sGC in the presence of increasing NO concentration (●) and CO concentration (■). Saturated solutions of CO and NO gas were diluted to the appropriate concentrations in the assay mixtures prior to adding enzyme. Assays were performed as described in the text. Data are plotted as the mean of triplicate determinations; the variation between triplicate data points is less than the size of the plotted points.

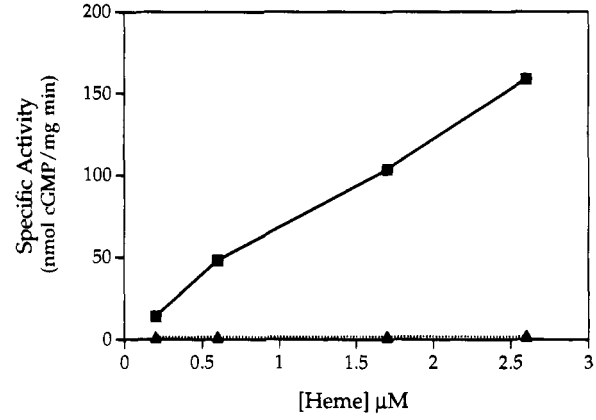
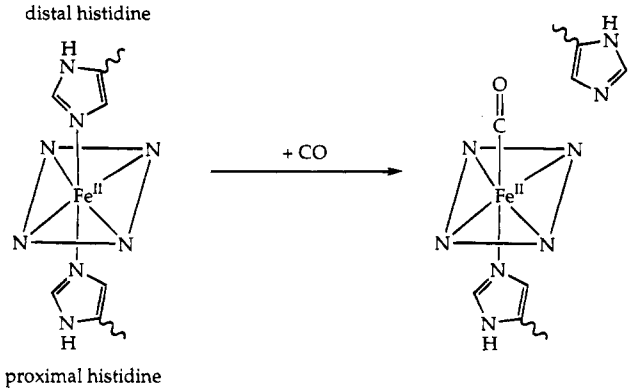


FIGURE 7: Activation of sGC by CO and NO as a function of the heme content of the protein. Activity of sGC in the presence of 100 μM CO (▲) and NO (■). As-isolated partially-purified sGC was reconstituted with varying amounts of heme according to the protocol described in the text. Heme content and enzyme activity were measured as described in the text. Data are plotted as the mean of triplicate determinations; the variation between triplicate data points is less than the size of the plotted points.

1.2−1.4-fold) in both 100000*g* supernatant (data not shown) and partially-purified sGC with up to 100 μM CO (Figure 6). This minimal activation is less than the 12-fold activation by CO in 10000*g* supernatant or 2.4- and 4-fold activation by CO observed in partially-purified sGC by Brune et al. (1990) and in purified sGC by Stone and Marletta (1994), respectively. It should be noted that both previous observations of limited activation by CO (Brune et al., 1990; Stone & Marletta, 1994) were made in CO-saturated solutions, a condition that is not likely to be physiologically relevant. The negligible activation of sGC by CO we observed is consistent with previous observations on the enzyme (Wolin et al., 1982).

Activation of sGC by NO only occurs when heme is present in the enzyme; on this basis it was proposed that the heme iron was the site of NO binding (Ignarro et al., 1982a; Craven & DeRubertis, 1983). Consistent with this hypothesis, we observe that the extent of activation of sGC is proportional to the heme content of the protein (Figure 7). Brune et al. (1990) observed that activation of sGC by CO

Scheme 1



decreased with increasing purification of the enzyme. This decrease could possibly have been due to the loss of heme from the enzyme during the purification process, as suggested by the concomitant decrease in activation by NO (Brune et al., 1990). Since it was possible that the lack of activation by CO that we observed was due to heme deficiency in the enzyme, we determined the effect of heme reconstitution on CO activation. As illustrated in Figure 7, we observe negligible CO activation irrespective of the heme content in the enzyme.

The negligible to minimal extent of CO activation of sGC suggests that CO is unlikely to be a natural effector of the enzyme. The spectroscopic data presented herein confirm the previous observation (Yu et al., 1994) that when CO binds to Fe(II)sGC, the distal histidine–iron bond is broken while the proximal histidine–iron bond remains intact (Scheme 1). The lack of CO activation is consistent with the proposed mechanism for NO activation where NO binding breaks the bond between the ferrous iron and the proximal histidine (Wolin et al., 1982; Traylor & Sharma, 1992; Traylor et al., 1993; Yu et al., 1994). The displacement of one histidine ligand from the heme iron upon binding CO does, however, suggest a mechanism by which CO weakly activates sGC. Slight activation of sGC could result in the doubling of cGMP levels that has been observed in platelets in response to CO (Utz & Ullrich, 1991); alternatively, CO may increase cGMP levels via an alternate pathway. Precontracted vascular tissue is relaxed by both CO and NO, but is significantly less responsive to CO than NO (Vedernikov et al., 1989; Furchgott & Jothianandan, 1991), suggesting a weak physiological response to CO.

Cyanide anion, which may bind weakly to Fe(III)sGC (*vide supra*), is a potent inhibitor of activation by SNP, but it failed to inhibit NO activation of sGC (Figure 8). A cyanide concentration of 1 mM completely abolished sGC activation by 50 μM SNP but had no effect on sGC activation by 50 μM NO. Concentrations of CN^- above 1 mM inhibited the basal activity of sGC; the activity at 10 mM CN^- was 70–80% of that at 1 mM CN^-. The ability of CN^- to inhibit activation of sGC by SNP is in complete agreement with the observation that generation of NO from SNP requires reduction of the nitroprusside anion and subsequent loss of a cyanide ligand (Glidewell & Johnson, 1987; Bates et al., 1991). The addition of excess CN^- blocks, by mass action, the release of CN^-, and thereby prevents formation of the intermediates necessary for NO release (Bates et al., 1991). Inhibition of sGC activation by SNP was previously observed (Wolin et al., 1982); however,

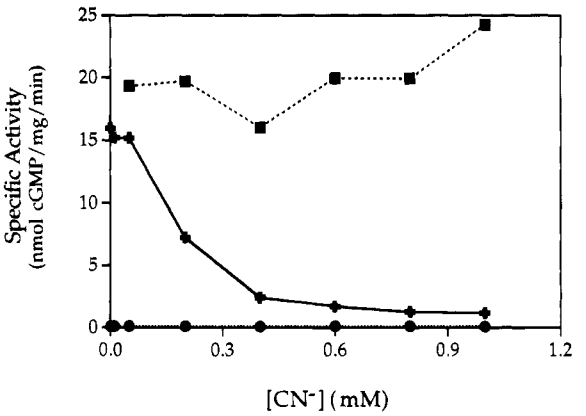


FIGURE 8: The effect of cyanide anion on the activation of sGC by NO and SNP. Soluble guanylyl cyclase activation by NO and SNP in the presence of varying concentrations of CN^-. Assay conditions were as described in the Experimental Procedures section; reactions were initiated by the addition of enzyme to an assay mixture containing the activator and CN^-. Control sGC activity as a function of $[CN^-]$ in the absence of an activator (●). Activation of sGC by NO (50 μM) as a function of $[CN^-]$ (■). Activation of sGC by SNP (50 μM) as a function of $[CN^-]$ (+). Data are plotted as the mean of triplicate determinations; the variation between triplicate data points is less than the size of the plotted points.

in contrast to the observations of Wolin et al. (1982) we do not observe inhibition of NO activation by CN^-. We noted inhibition of the basal activity of the enzyme at the high concentrations of CN^- used in the previous experiments (Wolin et al., 1982), which may explain the differences. The failure of CN^- to block activation of sGC by NO is consistent with the much greater affinity of NO for ferrous heme (Traylor & Sharma, 1992) and with our failure to observe binding of CN^- to sGC in the concentration range used in the enzyme assays. The present observations suggest that SNP activation of sGC does not proceed directly as does NO activation of sGC. Further support for this conclusion comes from electronic absorption spectra which show that the addition of SNP to sGC in the absence of thiols does not yield Fe(II)sGC(NO), with characteristic Soret absorption at 398 nm; rather, it forms an adduct which has a very broad band around 440 nm (data not shown).

CONCLUSIONS

Ferrous soluble guanylyl cyclase has been shown herein to exhibit MCD and electronic absorption spectroscopic properties similar to those of other histidine-ligated hemoproteins. The resting ferrous state of the enzyme likely exists as an admixture of five- and six-coordinate histidine/bis-histidine ligated species. These results are completely consistent with recent resonance Raman data (Yu et al., 1994). In addition, CO has been shown to bind to the heme, but no evidence for CO-mediated activation of sGC was observed. The enzymological and spectroscopic data presented shed new light on the possible mechanism of activation of sGC by NO. The endogenous heme in sGC is bound, in a mixture of five- and six-coordinate forms, to histidine ligand(s) that can be displaced upon binding exogenous ligands. Importantly, our observation that the Fe(II)sGC(CO) adduct is not competent to activate the enzyme is consistent with the requirement for the displacement of the proximal histidine upon NO binding as the trigger for activation (Wolin et al., 1982; Traylor & Sharma, 1992; Yu

et al., 1994). Further MCD studies of Fe(III)sGC and Fe(II)sGC(NO) are in progress.

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