# Binding of Nitric Oxide and Carbon Monoxide to Soluble Guanylate Cyclase As Observed with Resonance Raman Spectroscopy†

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ABSTRACT: Resonance Raman spectra have been recorded for the ferrous heme of soluble guanylate cyclase (sGC), the only receptor known thus far for ·NO. On the basis of the frequencies of porphyrin core sensitive vibrations in the high frequency region of the Raman spectrum, we conclude that the ferrous heme is five-coordinate, high spin, when no exogenous ligands are present. We assign a prominent vibration that occurs at 204 $cm^{-1}$ in the reduced enzyme to the heme $Fe^{2+}$-proximal histidine stretching vibration. In the ·NO bound form of the enzyme, the heme $Fe^{2+}$ retains a five-coordinate geometry. Assuming that ·NO binds to the distal side of the heme, this observation indicates that the weak Fe−His bond breaks when ·NO binds. Two isotope-sensitive vibrations are observed in the ·NO bound enzyme, one at 1677 $cm^{-1}$, attributed to the N−O stretching vibration, and one at 525 $cm^{-1}$, attributed to the Fe−NO stretching vibration. When CO is bound to the ferrous heme, the heme ligation is six-coordinate. From this, we conclude that the Fe−His bond is still intact and that, if cleavage of the Fe−proximal ligand bond is necessary for complete activation of sGC, then CO should only weakly activate the enzyme, which has been shown to be the case. In the carbonmonoxy enzyme, the Fe−CO stretching vibration is observed at 472 $cm^{-1}$ and the Fe−C−O bending vibration is detected at 562 $cm^{-1}$. These frequencies are the lowest yet observed for the Fe−CO stretching and Fe−C−O bending modes in heme proteins or model systems with imidazole as the proximal ligand and suggest that there is significant negative polarity in the distal pocket. The negative polarity and the low frequency of the Fe−His stretching vibration may account for the very low $O_2$ affinity of sGC.

The discovery of nitric oxide (·NO)[1] as an intercellular regulatory agent of a variety of physiological events has generated a great deal of interest. Intercellular communication with ·NO is mediated through direct activation of soluble guanylate cyclase (sGC), which is to date the only known receptor for ·NO. Upon ·NO binding, sGC catalyzes the conversion of GTP to cGMP, important for many physiological events such as vascular smooth muscle relaxation (Ignarro & Kadowitz, 1985), platelet aggregation (Azuma et al., 1986; Furlong et al., 1987), and neuronal communication (Garthwaite et al., 1988; Bredt & Snyder, 1989).

sGC is a heterodimer with a total mass of about 150 kDa consisting of α and β subunits that share considerable homology. Although it was reported some time ago that sGC is a hemoprotein, the stoichiometry of 1.5 hemes per heterodimer has only recently been determined (Stone & Marletta, 1995a). This suggests that, in the native enzyme, there is one heme per monomer. It is likely that the heme is the ·NO binding site because the presence of this cofactor is absolutely required for activation of sGC (Ignarro et al., 1982a; Craven & DeRubertis, 1983; Ignarro, 1989). On the basis of the absorption spectrum and on EPR measurements, Stone et al. concluded that a five-coordinate heme is formed in the presence of ·NO (Stone & Marletta, 1994; Stone et al., 1995). Since the heme Fe in the unactivated enzyme is five-coordinate with histidine as the most likely axial ligand (Stone & Marletta, 1994), it appears that, upon binding of ·NO, the bond between the proximal histidine ligand and the Fe is broken. The observation that metal free protoporphyrin IX can activate the enzyme in the absence of ·NO (Ignarro et al., 1982b) can be explained by the absence of a central metal atom in the free base porphyrin to which an amino acid residue can ligate, thereby resembling the complex formed after ·NO coordination.

The heme environment in sGC must be distinct from that of other heme-containing enzymes. Typically, ferrous hemoproteins, such as hemoglobin, that form nitrosyl complexes are five-coordinate in their reduced states and, in an aerobic environment, rapidly bind oxygen. Reaction with ·NO then leads to the formation of ferric heme and nitrate (Doyle & Hoekstra, 1981). The heme environment of sGC, however, is such that the affinity for $O_2$ is extremely low (Stone & Marletta, 1994). Ferrous nitrosyl hemes typically are stable complexes with low rates for dissociation (Hille

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[1] Abbreviations: sGC, soluble guanylate cyclase; Mb, myoglobin; Hb, hemoglobin; PPIX, protoporhyrin IX; TEA, triethanolamine; DTT, dithiothreitol; CO, carbon monoxide; ·NO, nitric oxide; GTP, guanosine 5′-triphosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; IHP, inositol hexaphosphate; EPR, electron paramagnetic resonance.

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et al., 1979; Sharma & Ranney, 1978). It is therefore not clear whether simple dissociation can account for deactivation of the enzyme. While it was initially thought that the ·NO off-rate would be too slow to account for deactivation of the enzyme known to occur in *in vivo*, pre-steady-state kinetic measurements of ·NO binding have led to a model that predicts a relatively fast off-rate for ·NO (Stone & Marletta, 1996). This finding once again points to a highly unusual heme environment.

Resonance Raman spectroscopy is a technique ideally suited to study heme groups in proteins since it allows detailed study of the heme environment without interference from vibrations in the rest of the enzyme. A resonance Raman study of sGC has recently been reported and concluded that the enzyme as isolated is six-coordinate (Yu et al., 1994). The data that we present in this report indicate that under air the heme is five-coordinate, supporting the absorption spectroscopy (Stone & Marletta, 1994, 1995a; Stone et al., 1995). We detect a stretching vibration at 204 $cm^{-1}$ that we assign to $\nu$(Fe−His). From the low frequency for this mode, we conclude that the bond between the proximal histidine and the heme Fe in the ferrous state is very weak. Isotopic substitutions have been used to identify vibrations due to CO and ·NO following their binding to the heme Fe. The frequency of the Fe−CO stretching vibration is extremely low, more than 30 $cm^{-1}$ lower than that observed for other comparable heme-containing enzymes (Ray et al., 1994), and reflects the unusual heme environment in sGC. We suggest that the polarity of the distal heme pocket is responsible for this and discuss the role of the distal environment in the inhibition of $O_2$ binding. Our results indicate that ·NO is more effective than CO in activating sGC due to the ability of ·NO, but not CO, to break the bond between the heme Fe and the proximal histidine.

## MATERIALS AND METHODS

sGC was isolated from bovine lung as described recently by Stone and Marletta (1995a). The heme content of this preparation was determined to be 1.5 heme per heterodimer. The buffer in which the enzyme was dissolved contained 50 mM TEA, pH 7.4, 10 mM DTT, and 0.4 M NaCl. When frozen, 30% glycerol was added for stability. For the experiments, the glycerol content ranged from 5 to 30%. The concentration of the protein in the sample used for the experiments was 5−10 $\mu$M. $^{12}C^{16}O$ (99.5%) and ·$^{14}N^{16}O$ (99.0%) were obtained from Matheson. $^{13}C^{16}O$ (99%) was purchased from Cambridge Isotopes. $^{13}C^{18}O$ (97%) and ·$^{15}N^{18}O$ (99.4 %) were obtained from Isotech, Inc. (Miamisburg, OH). Both forms of ·NO were purified of other nitrogen oxides by passage through saturated KOH.

Resonance Raman spectra were obtained at 10−20 °C. To obtain Raman spectra of ferrous sGC and of ferrous−CO sGC, a dye laser (Coherent CR599) with Stilbene 420 as the dye was used, and this apparatus was pumped by an $Ar^+$ laser (Coherent Innova 200). A $Kr^+$ laser (Coherent K-90) was used for excitation of nitrosyl sGC at 406.7 nm. The resonance Raman scattering was detected with a spectrometer (Spex 1877 Triplemate) in combination with a liquid nitrogen cooled CCD detector (EG&G OMA 4, Model 1530-CUV-1024S). To reduce potential effects of photo-induced degradation, samples were contained in a spinning cell. No significant degradation of the various samples was observed, as judged from the resonance Raman spectra, even after prolonged excitation with laser light. In the case of nitrosyl sGC, the sample was made anaerobic by flushing with argon prior to addition of ·NO to the head space of the cell to a final concentration of 2%. Absorption spectra recorded after the experiments were identical to those obtained before the experiments. The laser light was focused into the cell so that the laser power ranged from 0.5 mW (ferrous−CO) to 20 mW (ferrous−NO).

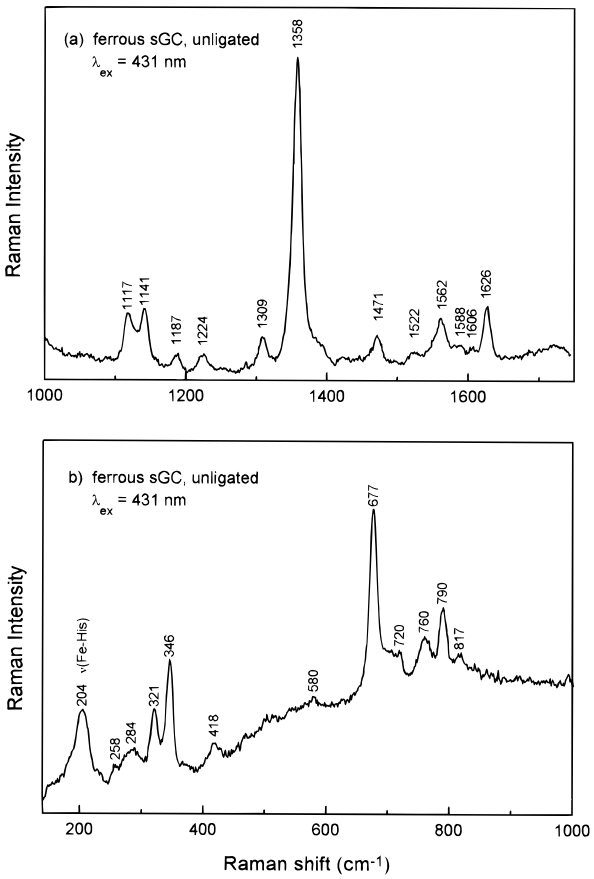


FIGURE 1: High (a) and low (b) frequency Raman spectra obtained for ferrous sGC with 431 nm laser excitation. Signals resulting from the buffer were subtracted. To flatten the baseline, a straight line was subtracted from the raw data.

## RESULTS

The resonance Raman spectrum of ferrous sGC, obtained with excitation at the 431 nm maximum of the Soret absorption band (Stone & Marletta, 1994), is shown in Figure 1 in the high (a) and low (b) frequency regions. The spectrum was obtained in a spinning cell, but a measurement performed on a sample in a capillary gave identical results (data not shown). Moreover, varying the laser power from 0.5 to 10 mW with the spinning cell arrangement produced no variation in the spectrum. The high frequency spectrum (a) is useful for determination of the spin and coordination state of the heme. The modes in that region arise from heme macrocycle vibrations that are sensitive to the core size of the heme and the valence state of the iron. For ferrous sGC the spectrum is very similar to that of the model compound (2-MeImH)Fe(II) protoporphyrin IX (Choi et al., 1983), as can be seen from the compilation of selected modes in Table 1. In the model compound, the heme Fe is five-coordinate, high-spin, and we thus infer a five-coordinate, high-spin state

Table 1: Resonance Raman Frequencies and Mode Assignments for Ferrous sGC

| | coord. | spin[a] | $\nu_{CC}$ | $\nu_{10}$ | $\nu_{37}$ | $\nu_2$ | $\nu_3$ | $\nu_4$ | $\nu$(Fe−His) | reference |
|---|---|---|---|---|---|---|---|---|---|---|
| sGC | 5 | hs | 1626 | 1606 | 1588 | 1562 | 1471 | 1358 | 204 | this work |
| (2-MeImH)Fe(II) PP | 5 | hs | 1622 | 1604 | 1583 | 1562 | 1471 | 1357 | 204 | 1 |
| (ImH)2 Fe(II) PP | 6 | ls | 1620 | 1617 | 1604 | 1584 | 1493 | 1359 | 201 | 1 |
| deoxy-Mb | 5 | hs | 1618 | 1607 | 1584 | 1563 | 1471 | 1357 | 220 | 2, 3 |

[a] hs, high spin; ls, low spin; 1, Choi et al. (1983); 2, Choi et al. (1982); 3, Rousseau and Friedman (1988).

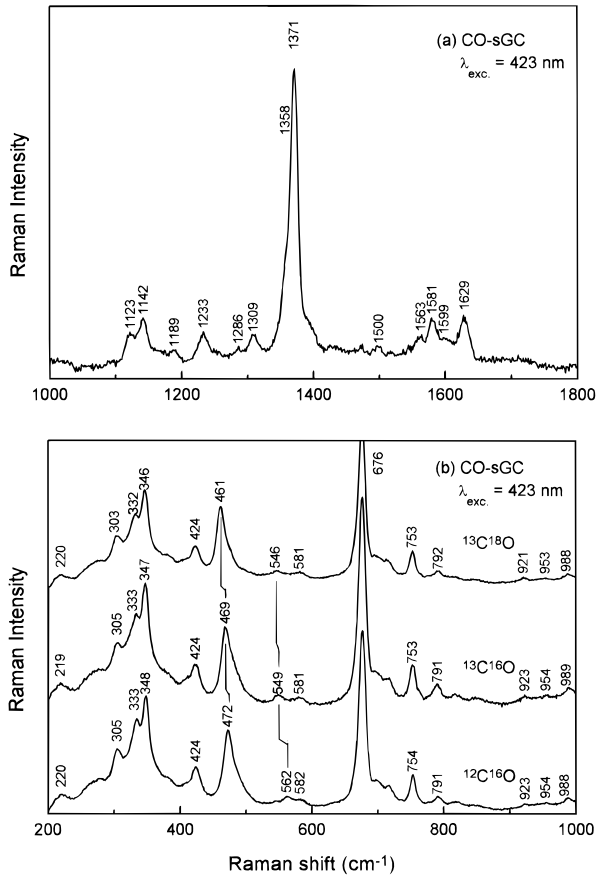


FIGURE 2: High (a) and low (b) frequency Raman spectra for carbonmonoxy sGC with 423 nm laser excitation. Panel b shows spectra obtained with isotopic substitutions for CO. To flatten the baseline, a straight line was subtracted from the raw data.

for the heme Fe of reduced, unliganded sGC. The low frequency spectrum of ferrous sGC (Figure 1b) shows a mode at 204 $cm^{-1}$. On the basis of UV−visible spectroscopy and redox potential considerations, histidine has been inferred as the proximal ligand in the reduced enzyme (Stone & Marletta, 1994). The frequency and intensity of the 204 $cm^{-1}$ vibration is fully consistent with this earlier work and we assign this mode as arising from $\nu$(Fe−His).

Figure 2 shows the high (a) and low (b) frequency resonance Raman spectrum of sGC in the presence of CO, obtained with 423 nm laser light. CO is easily photolyzed from the heme. To avoid photolysis, we adjusted the laser power and minimized the shoulder on the low frequency side of the 1371 $cm^{-1}$ $\nu_4$ vibration, as the shoulder originates from sGC heme centers from which CO has been photodissociated. The spectrum of the carbonmonoxy-bound enzyme shows significant differences relative to that of the unligated, ferrous enzyme as a result of coordination of CO to the heme. As shown in Table 2, the high frequency modes for carbonmonoxy sGC correlate well with those of CO−myoglobin (CO−Mb) and CO−hemoglobin (CO−Hb), and therefore we conclude that the heme Fe of sGC adopts a six-coordinate, low-spin configuration upon CO coordination.

The low frequency spectrum of the carbonmonoxy enzyme (Figure 2b) shows two modes, one at 472 $cm^{-1}$ and the other at 562 $cm^{-1}$, that are sensitive to CO isotopic substitution. Accordingly, we assign these modes as originating from vibrations involving Fe−CO stretching or Fe−C−O bending motions. Assignment of these modes is straightforward because the isotopic shifts follow well established shift patterns (Li et al., 1988). The mode at 472 $cm^{-1}$ shifts to 469 $cm^{-1}$ with $^{13}C^{16}O$ and to 461 $cm^{-1}$ with $^{13}C^{18}O$. These shifts are characteristic for the Fe−C stretching mode [$\nu$(Fe−CO)]. The stretching mode shows some asymmetry, suggesting that there may be slight variations in the way in which CO is bound to the heme. The mode at 562 $cm^{-1}$ shifts to 549 $cm^{-1}$ with $^{13}C^{16}O$ and to 546 $cm^{-1}$ with $^{13}C^{18}O$. On the basis of these isotopic shifts, we attribute it to the Fe−C−O bend [$\delta$(Fe−C−O)].

Figure 3 shows the spectrum of the nitrosyl adduct to sGC in the high (a) and low (b) frequency regions. The high frequency spectrum is almost identical to that of Fe(II)−NO protoporhyrin IX (see Table 3). In this model heme compound, the heme Fe is five-coordinate, and, accordingly, we deduce that the heme of nitrosyl sGC is also five-coordinate. In the low frequency spectrum there is a mode at 518 $cm^{-1}$ that shows sensitivity to isotopic exchange of ·NO. If $^{15}N^{18}O$ is used as the ligand, this mode shifts down to 511 $cm^{-1}$. This mode, however, also broadens for the isotope, probably reflecting the occurrence of an underlying mode that is isotope insensitive. The difference spectrum, obtained by subtraction of the spectrum with $^{15}N^{18}O$ from that with $^{14}N^{16}O$, shows positive and negative peaks at 525 and 509 $cm^{-1}$, respectively. We attribute this isotope effect to a mode originating from the Fe−NO stretching motion. The isotopic shift is of similar magnitude to that previously observed for six-coordinate nitrosyl iron porphyrins (Lipscomb et al., 1993). In the high frequency region of the spectrum, there is a weak mode at 1677 $cm^{-1}$ that shifts to 1607 $cm^{-1}$ with $^{15}N^{18}O$. For NO−Hb + IHP the N−O stretching mode occurs at 1668 $cm^{-1}$ (Maxwell & Caughey, 1976), not far from the mode observed for sGC. We thus attribute the 1677 $cm^{-1}$ mode to the N−O stretch. If we assume that N−O stretching can be described by a harmonic oscillator model, we expect an isotopic shift of about 73 $cm^{-1}$, which agrees very well with our observation of the 1607 $cm^{-1}$ mode in the isotopomer.

## DISCUSSION

The Raman spectra we have obtained for ferrous sGC, CO−sGC, and NO−sGC provide a wealth of information on the heme environment that allows us to obtain new insights into ligand binding to the heme. We discuss this below for each of the three forms of enzyme that we have investigated.

Table 2: Resonance Raman Frequencies and Mode Assignments for Carbonmonoxy sGC and Literature Values for Other Heme Proteins

| | coord | $\nu_{CC}$ | $\nu_{10}$ | $\nu_2$ | $\nu_3$ | $\nu_4$ | $\nu$(Fe−CO) | $\delta$(Fe−C−O) | $\nu$(C−O) | reference |
|---|---|---|---|---|---|---|---|---|---|---|
| CO−sGC | 6 | n.o.[a] | 1629 | 1581 | 1500 | 1371 | 472 | 562 | n.o. | this work |
| CO−Mb | 6 | 1622 | 1637 | 1587 | 1498 | 1372 | 512 | 577 | 1944 | 1, 2 |
| CO−Hb A | 6 | 1622 | 1633 | 1586 | 1500 | 1372 | 507 | 578 | 1951 | 1 |

[a] n.o., not observed; 1, Tsubaki et al. (1982); 2, Rimai et al. (1975).

Table 3: Resonance Raman and Infrared Frequencies and Mode Assignments for Nitrosyl sGC and Literature Values for Other Heme Proteins and Model Systems

| | coord. | $\nu_{CC}$ | $\nu_{10}$ | $\nu_2$ | $\nu_3$ | $\nu_4$ | $\nu$(Fe−NO) | $\nu$(N−O) | reference |
|---|---|---|---|---|---|---|---|---|---|
| NO−sGC | 5 | n.o. | 1646 | 1584 | 1509 | 1375 | 525 | 1677 | this work |
| NO−Mb | 6 | 1624 | 1638 | 1584 | 1501 | 1375 | 554[a] | 1624 | 1, 3 |
| NO−Hb A | 6 | 1622 | 1634 | 1585 | 1500 | *b* | 551[a] | 1622 | 1, 3 |
| NO−(PP) Fe(II) | 5 | n.o. | 1648 | 1586 | 1508 | 1377 | n.o. | n.o. | 1 |
| NO−Mb + SDS | 5 | | 1646 | 1587 | 1512 | 1376 | n.o. | n.o. | 4 |
| NO−Fe(II) OEP | 6 | | | | | | 527 | | 5 |
| NO−Hb A + IHP | 5 | | 1645 | 1600 | 1508 | 1378 | | 1668 | 2, 7 |

[a] Based on the isotopic shift pattern Benko and Yu (1983) argue that this is the bending mode. [b] Value not reported; n.o., not observed; 1, Tsubaki and Yu (1982); 2, Maxwell and Caughey (1976); 3, Benko and Yu (1983); 4, Mackin et al. (1983); 5, Lipscomb et al. (1993); 6. Choi et al. (1991); 7. Nagai et al. (1980).

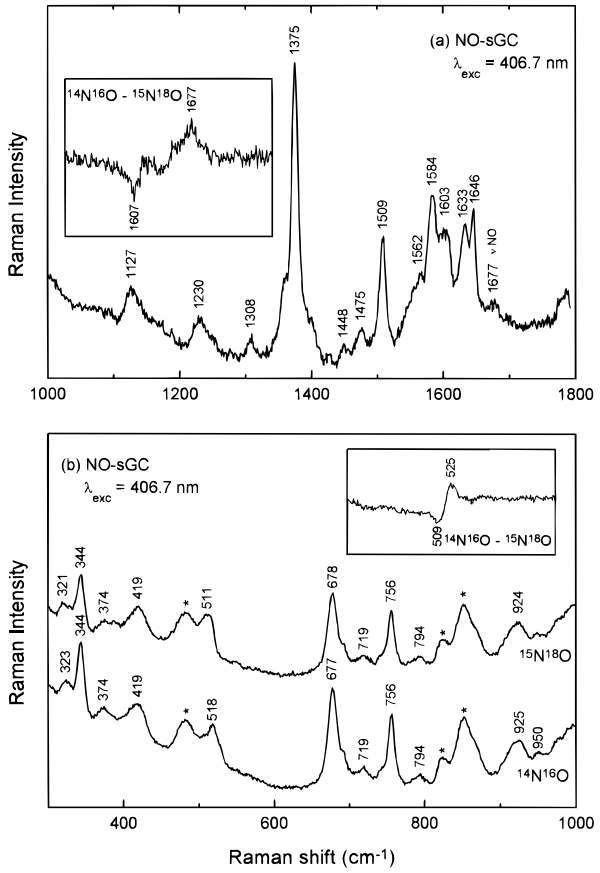


FIGURE 3: High (a) and low (b) frequency Raman spectra of nitrosyl sGC recorded with 406.7 nm laser excitation. The signals originating from the buffer were subtracted from the high frequency spectrum. In the low frequency spectrum these signals are indicated by asterisks. The insets in panels a and b show the difference spectrum obtained when the spectrum with $^{15}N^{18}O$ is subtracted from that with $^{14}N^{16}O$. To flatten the baseline, a straight line was subtracted from the raw data.

*Ferrous sGC.* sGC as isolated exists in the ferrous oxidation state and is very stable in air (Stone & Marletta, 1994, 1995a). From the Raman data in Figure 1 and the correlations in Table 1, we conclude that this form of the enzyme in the absence of ligands adopts a five-coordinate, high-spin configuration. We assign the mode at 204 $cm^{-1}$ for ferrous sGC in Figure 1 to $\nu$(Fe−His). The frequency of this mode is lower than that observed for most enzymes and is very similar to Fe(II)PPDME(2-MeIM) in DMF (Kitagawa, 1988). Some lower frequencies have been observed for cytochrome $ba_3$ [193 $cm^{-1}$, Oertling et al. (1994)] or cytochrome $aa_3$ [194 $cm^{-1}$, Lauraeus et al. (1992)], although there is some uncertainty as to whether these modes are actually Fe−His stretching vibrations, or whether they are due to torsional motions involving the Fe−His bond. An important determinant of the Fe−His frequency is the hydrogen bonding status of the His $N_\delta$ hydrogen. If there is no hydrogen bond, the stretching frequency is at about 200 $cm^{-1}$. If a hydrogen bond is present, the frequency is typically 20 $cm^{-1}$ higher. If the imidazole proton is removed completely to produce the imidazolate form of the ligand, the stretching frequency is even higher, at about 235 $cm^{-1}$ (Kitagawa, 1988). Because the frequency of sGC is close to 200 $cm^{-1}$, our results suggest that the proximal histidine is not hydrogen bonded to other amino acids or to intercollated solvent $H_2O$ and is neutral. The conclusion that the proximal histidine is neutral agrees with the stability of the ferrous oxidation state, suggesting a relatively high oxidation potential for the enzyme (Stone & Marletta, 1994). It has further been suggested that the angle of the histidine relative to the heme nitrogens influences the strength of the Fe−His bond (Lauraeus et al., 1993), but our data do not give specific information on this issue.

To some extent the weak Fe−His bond may be responsible for the low $O_2$ affinity of sGC. For heme model compounds, a correlation was found between the frequency of the Fe−His bond and the frequency of the Fe−O bond (Oertling et al., 1990). If the strength of the Fe−His bond increases, there is an accompanying increase in the strength of the Fe−O bond, which reflects increased $\pi$ donation from imidazole that enhances Fe($d_\pi$) donation to the $O_2(\pi^*)$ orbital and strengthens the Fe−O bond. For proteins, such a trend was not clearly evident, probably because distal effects are more important (Oertling et al., 1990; see below). However, on the basis of the strength of the Fe-His bond, we expect the Fe−O bond to be weak for sGC. It is noted that, although a clear relationship between the oxygen affinity and the Fe−His frequency exists for several hemoglobins from

Kitagawa's work (1988) showing that the oxygen affinity is enhanced with increasing Fe−His frequency, the relationship between $O_2$ binding affinity and Fe−O bond strength is not necessarily straightforward (see below).

*CO−sGC.* Carbonmonoxy sGC is six-coordinate as determined by similarities in its Raman spectrum to that of CO−myoglobin and CO−hemoglobin (Rimai et al., 1975; Tsubaki et al., 1982). This indicates that CO will ligate as a sixth ligand to the heme. The isotopic shifts of the mode at 472 $cm^{-1}$ show that this mode can be attributed to $\nu$(Fe−CO). The strength of the Fe−CO bond is, however, exceptional, because the stretching frequency of 472 $cm^{-1}$ is very low. It is by far the lowest frequency observed for a heme with histidine as the proximal ligand. Fe−CO stretching frequencies for model compounds with CO bound parallel to the heme *z*-axis are at about 490 $cm^{-1}$, and for many proteins the frequencies rise above 500 $cm^{-1}$ (Li & Spiro, 1988). The frequency for $\nu$(Fe−CO) in CO−sGC is more similar to those of the cytochrome P-450 hemes, which have thiolate, not imidazole, as the proximal ligand. Determination of the C−O stretching frequency [$\nu$(C−O)] is useful, since an inverse linear relationship between $\nu$(Fe−CO) and $\nu$(C−O) has been established for a series of heme proteins and model systems (Kerr & Yu, 1988; Tsubaki & Ichikawa, 1985; Paul et al., 1985; Tsubaki et al., 1986; Uno et al., 1987). The underlying basis for this relationship is Fe $d_\pi \rightarrow$ CO $\pi^*$ back-donation. As this increases, the Fe−C bond order increases and the C−O bond order decreases. With $\nu$(Fe−CO) equal to 472 $cm^{-1}$, the frequency predicted by this correlation for $\nu$(C−O) for sGC is approximately 2000 $cm^{-1}$. Unfortunately, we have not been able to test this prediction as attempts to detect a Raman active C−O stretching vibration at about 2000 $cm^{-1}$ have been unsuccessful owing to the low amount of protein available. Similarly, detection of $\nu$(C−O) in sGC by FTIR is not yet feasible for the same reason.

The weak Fe−C bond correlates with the exceptionally fast dissociation rate for CO (3.5 $s^{-1}$) recently determined by Stone and Marletta (1995b). Li et al. (1994) showed that there is a correlation between the C−O stretching frequencies in a series of mutants of myoglobin, and thus $\nu$(Fe−CO), and the CO dissociation rates. Most myoglobin mutants have dissociation rates between 0.1 and 0.005 $s^{-1}$, and these decrease as $\nu$(C−O) decreases. Even though we could not measure the C−O stretching frequency, we estimate it above to be about 2000 $cm^{-1}$. From this estimate of $\nu$(C−O) and the myoglobin work, we calculate a rate of about 0.3 $s^{-1}$ for the CO dissociation rate constant in sGC. This value is still a factor of 10 lower than that measured experimentally. Nonetheless, the vibrational data provide a molecular rationale for the extremely fast CO dissociation rate constant.

Kerr at al. (1983) showed that the extent of $\pi$ back-donation and the frequency of the Fe−CO bond depend on the strength of the trans ligand. If the ligand is weak or absent, relatively high frequencies are found for the Fe−CO stretch because the competition for the metal $\sigma$ orbital is not severe. On the basis of the weak proximal Fe−His bond we observe for reduced sGC, we expect a relatively strong Fe−CO bond, opposite to what we observed. From this, we conclude that proximal effects alone cannot account for our observations, and that the environment in the distal pocket is largely responsible for the low $\nu$(Fe−CO).

Until recently, changes of $\nu$(Fe−CO) were attributed to steric hindrance in the distal pocket. This would lead to a bent and tilted geometry of CO, an increase of the Fe−CO stretching frequency, and Raman activity for the bending mode. Recent measurements (Nagai et al., 1987; Augsburger et al., 1991; Sakan et al., 1993; Lin et al., 1990; Lian et al., 1993; Li et al., 1994; Park et al., 1991; Ray et al., 1994), however, indicate that steric effects play only a minor role and that the major factor governing the frequencies of the CO−ligand is the electrostatic potential surrounding the ligand. Steric effects are important for determination of the association rate constant but, contrary to previous assumptions, are now thought not to affect the binding geometry to a large extent. Positive charges near the oxygen atom of CO, as in the case of the E7 distal histidine in myoglobin, will increase the back bonding, decrease $\nu$(C−O), and increase $\nu$(Fe−CO). If there are no distal positive charges, the back-bonding effects are smaller, and a frequency of about 490 $cm^{-1}$ is observed for the Fe−CO stretch (Ray et al., 1994). The frequency can be even lower if a negative charge is placed next to the CO, as in the case of the double mutant of pig Mb [H64V-V68T] that has the Fe−CO stretching frequency at 479 $cm^{-1}$ (Biram et al., 1991). Back-bonding is inhibited in this case. Measurements on distal mutants of myoglobin (Sakan et al., 1993) and hemoglobin (Lin et al., 1990) showed that there is a linear correlation between the hydrophobicity of the distal amino acid, expressed by its hydropathy index (Kyte & Doolittle, 1982), and $\nu$(Fe−CO). For sGC we found a $\nu$(Fe−CO) lower than that for any other histidyl ligated system, and, from the above reasoning, we conclude that the polarity of the distal pocket in sGC is negative.

Negative polarity will have interesting consequences for the $O_2$ binding affinity. It was shown by Smerdon et al. (1991) that negative polarity in the distal pocket destabilizes the binding of $O_2$. They performed measurements on the binding of different ligands to a mutant of pig Mb in which the nonpolar amino acid $Val^{68}$ was replaced by the polar threonine residue. Since Val is isosteric with Thr, all changes in oxygen binding were attributed to polar effects. Under normal circumstances, interactions of $O_2$ with $His^{64}$ stabilize bound $O_2$ by the formation of a hydrogen bond between the imidazole and the terminal oxygen atom. Even though threonine can also form a hydrogen bond, it is clear from the crystal structure that its hydroxy proton interacts with a carbonyl group of the protein backbone and not with $O_2$. This leaves the nonbonded electron pairs of the $\beta$-hydroxy group oriented toward the bound oxygen, which will destabilize oxygen binding, especially because there is a partial charge on the terminal oxygen atom of the bound oxygen. For the CO adduct the destabilizing effect is less, because the charge displacement due to electron transfer from Fe to the bound ligand is diminished for CO, which has higher lying $\pi^*$ orbitals than $O_2$ (Smerdon et al., 1991; Quillin et al., 1993). For the V68T mutant the affinity for oxygen was decreased 17 times and that for CO 5 times relative to the wild type (Smerdon et al., 1991). For CO, the decrease in binding affinity was attributed to a large increase in the dissociation rate constant, similar to what is observed for sGC (Stone & Marletta, 1995b). Negative polarity in the distal pocket provides an explanation for the observation that sGC does not bind oxygen with high affinity and is consistent with the fast CO-dissociation rate constant.

The mode at 562 $cm^{-1}$ in Figure 2b was attributed to the Fe−C−O bend. The observation of the bend is important because it indicates that CO is bound off-axis to the heme Fe (Li & Spiro, 1988). Moreover, Ray et al. (1994) concluded that observation of this mode is correlated with polarity in the distal pocket. As for $\nu$(Fe−CO), $\delta$(Fe−C−O) is also at a lower frequency than that of any other imidazole ligated heme and also more similar to those in carbonmonoxy ligated P-450 type enzymes. The difference between the CO-bending and stretching vibrational frequencies is about 90 $cm^{-1}$. This is larger than that observed for other hemes, which range from 50 to 80 $cm^{-1}$ (Li & Spiro, 1988). Normal coordinate calculations (Li & Spiro, 1988) showed that the stretching and bending frequency separation increases with the bending angle. This suggests that the bending of CO is larger for sGC than for other hemes. However, for those calculations it was assumed that the force constants remain the same when the angle is changed. If we consider recent insights concerning back-bonding, as discussed above, this assumption is questionable. Even though the bending frequency for sGC is very low, it does not deviate significantly from the linear correlation that exists between $\nu$(Fe−CO) and $\delta$(Fe−C−O) observed for a variety of model hemes and heme enzymes (Ray et al., 1994; Li & Spiro, 1988).

Jewsbury et al. (1994, 1995) recently performed *ab initio* calculations that suggest that the proximal ligand is a major factor in determining Fe−C−O distortions. With histidine as the proximal ligand, rotation of the histidine plane relative to the porphyrin has a large effect on the Fe−C−O distortion. From these calculations, they concluded that amino acids in the distal pocket only fine-tune the distortion. Our results do not support these conclusions, as we have no indications form our spectra that the proximal histidine causes a distortion of the CO orientation in sGC.

*NO−sGC.* From the resemblance of the Raman spectrum of nitrosyl−sGC in the high frequency region to that of the pentacoordinated Fe(II)−NO protoporphyrin IX complex, we conclude that the Fe of NO−sGC is five-coordinate. The only difference with the model system is that we observed the N−O stretching frequency at 1677 $cm^{-1}$, whereas in the model systems no frequencies assigned to N−O were observed in the Raman spectrum. Maxwell and Caughey (1976), however, reported that the N−O stretching vibration in the IR spectrum occurs at 1675 $cm^{-1}$ for five-coordinate model hemes, whereas the frequency is at about 1625 $cm^{-1}$ for six-coordinate hemes. A frequency of 525 $cm^{-1}$ for the Fe−NO stretching vibration was reported for a five-coordinate model porphyrin (Choi et al., 1991). Our assignments of $\nu$(N−O) at 1677 $cm^{-1}$ and $\nu$(Fe−NO) at 525 $cm^{-1}$ are clearly consistent with five-coordination for the Fe in sGC (Figure 3). In other five-coordinate proteins and model complexes, however, no Raman active NO-sensitive vibrations were observed, and it was assumed that five-coordinate complexes do not show enhancement of NO sensitive modes (Yu et al., 1994; Mackin et al., 1983; Tsubaki & Yu, 1982). Failure to observe Raman active Fe−NO vibrations may partly be a matter of signal-to-noise. The measurements performed on Fe protoporphyrin IX (Tsubaki & Yu, 1982) and on myoglobin (Mackin et al., 1983) were hampered by fluorescence, which limited detectability. Nonetheless, the isotopic shifts that we observe for the Fe−NO stretching vibration at 525 $cm^{-1}$ and the N−O stretching vibration at 1677 $cm^{-1}$ clearly show that NO-sensitive modes can be resonance enhanced for five-coordinate nitrosyl complexes.

Mackin et al. (1983) observed a mode at 525 $cm^{-1}$ in nitrosyl−Mb but saw no isotope effect. We note that, in addition to the NO-isotope-sensitive mode at 525 $cm^{-1}$, sGC also exhibits a mode around 520 $cm^{-1}$ that shows no isotope sensitivity. The Fe−NO stretching vibration is similar to that observed in six-coordinate complexes with various proximal ligands [∼525 $cm^{-1}$, Lipscomb et al. (1993)] and to six-coordinate NO−Fe−NO complexes [519 $cm^{-1}$, Lipscomb et al. (1993)]. Lipscomb et al. (1993) noticed that the Fe−NO bond strength was almost independent of the strength of the trans ligand. It may therefore not be surprising that five- and six-coordinate complexes have similar Fe−NO stretching frequencies. A frequency of 480 $cm^{-1}$ for the Fe-NO stretching vibration was obtained with IR for a solid-state five-coordinate nitrosyl heme (Fuchsman et al., 1974). The N−O stretching frequency of this heme is about 10−20 $cm^{-1}$ lower than that obtained in solution, but the influence of drying on the complex is not clear. In six-coordinate heme proteins, the Fe−NO stretching frequencies are at about 550 $cm^{-1}$ (Table 1), almost 30 $cm^{-1}$ higher than of sGC. Even though the literature data on ·NO binding is not as extensive as that on CO, the difference can be explained with reasoning analogous to that used above for the low Fe−CO stretching frequency.

Our conclusion that NO−sGC is five-coordinate agrees with recent EPR results that showed that the spectrum of nitrosyl−sGC is almost identical with that of a five-coordinate model compound (Stone et al., 1995). The EPR spectrum showed that a small fraction of the nitrosyl heme is six-coordinate. In the resonance Raman spectrum in Figure 3, this fraction was not observed by means of heme core modes indicating a six-coordinate complex. The absorption spectrum of nitrosyl−sGC is also indicative of a five-coordinate ferrous nitrosyl heme (Stone & Marletta, 1994). This coordination implies that ligation of ·NO causes the Fe−His bond to break. Supposedly, this is the first step in the activation of the enzyme (Stone & Marletta, 1994). According to recent stopped-flow measurements, ·NO first binds to the heme to form a six-coordinate nitrosyl complex that converts relatively slowly to the five-coordinate complex. Breaking of the Fe−His bond by ·NO is possible because the binding of ·NO can dramatically lower the binding affinity for the proximal histidine (Traylor & Sharma, 1992). In the T-state structure of nitrosylhemoglobin, the Fe−His bonds are broken in the α subunits, but not in the $\beta$ subunits (Nagai et al., 1980). This correlates with the strengths of the Fe−His bond. In the α subunit, the Fe−His stretching frequency is 203 $cm^{-1}$, whereas in the $\beta$ subunit this mode occurs at 217 $cm^{-1}$ (Nagai & Kitagawa, 1980). The $\nu$(Fe−His) frequency of the α subunit is similar to that we observe for sGC, and, for ·NO induced cleavage, the Fe−His bond apparently has to be weak. If breaking of the Fe−His bond is assumed to be critical for activation of the enzyme, it is clear why CO only weakly activates the enzyme (Stone & Marletta, 1994). When CO is bound, the heme Fe adopts a six-coordinate structure and the Fe−His bond remains intact.

For the nitrosyl complex, we did not observe a low frequency Fe−N−O bending mode. Unlike the situation for CO, this observation may not have ramifications for the geometry of the nitrosyl complex, since a bending vibration

was likewise not observed in six-coordinate strapped heme nitrosyl complexes (Lipscomb et al., 1993). There are no ferrous nitrosylhemes known for which both the Fe−N stretch and the Fe−N−O bending vibrations have been observed by Raman spectroscopy. Even without a bending mode, however, we can assume that nitrosyl ferrous sGC has a bent geometry (Scheidt & Piciulo, 1976; Scheidt & Frisse, 1975; Enemark & Feltham, 1974).

The isotopic shift of the Fe−NO stretching mode is about 16 $cm^{-1}$ for the $^{15}N^{18}O$ isotope. This shift is similar to that observed in six-coordinate model systems. Those models also showed Fe−NO stretches at about 525 $cm^{-1}$. On the basis of the limited amount of information on nitrosyl complexes in the literature, we propose that the nitrosyl complex of sGC is similar to other five-coordinate nitrosyl complexes and that its NO geometry is like that found in both five- and six-coordinate complexes.

Our resonance Raman spectra differ significantly from those recently obtained by Yu et al. (1994) with a partially purified, reconstituted preparation. They observed that the spectrum of ferrous sGC depended on the laser power incident on the sample and concluded that the heme Fe in the reduced, unligated enzyme is six-coordinated, with one of the heme ligands easily photolyzed. In contrast, we did not find a significant power dependence in the Raman spectrum of ferrous sGC. Burstyn et al. (1995) suggested that glycerol can influence the spin state and coordination number of the heme Fe and may account for differences observed between the native and reconstituted enzyme, but Stone and Marletta (1994, 1995a) showed that glycerol has no effect on the absorption spectrum, and thus on the heme environment, for the sGC preparation used in this work. Our results strongly indicate that reconstitution does not result in the same heme environment found in the native enzyme, assuming that the signal observed in their partially purified preparation is due only to sGC.

The mechanism of deactivation of sGC is still poorly understood. While initially thought to be not feasable, recent experiments suggest that deactivation could be due to simple dissociation of ·NO (Stone & Marletta, 1996). It has been suggested that there may be a distal amino acid residue involved in dissociation of ·NO from the ferric heme of cytochrome $cd_1$ (Fülöp et al., 1995). Our resonance Raman results, however, show no evidence of a functionally specialized distal amino acid residue involved in deactivation, as the $\nu$(Fe−NO) and $\nu$(N−O) frequencies are close to those observed in unperturbed, five-coordinate heme nitrosyl model compounds. The polarity effects that we propose to account for the low Fe−CO frequency may well facilitate dissociation of ·NO from the heme.

Figure 4 summarizes our results. The ferrous heme of sGC is five-coordinate with a weak bond to a histidine ligand. When CO is bound to the ferrous heme, it assumes a slightly bent or tilted geometry but does not cleave the Fe−His bond. The frequency of the Fe−CO vibration is low, probably as a result of negative polarity in the distal pocket. Because the Fe−His bond is weak, ·NO ruptures this bond when it binds.

Our results give us some new information about the mechanism of $O_2$ binding to sGC. From our Raman measurements, we detect two mechanisms that reduce the oxygen affinity. First, the weak Fe−His bond will decrease

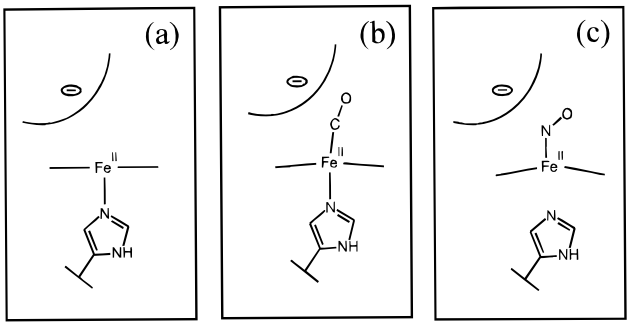


FIGURE 4: Models for (a) reduced, (b) CO-ligated, and (c) NO-ligated sGC. The view is parallel to the heme plane to emphasize the Fe axial ligation in the various species. The CO adduct is slightly bent and tilted, but the bond between the heme Fe and the proximal histidine is intact. With NO, the Fe−His bond is broken. The negative charge indicates the negative polarity of the distal pocket.

the oxygen affinity (Oertling et al., 1990). Second, negative polarity in the distal heme pocket destabilizes $O_2$ binding. Our results do not give specific information about a third factor that may decrease the $O_2$ affinity of sGC, that is, steric hindrance in the distal pocket. Stone and Marletta (1995b) recently determined that the CO association rate constant for sGC is $3.6 \times 10^4$ $M^{-1}$ $s^{-1}$. This value is comparable to that observed for hemoglobin or myoglobin (Antonini & Brunori, 1971) and significantly larger than that for cytochrome $c'$, for which steric hindrance in the distal pocket was given as a possible reason for lack of oxygen binding (Kassner, 1991). As discussed above and by Ray et al. (1994), steric hindrance strongly influences the association rate constants. The relatively high association constant of CO for sGC suggests that steric hindrance may not be a primary reason for the low $O_2$ affinity of sGC.

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