Effects of GTP on Bound Nitric Oxide of Soluble Guanylate Cyclase Probed by Resonance Raman Spectroscopy†

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ABSTRACT: Soluble guanylate cyclase (sGC) was isolated from bovine lung, and its resonance Raman (RR) spectra were investigated for the reduced, CO-bound (CO-sGC), NO-bound (NO-sGC), oxidized, and oxidized NO-bound forms in the presence and absence of GTP. The enzyme was purified by more than 12 000-fold in terms of specific activity than the supernatant of homogenates, and the heme content was determined with the pyridine hemochoromogen method and Bradford's protein assay to be 0.8 per heterodimer (α, M_r = 74 000; β, M_r = 69 000). The RR spectra of sGC and CO-sGC including the Fe−His stretch at 203 cm^{-1} and the Fe−CO stretch at 473 cm^{-1} were unaltered by binding of GTP and cGMP, but apparent RR spectra of NO-sGC in the presence of GTP changed with time and concentrations of GTP. In the absence of GTP, the RR bands of the N−O stretch (ν_{NO}) and the Fe−NO stretch (ν_{Fe-NO}) were observed at 1681 and 521 cm^{-1}, respectively. In its presence, however, two ν_{NO} bands were observed at 1700 and 1681 cm^{-1}, which exhibited ^{15}NO isotopic frequency shifts of 32 and 34 cm^{-1}, respectively. Similar Raman spectral changes were observed with the same amount of cGMP but not with PP_i or GTP analogues including ATP, GMPPNP, and GTPγS. This suggests that GTP or cGMP binds to the distal side of the heme in the proximity of bound NO, possibly regulating NO binding.

The physiological receptor of nitric oxide (NO) synthesized by NO synthase (NOS)[1] from L-arginine is a soluble guanylate cyclase (sGC, EC 4.6.1.2.), which is a protoheme-containing protein with M_r = 150 000 and catalyzes the conversion of GTP to cGMP [see Bredt and Snyder (1994), Marletta (1994), and Nathan (1992) for a review]. Since cGMP acts as a second messenger in cells of animal tissues, NO plays important physiological roles in mammalian smooth muscles (Ignarro & Kadowitz, 1985), platelets (Azuma et al., 1986; Furlong et al., 1987; Radomski et al., 1987), brain (Garthwaite et al., 1988; Bredt & Snyder, 1989), and macrophages (Marletta et al., 1988; Stuehr & Nathan, 1989; Hibbs et al., 1989). The catalytic activity of sGC requires the presence of either Mg^{2+} or Mn^{2+} in addition to hemes in excess of GTP (Ignarro et al., 1982a) and is significantly promoted by binding of NO.

The sGC is a complex of two similar, but not identical, polypeptides (Garbers, 1979; Kamisaki et al., 1986). The stoichiometry of the number of hemes per heterodimer was recently determined to be 1.5 (Stone & Marletta, 1995a). The heme iron in the unactivated enzyme adopts the five-coordinate high-spin state with a histidine residue (proximal His) as the most likely axial ligand (Stone & Marletta, 1994), but in the presence of NO the absorption and EPR spectra suggested the formation of the five-coordinate heme with NO as the axial ligand (Stone et al., 1995). Therefore, binding of NO to the trans position of the proximal His causes cleavage of the Fe−His bond. In the presence of CO, however, sGC forms a six-coordinate heme with CO and His as axial ligands, and CO slightly activates the catalytic activity (Brüne et al., 1990; Stone & Marletta, 1994; Bustyn et al., 1995).

It is also reported that the free base protoporphyrin IX (PPIX) (Ignarro et al., 1982b) and arachidonic acid (Ignarro & Wood, 1987) can also stimulate the guanylate cyclase activity for heme-deficient sGC. Although the membrane-bound and particulate guanylate cyclases do not contain a heme group, the C-terminal sides in the catalytically active domain of these enzymes have an amino acid sequence similar to that of sGC, and in addition, they have the consensus sequence of natriuretic peptide receptors and kinases (Koesling et al., 1988, 1990; Nakane et al., 1988, 1990). Our studies revealed that sGC was modified by an ADP-ribosyltransferase similar to general G-proteins, which shuttle between the GTP-bound active form and the GDP-bound inactive form (Pennington, 1994), and the ADP-ribosylated sGC exhibited an increased specific activity (Tomita et al., 1997). These features suggest that a regulation mechanism of the catalytic activity of sGC is not straightforward.

Resonance Raman (RR) spectroscopy can provide detailed structural information on the heme groups of proteins. As

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[1] Abbreviations: sGC, soluble guanylate cyclase; GTP, guanosine 5′-triphosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; PP_i, pyrophosphate; PPIX, protoporphyrin IX; TEA, triethanolamine; DTT, dithiothreitol; GDP, guanosine 5′-diphosphate; GMPPNP, 5′-guanylyl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Hb, hemoglobin; P-450$_{cam}$, cytochrome P-450 isolated from *Pseudomona putida*, the product of the CYP101 gene; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SNP, sodium nitroprusside; ν_{NO}, N−O stretching; ν_{Fe-NO}, Fe−NO stretching; δ_{FeNO}, Fe−N−O bending; ν_{Fe-His}, Fe−histidine stretching; ν_{Fe-CO}, Fe−CO stretching; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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to NO adducts of heme proteins, this technique allows observation of the Fe−NO stretching (ν_{Fe-NO}) and bending (δ_{FeNO}) modes (Kerr & Yu, 1988). In fact, Nagai et al. (1980) demonstrated from RR study of NO adducts of hemoglobin (Hb) that the Fe−His bond of the α subunit is cleaved in the T structure but not in the R structure while the Fe−His bond of the β subunit retains the bonding irrespective of the T or R structure. Hu and Kincaid (1991a,b) pointed out that the ν_{Fe-NO} and δ_{FeNO} RR bands of NO-P450$_{cam}$ sensitively reflect the size of the substrate bound to the distal side of the heme via direct interactions between NO and the substrate. Recent applications of RR spectroscopy to sGC revealed the Fe^{II}−His stretching (ν_{Fe-His}) band at 204 cm^{-1} for the enzyme as isolated and the Fe^{II}−CO stretching (ν_{Fe-CO}) band at 472 cm^{-1} (Deinum et al., 1996), which are distinctly lower than those of other heme proteins (Kerr & Yu, 1988), although Yu et al. (1994) reported somewhat controversial results. There has been no RR study of the substrate effects on the bound NO of sGC. If the substrate-binding site of sGC is near the heme group like P450$_{cam}$, the presence of GTP may influence some NO-associated RR bands of the NO adduct of sGC. Accordingly, in the present study, we modified the purification procedure to get a larger amount of enzyme and characterized the enzyme obtained with the new method first. Then, we focused our attention on the effects of GTP binding to sGC by investigating the RR spectra of its CO (CO-sGC) and NO adducts (NO-sGC), and point out that the N−O stretching (ν_{NO}), but not the Fe−NO sretching (ν_{Fe-NO}), frequency is influenced by binding of GTP or cGMP.

MATERIALS AND METHODS

The sGC enzyme was purified from fresh bovine lung with a method modified from that of Stone and Marletta (1994, 1995a,b). The minced meat (4 kg) was homogenized into 4 L of buffer (25 mM TEA-HCl, 50 mM NaCl, 5 mM DTT, 1 mM EDTA, 1 mM benzamidine, 1 mg/mL leupeptin, and pepstatin A, pH 7.4) and was centrifuged at 10000*g* for 20 min first. The supernatant was further centrifuged at 100000*g* for 30 min, and its supernatant was put into a DEAE-Fast Flow column (1000 mL, Pharmacia). After thorough washing with the homogenizing buffer, the adsorbates were eluted with a linear gradient of NaCl (from 0.05 to 1.05 M), and a fraction containing NO-sensitive guanylate cyclase activity was collected.

Ammonium sulfate was added to the collected fraction up to 23% saturation, and the resulting precipitate was removed by centrifugation at 15000*g* for 20 min. Ammonium sulfate was further added into the supernatant to 41% saturation. The precipitate was collected by centrifugation and was suspended in a small volume of buffer A (25 mM TEA-HCl, 5 mM DTT, 1 mM benzamidine, 1 mg/mL leupeptin, and pepstatin A, pH 7.4). The suspension was dialyzed against buffer A overnight. After addition of $MnCl_2$ to 4 mM, the dialysate was centrifuged at 15000*g* for 20 min, and the supernatant was put into a GTP−agarose column (100 mL), which had been equilibrated with buffer A containing 4 mM $MnCl_2$, and eluted with a linear gradient from buffer A containing 4 mM $MnCl_2$ to buffer A containing 6 mM ATP. The fraction containing NO-sensitive GC activity was concentrated by an Amicon-50 concentrator and was dialyzed against buffer A. The dialysate was put into a hydroxyapatite column (30 mL) equilibrated with buffer A, and eluted with a linear gradient of phosphate (from 0 to 200 mM). The fraction containing NO-sensitive GC activity was concentrated with Amicon-50 and further subjected to a gel-filtration column (Superdex 200 pg, Pharmacia) that had been equilibrated with 25 mM TEA-HCl (pH 7.4) containing 5 mM DTT. The fraction containing sGC was concentrated again with Amicon-50. Finally, 3.2 mg of the purified enzyme was obtained. If necessary, glycerol was added into the concentrated sGC preparation at a final concentration of 20%. When the sGC preparation was kept in a freezer at 77 K, the activity of sGC was stable for 3 weeks.

The activity of sGC was routinely measured before every Raman measurement in the following way. The sample was incubated with 50 mM Tris-HCl (pH 7.4) containing 1 mM theophilline, 1 mM DTT, 1 mM GTP, and 1 mM Mg^{2+} (or Mn^{2+}) at 37 °C for 10 min. The enzyme activity under the condition mentioned above was constant for 15 min, at least, and the amount of products increased linearly. After incubation, the reaction was stopped by addition of 9 volumes of ethanol, and the solution was centrifuged. The supernatant was dried in an evaporator, and the dried residuals were dissolved in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. The amount of cGMP present in this solution was measured by a [^{3}H]cyclic GMP immunoassay system (Amersham, TRK500) or the high-pressure liquid chromatography method in the following way.

[α-^{32}P]GTP was substituted for the cold GTP in the incubation mixture, and the reaction was stopped by the addition of 5% perchloric acid. After centrifugation, the pH of the supernatant was adjusted to 4.0 with KOH. The resulting precipitate was removed by centrifugation. The supernatant was injected into a high-pressure column (C18 Inartsil, 4.6 × 150 mm) which had been equilibrated with 20 mM sodium phosphate buffer (pH 3.5) containing 5 mM tetra-*n*-butylammonium bromide, at a flow rate of 1.0 mL/min. The adsorbates were eluted with a linear gradient of acetonitrile (from 0 to 60%). The effluent was monitored with a UV detector at 260 nm and a β-ray detector (A-150, Packard Japan). The cGMP retained in the column was eluted 8.0 min after sample injection.

The heme content of the purified enzyme was determined by the pyridine hemochromogen method (de Duve, 1948); 300 mL of the solutions with different concentrations of enzyme was mixed with 100 mL of pyridine and 4.5 mL of 8 M NaOH (White & Marletta, 1992) and was reduced with sodium dithionite under a N_2 atmosphere. The absorption spectra between 500 and 600 nm were measured within 2 min following the reduction, and the absorbance differences between 540 and 556 nm were determined. The same procedure was applied to myoglobin solutions with various concentrations, and a calibration line for absorbances *vs* heme contents under the present solution conditions was established. The protein concentration was determined with the method of Bradford (1976).

For Raman experiments, the purified sGC was diluted to 30 μM (heme), under the assumption of ϵ_{mM}(heme) = 105 cm^{-1} at 431 nm (Stone & Marletta, 1995a), with 50 mM TEA buffer, pH 7.4, containing 5 mM DTT and 20% glycerol (if necessary). To obtain NO-sGC or CO-sGC, about 50 mL of the solution was put into an air-tight Raman spinning cell (3000 rpm), and after repetitions of degassing (to 0.1 mmHg) and filling with pure N_2 gas, NO or CO gas,

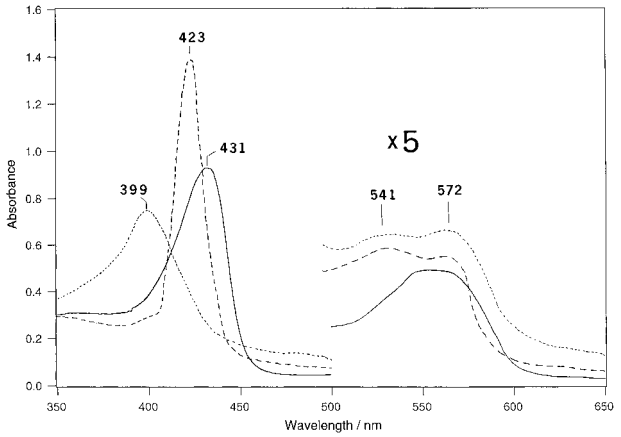

FIGURE 1: Absorption spectra of reduced sGC (—) , CO-sGC (- - -), and NO-sGC (···). The enzyme was dissolved in 25 mM TEA buffer, pH 7.4, and the heme concentration was 10 μM. The path length was 10 mm.

respectively, was incorporated into the upper space of the Raman cell through a rubber septum by an air-tight syringe. The NO gas, which was always passed through 1 M KOH before use to remove other nitrogen oxides, was injected to make the final concentration 2% (v/v). Ferric sGC was prepared by adding 300 mM ferricyanide to the purified preparation in 50 mM HEPES, pH 7.8, and the solution was subjected to gel filtration with a Sephadex G-25 column to remove the remaining ferricyanide.

Raman scattering was excited with the 406.7 nm line of a Kr ion laser (Spectra Physics, Model 2016) or the 441.6 nm line of a He-Cd laser (Kinmon Electrics, CD4805R). The scattered light at right angle was dispersed with a single polychromator (Ritsu Oyokogaku, DG-1000) and detected by a cooled CCD detector (Astromed) or was dispersed with a double monochromator (JEOL 400D) and detected with a photomultiplier (HPKK, R943-02). Raman shifts were calibrated with toluene or carbon tetrachloride observed with the same spinning cell as used for sGC; the bands of toluene at 1604.4, 1379, 1210.5, 785.3, 621.7, and 521.1 cm^{-1} and those of CCl_4 at 459, 314.2, and 218.4 cm^{-1} were used as standards. Accuracy of frequencies is ± 1 cm^{-1}. Sample temperature was kept at 20 °C by flushing with cold N_2 gas.

UV−visible absorption spectra were measured with a spectrophotometer (Hitachi, U-3210) before and after Raman measurements, and the integrity of the enzyme was confirmed. The isotope purity of $^{15}N^{16}O$ (Shoko Tsusho) was 99 atom %. GTP, GDP, 5′-guanylyl imidodiphosphate (GMPPNP), and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) were purchased from Nacalai Tesque.

RESULTS

The present sGC was purified more than 12 000-fold as compared with the lung extract in terms of specific activity. The visible absorption spectra of the purified sGC, CO-sGC, and NO-sGC are shown in Figure 1. The Soret peak was observed at 431 nm for reduced sGC, at 423 nm for CO-sGC, at 399 nm for NO-sGC, at 425 nm for nitrosomethane-sGC (not shown), and at 391 nm for the ferric unligated state (not shown). Gel filtration by a TSK 3000SW column gave a molecular mass of ca. 140 kDa for the purified sGC. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) of the purified sGC gave two bands at 69 000 and 74 000 Da when the running gel contained 10% acrylamide. The amount of hemes contained in the sample was determined with the pyridine hemochromogen method using the calibration line constructed with known concentrations of myoglobin. The protein content of the enzyme solution was determined with Bradford's method and the molecular weight determined with SDS−PAGE. These measurements gave 0.8 heme/dimer as the heme content of the present sGC. This is a noncorrected value, but its validity depends on the validity of the protein content thus determined.

The enzyme assay of the present preparation gave $K_m = 44 \pm 8$ mM for GTP and $V_{max} = 77 \pm 10$ nmol min^{-1} (mg of protein)$^{-1}$ in the presence of $MgCl_2$, which are referred to as the basal activity of this preparation. The activity was enhanced by 50- and 200-fold upon addition of PPIX (1 μM) and sodium nitroprusside (SNP, 100 μM), respectively. Upon replacement of $MgCl_2$ with $MnCl_2$, the activity was raised by 10-fold, and this was due to a ~10-fold increase of V_{max} [710 $\pm$ 55 nmol min^{-1} (mg of protein)$^{-1}$, while the K_m for GTP did not change (44 $\pm$ 8 mM for four different preparations). When NO gas was introduced into the reaction mixture, the amount of the product (cGMP) was drastically increased (more than 150-fold) compared with that under basal conditions. In fact, the V_{max} of sGC was increased by 150-fold [11 500 $\pm$ 900 nmol min^{-1} (mg of protein)$^{-1}$ and the K_m for GTP was decreased to about half (22 $\pm$ 4 mM) by incorporation of NO gas. Thus, this preparation is essentially the same as that reported by Stone and Marletta (1995a,b).

Figure 2 shows the RR spectra in the 1300−1800 cm^{-1} region of unligated ferrous (A), CO-bound (B), NO-bound ferrous (C), unligated ferric (D), and NO-bound ferric (E) forms of sGC excited at 406.7 nm. Spectrum (A) remained unaltered even in the presence of air. Spectra (C−E) were examined under the higher resolution of the spectrometer using the second-order diffraction of the grating, and, accordingly, a single band reported at 1603 cm^{-1} for NO-sGC (Deinum et al., 1996) is resolved into two bands at 1604 and 1598 cm^{-1} in spectrum (C). Spectrum (D) was observed for the ferric sGC 30 min after gel filtration. Only spectrum (E) was observed in the presence of GTP and $MgCl_2$. A weak band seems to be present at 1700 cm^{-1} in spectrum (E). Although the intensity of the band at 1679 cm^{-1} is more intense in spectrum (C) than in spectrum (E), RR spectra (C) and (E) are apparently alike, suggesting that ferric NO-sGC is reduced in the presence of much excess of NO.

Spectra (A) through (D) were observed in the absence of GTP, but the RR spectra of unligated ferrous and ferric sGC and CO-sGC in the presence of 10 mM GTP were almost the same as those shown in Figure 2. The ν_4 band appears at 1356, 1369, 1375, and 1373 cm^{-1} for ferrous sGC, CO-sGC, NO-sGC, and ferric sGC, respectively, ν_3 occurs at 1472, 1498, 1509, and 1494 cm^{-1}, and ν_{10} is found at 1625, 1627, 1646, and 1632 cm^{-1}, respectively. These frequencies are specific to the four forms and well reproduce the characteristics of reported RR spectra excited at 431 nm (Deinum et al., 1996) except for the relative intensities of RR bands. The bands at 1470 cm^{-1} in spectra (B) and (C) are due to the CO-photodissociated species and glycerol present (20% v/v), respectively.

Figure 3 shows the effects of GTP on the RR spectra of reduced NO-sGC. Spectra (A) and (B) were observed for the ^{14}NO and ^{15}NO adducts, respectively, in the absence of GTP, and trace (C) depicts their difference (A − B). A

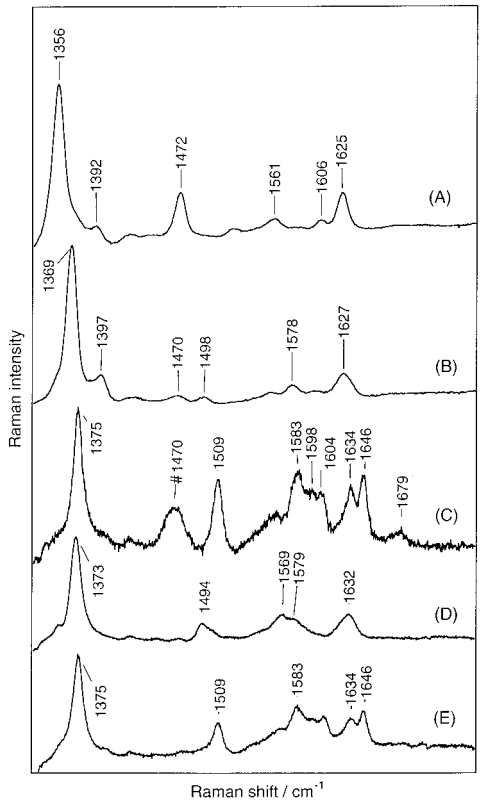

FIGURE 2: RR spectra in the 1250−1800 cm^{-1} region of sGC (A), CO-sGC (B), NO-sGC (C), ferric sGC 30 min after gel filtration (D), and ferric NO-sGC 10 min after the addition of NO to the ferric sGC (E). Spectra (C−E) were observed under higher resolution using the second-order diffraction of the grating. Raman bands marked by # arise from glycerol. Experimental conditions for spectra (A) and (B): solvent, 25 mM TEA buffer, pH 7.8; concentration, 30 μM heme; temperature, 20 °C; excitation, 406.7 nm, 4.5 mW at sample; slit, 200 μm; accumulation time, 5 min. For spectrum (C): solvent, 25 mM TEA buffer, pH 7.4, containing 5 mM DTT and 20% (v/v) glycerol; concentration, 10 μM heme; excitation, 406.7 nm, 3.5 mW at sample; slit, 200 μm; accumulation time, 5 min. For spectra (D, E): solvent, 50 mM HEPES, pH 7.8; concentration, 20 μM heme; excitation, 406.7 nm, 4.5 mW at sample; slit, 200 μm; accumulation time, 5 min. Only spectrum (E) was observed in the presence of 10 mM GTP and 4 mM $MgCl_2$, but others were observed in their absence.

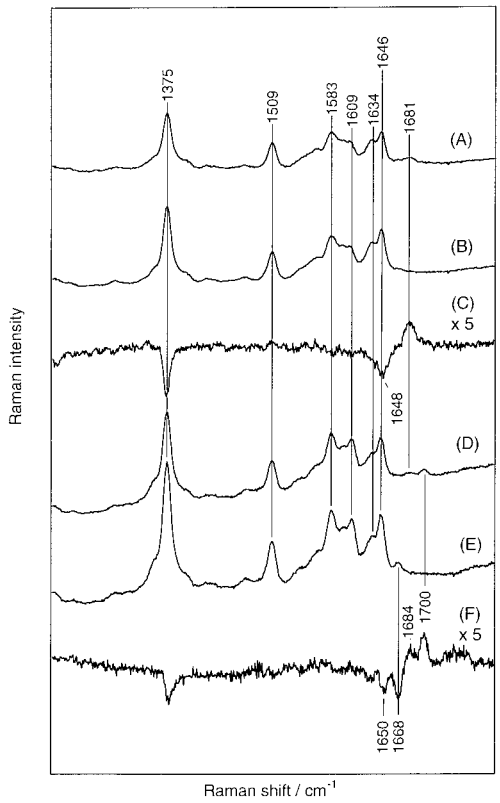

FIGURE 3: RR spectra in the 1250−1800 cm^{-1} region of NO-sGC in the absence (A−C) and presence (D−F) of GTP and $MgCl_2$. (A) ^{14}NO-sGC; (B) ^{15}NO-sGC; (C) difference, spectrum (A) − spectrum (B); (D) ^{14}NO-sGC; (E) ^{15}NO-sGC; and (F) difference, spectrum (D) − spectrum (E). The ordinate scales of spectra (C) and (F) are expanded 5 times. Solvent, 25 mM TEA buffer, pH 7.8 [containing 10 mM GTP and 4 mM $MgCl_2$ for (D) and (E)]; concentration, 30 μM heme; temperature, 20 °C; excitation, 406.7 nm, 4.5 mW at sample; slit, 200 μm; accumulation time, 5 min

single differential peak appears at 1681/1648 cm^{-1} for ^{14}NO-sGC/^{15}NO-sGC. Spectra (D) and (E) were observed for the ^{14}NO and ^{15}NO adducts, respectively, in the presence of GTP and $MgCl_2$. There are two bands at 1681 and 1700 cm^{-1} in spectrum (D). It was reproducibly observed for different preparations. When the differences between spectra (D) and (E) were calculated, there appeared two positive peaks at 1684 and 1700 cm^{-1} and two troughs at 1650 and 1668 cm^{-1} as illustrated by trace (F). The frequency shifts between the positive and negative peaks are 32 and 34 cm^{-1} for the high- and low-frequency counterparts, respectively, indicating that both bands arise from the ν_{NO} mode.

The 1684/1650 cm^{-1} pair is practically the same as that observed in the absence of GTP, but the two kinds of ν_{NO} bands may not correspond to the GTP-unbound and bound forms, because the concentration of GTP is much higher than the K_m value. The relative intensity of the 1681 and 1700 cm^{-1} bands somewhat changed with time presumably owing to consumption of GTP. However, after the observation of RR spectra, GTP was still present. The additional ν_{NO} band could not be recognized upon addition of various GTP analogues (10 mM) including ATP, GDP, GMPPNP, and GTPγS which inhibited cGMP formation from GTP by more than 50% of that in their absence. However, when 10 mM cGMP was present, the additional band was observed. PP_i did not give the band. Therefore, it is highly likely that the observed effect of GTP is caused by the product of enzymatic reaction. When the laser power was raised, the spectrum of ferric unligated sGC (Figure 2D) increasingly contributed to the observed spectrum only in the presence of GTP, suggesting the occurrence of oxidative photodissociation of NO in the presence of GTP.

To explore the corresponding ν_{Fe-NO} bands, the RR spectra in the lower frequency region were examined under the same conditions as those for Figure 3, and the results are shown in Figure 4, where the RR spectra of ^{14}NO-sGC (A) and ^{15}NO-sGC (B) in the absence of GTP, of ^{14}NO-sGC (D) and ^{15}NO-sGC (E) in the presence of GTP and $MgCl_2$, and their differences (C) (=A − B) and (F) (=D − E) are displayed. The isotope-difference spectrum (C) indicates the presence of a single ν_{Fe-NO} band at 521 and 512 cm^{-1} for the ^{14}NO and ^{15}NO adducts, respectively, in the absence of GTP. Unexpectedly, no additional isotope-sensitive band was recognized in spectrum (F), and it was the same for the presence of cGMP and $MgCl_2$. Consequently, there are no clear GTP binding effects on the Fe−NO bond of NO-sGC.

Figure 5 shows the the RR spectra in a low-frequency region of ferrous sGC (A), and CO-sGC (B) excited at 406.7 nm, while the inset delineates the RR spectrum of ferrous sGC excited at 441.6 nm. The Fe−His stretching (ν_{Fe-His})

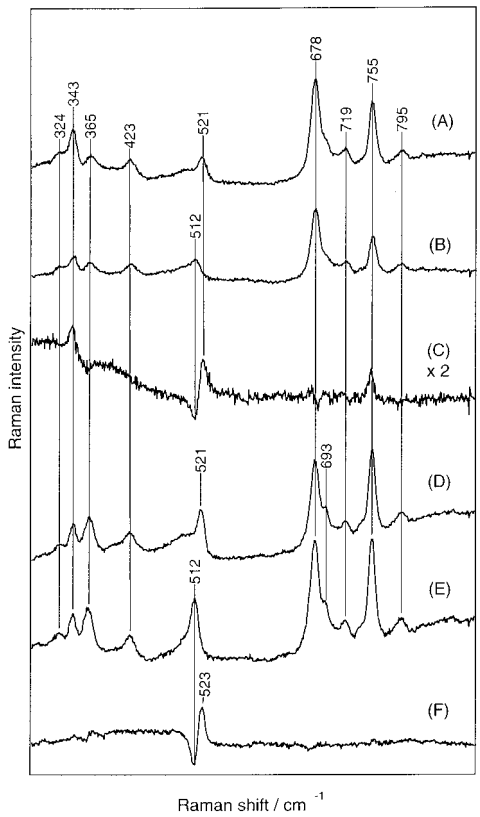

FIGURE 4: RR spectra in the 250−850 cm^{-1} region of NO-sGC in the absence (A−C) and presence (D−F) of GTP and $MgCl_2$. (A) ^{14}NO-sGC; (B) ^{15}NO-sGC; (C) difference, spectrum (A) − spectrum (B); (D) ^{14}NO-sGC; (E) ^{15}NO-sGC; and (F) difference, spectrum (D) − spectrum (E). The ordinate scale of spectrum (C) is expanded 2 times, but that of spectrum (F) is not expanded. Experimental conditions are the same as those for Figure 3.

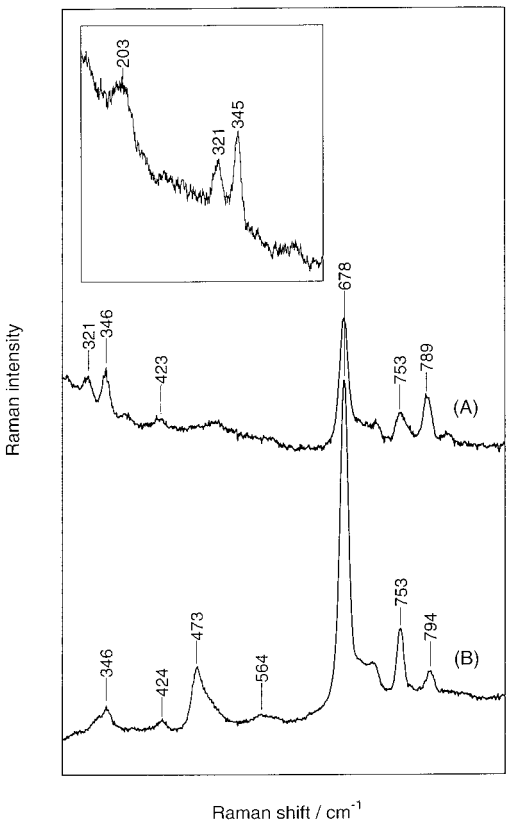

FIGURE 5: RR spectra in the 250−850 cm^{-1} region of ferrous sGC (A) and CO-sGC (B) in the absence of GTP and $MgCl_2$ excited at 406.7 nm. The inset shows the RR spectrum of ferrous sGC excited at 441.6 nm,which was measured with the scanning spectrometer and detected with a photomultiplier. Solvent, 25 mM TEA buffer, pH 7.4; concentration, 30 μM heme; excitation, 406.7 nm, 3.5 mW at sample; accumulation time, 5 min.

band was not enhanced for the ferrous sGC upon excitation at 406.7 nm, but it was observed at 203 cm^{-1} for this preparation upon excitation at 441.6 nm. The ν_{Fe-His} band appears broad and weak compared with that of deoxyMb and is reminiscent of the ν_{Fe-His} band in the α subunit of T-structure deoxyHb (Nagai & Kitagawa, 1980), suggesting the presence of some strain in the Fe−His bond. The RR spectrum of unligated sGC including ν_{Fe-His} was unaltered by addition of $MnCl_2$, cGMP, and GTP. Upon introduction of CO to the sample of spectrum (A), the relative intensities of the Raman bands were altered as illustrated by spectrum (B), and the Fe−CO stretching (ν_{Fe-CO}) band appeared at unusually low frequency (473 cm^{-1}) as pointed out previously (Deinum et al., 1996). This frequency remained unaltered upon addition of 10 mM cGMP and GTP. The RR spectral patterns of CO-sGC and NO-sGC (Figure 4) in the 300−800 cm^{-1} region are alike except for the Fe−ligand bands in contrast with their distinct differences in the higher frequency region [(B) and (C) in Figure 2].

DISCUSSION

Binding of GTP and $MgCl_2$ to sGC yielded little effect on the RR spectrum including the ν_{Fe-His} band. Generally the ν_{Fe-His} frequency is sensitive to the tertiary and quaternary structures of Hb and the hydrogen bond strength of the proximal His of peroxidases (Kitagawa, 1988). This means that substrate binding scarcely alters the tertiary structure of sGC protein in the heme proximal side. The ν_{Fe-CO} frequency of CO-sGC and the ν_{Fe-NO} frequency of NO-sGC and their visible absorption spectra also remained unaltered upon the addition of GTP or cGMP, indicating that the geometry of bound CO and NO and their heme structures are scarcely affected by binding of GTP or cGMP. Only the ν_{NO} frequency was appreciably influenced. These spectral changes were not observed with various GTP analogues such as ATP, GDP, GMPPNP, and GTPγS, which inhibit the catalytic reaction but are not themselves converted to other compounds by sGC.

Spectrum (D) in Figure 3 gives two ν_{NO} RR bands, but one of them (1681 cm^{-1}) is very close to that in spectrum (A). This band corresponds to ν_{NO} of the five-coordinate heme with NO as an axial ligand. Maxwell and Caughey (1976) pointed out from IR studies of model compounds that the ν_{NO} frequency of the five-coordinate $NOFe^{II}$(PPIX) was sensitive to solvents: 1669, 1676, and 1684 cm^{-1} for $C_2H_4Cl_2$, $CHCl_3$, and CCl_4, respectively. The ν_{NO} frequency is greatly reduced to 1615−1635 cm^{-1} for six-coordinate complexes. In this study, we also confirmed the appearance of the ν_{NO} and ν_{Fe-NO} RR bands of the five-coordinate $NOFe^{II}$(PPIX) at 1669 and 527 cm^{-1} for the ^{14}NO adduct and at 1641 and 516 cm^{-1} for the ^{15}NO adduct in a 50 mM TEA aqueous solution containing 0.5% sodium cholate at neutral pH. The magnitudes of the ^{15}N isotopic frequency shifts for this model compound ($\Delta\nu_{NO} = 28$ and $\Delta\nu_{Fe-NO} = 11$ cm^{-1}) are close to those observed for NO-sGC in the absence of GTP ($\Delta\nu_{NO} = 32-34$ and $\Delta\nu_{Fe-NO} = 9-11$ cm^{-1}). Accordingly, the heme of the NO-sGC, which gives the ν_{NO} RR band at 1681cm^{-1}, is considered to adopt a five-

coordinate structure similar to that of $NOFe^{II}$(PPIX) in organic solvents.

The other component of ν_{NO} of NO-sGC in the presence of GTP or cGMP cannot be attributed to the six-coordinate structure judging from its frequency (1700 cm^{-1}). The higher frequency may suggest that the heme is of five-coordinate structure with NO at an axial position, and the bound NO is surrounded by more hydrophobic environments than in the case of the absence of GTP. Since cGMP brought about the same effect, it is highly likely that the observed GTP effect is practically caused by cGMP. In this case, cGMP does bind to the site different from the substrate binding site where GTP analogues bind, presumably to some part of the distal side of the heme pocket, and its tail extends over the liganded heme. The tail would not be so close to the heme as to have steric hindrance on the bound ligand but is not so far as some field effects cannot reach.

In the physiological conditions, the cGMP's produced through the enzymatic reaction go out of the heme pocket to work as the second messenger in cells. However, in the present closed system, the produced cGMP is accumulated, and it would physiologically correspond to the state of overproduction. Under such conditions, it is reasonable that cGMP binds to a regulation site, protecting the influx of NO to the heme iron of sGC. Since NO raises the enzymatic activity by a factor of 200−400 of the basal activity, it seems likely that cGMP regulates the activity of sGC by controlling NO binding to sGC. Determination of K_d values of NO to sGC in the presence of various GTP analogues is under progress in this laboratory.

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