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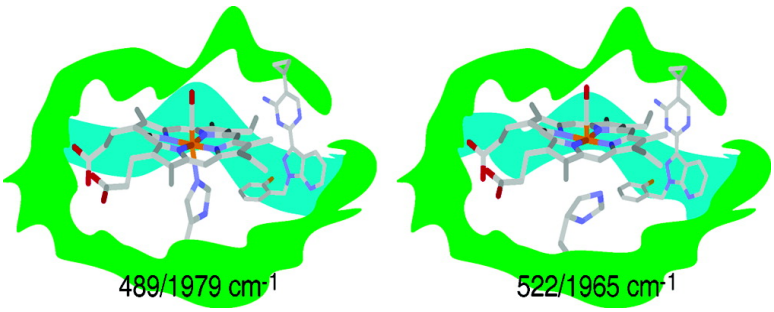

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Resonance Raman and Infrared Spectroscopic Studies of High-Output Forms of Human Soluble Guanylyl Cyclase†

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Abstract: The allosteric regulator BAY-41-2272 converts the CO adduct of soluble guanylyl cyclase (CO−sGC) enzyme from a low- to high-output form, with respect to production of cGMP. Resonance Raman (RR) and Fourier Transform Infrared (FTIR) spectroscopic techniques are used to show that the CO−sGC exists as major and minor conformers, both having ν(Fe−CO) and ν(C−O) modes characteristic of 6-coordinate species. It is further shown that addition of BAY-41-2272 to the CO adduct induces the transition of some fraction of the initial CO−heme adducts into two new CO−heme complexes, the fractional conversion being dependent on the temperature. One new complex displays vibrational modes characteristic of pentacoordinated CO-adduct, and its formation is not affected by temperature. The second complex, although slightly different from the original CO-adducts, is hexacoordinated, and its formation is facilitated by temperature. The production of substantial amounts of the 5-coordinate CO adduct upon addition of BAY-41-2272, reveals the fact that several out-of-plane heme deformation modes are simultaneously activated, an observation similar to that realized upon NO activation. While the precise nature of these modes will require elucidation by isotopic labeling experiments, by analogy with earlier studies of other heme proteins, several bands associated with modes attributable to peripheral substituent deformations and methine carbon movements are implicated. The documented formation of *two* new forms upon addition of Bay-41-2272 (a 5-coordinate and a new 6-coordinate form) is discussed with respect to the implications for enzyme activation.

Introduction

Soluble guanylyl cyclase (sGC) is the only member of the mammalian guanylyl cyclase family of proteins that is not membrane bound. These enzymes convert GTP into second messenger cGMP upon activation by various ligands.[1] Binding of its physiological activator, nitric oxide (NO), to sGC affects various processes, including smooth muscle relaxation, platelet reactivity, central and peripheral neurotransmission, and other effects;[1] i.e., physiological activation of cytosolic sGC is dependent on nitric oxide.

Soluble guanylyl cyclase is a heterodimeric hemoprotein[2] composed of one larger α subunit (∼80 kDa), one smaller β subunit (∼70 kDa) and one ferrous protoheme moiety.[3] The ferrous heme is coordinated only by the His105 residue of the β subunit[4,5] and is stabilized through interaction of its propionic acid groups with Tyr135 and Arg139 residues of the β subunit.[6]

Binding of nitric oxide to the heme iron triggers a series of intramolecular transformations that result in a several-hundred-fold activation of the enzyme's cGMP productivity.[7] The immediate product is a transient hexacoordinated heme-adduct, which is rapidly converted into a pentacoordinated nitrosyl-heme due to the cleavage of the coordination with proximal His105 ligand.[8] Formation of this pentacoordinated nitrosyl heme coincides in time with the increased output of cGMP, suggesting that scission of the Fe−N_{his} bond is the key event in the transition to the high-output NO-activated mode.[8] The enzyme in this state could be regarded as being in a high-output mode.

† Abbreviations used: sGC, soluble guanylyl cyclase; NO, nitric oxide; SNP, sodium nitroprusside; NOS, nitric oxide synthase; BAY-41-2272, 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]pyrimidin-4-ylamine; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole; cGMP, cyclic guanosine 3′,5′-monophosphate; IBMX, 3-isobutyl-1-methylxanthine.

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The enzyme is also activated 2−4-fold by carbon monoxide.[7] Binding of CO to the heme, however, results only in the formation of hexacoordinated carbonyl heme with preserved coordination with His105 residue and CO−sGC in the low-output mode; i.e., the hexacoordinated CO adduct is more stable than the corresponding NO adduct. This state of lower activation could be regarded as a low-output mode of the enzyme.

Of most interest in the present work is the fact that certain synthetic compounds, such as the originally discovered YC-1[9] and its subsequently discovered analogue, BAY-41-2272,[10] act as allosteric regulators, producing weak activation (5−10-fold) of sGC, again, a low-output mode. Interestingly, *combined* treatment with carbon monoxide and structurally related allosteric activators YC-1 or BAY-41-2272 strongly induces the enzyme, which displays a specific activity approaching that of the NO-stimulated sGC, suggesting that these allosteric regulators convert low-output CO−sGC enzyme into the high-output enzyme. While it was initially suggested that increased affinity of CO for the heme in the presence of YC-1 was at least partially responsible for the stimulation,[11,12] other studies found no changes in the on- and off-rates of CO binding in the presence of YC-1.[13] Moreover, YC-1 activator has a synergistic effect *even at saturating concentrations* of CO, when the increased CO affinity should not affect the enzyme activity.

The relationship between the coordination state of CO−heme and the increased enzymatic activity upon exposure to allosteric regulators is of direct interest and can be effectively probed by spectroscopic techniques, especially resonance Raman (RR) and Fourier transform infrared (FTIR). An early RR study of the YC-1 effect on the state of the CO−heme adduct suggested that CO binding stimulated activity, even though the RR data were interpreted to indicate that the carbonyl heme remained in a hexacoordinated state.[14] However, a later study, using a combined RR and infrared spectroscopy approach, presented direct spectroscopic evidence for a 5-coordinate heme CO fragment, implying that YC-1 facilitates the release of the proximal βHis105 residue;[15] these authors also noted that the presence of the 5-coordinate signature band in the spectra obtained in the earlier work[14] had gone unnoticed. More recent work by Kitagawa and co-workers[16] confirms the existence of both forms of CO adduct in the presence of YC-1. In any case, on the basis of the very weak RR and IR intensities for the 5-coordinate species observed in those studies, it appears that the addition of YC-1 causes only a very small fraction of the hexacoordinated Fe−CO adduct to convert to the active 5-coordinate form.

In the present work, employing a newly discovered stronger allosteric effector, BAY-41-2272,[10] RR and IR spectroscopic techniques are used to investigate the structure−function relationships for these types of effectors and to better define the structural changes occurring at the active site. The results of the spectroscopic measurements demonstrate that the CO adduct of sGC exists in two (6-coordinate) forms and that, upon the addition of BAY-41-2272, three types of CO adducts are present. One is apparently identical to the major 6-coordinate form present in the absence of BAY-41-2272, while a second 6-coordinate form is slightly different than those present in the absence of BAY-41-2272; a distinctly different form arises in the presence of BAY-41-2272 that exhibits vibrational bands characteristic of a 5-coordinate CO-adduct.

Material and Methods

Protein Preparation. Four liters of Sf9 cells was cultured at 27 °C in Grace medium with 10% (v/v) fetal calf serum, 1% (v/v) Pluronic 68, and 1% of penicillin and streptomycin. At a culture density of 2.0 $\times$ 10^6 cells/mL, baculoviruses carrying the α and β subunits of human sGC[17] were added to the culture at a multiplicity of infection of 5 and harvested 72 h postinfection by centrifugation. The cells were resuspended in the lysis buffer (25 mM triethanolamine (TEA), pH 7.5, 10% glycerol, 4 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 4 mM GSH, 1 mM PMSF, and 5 mg/mL each of pepstatin A, leupeptin, aprotinin, and chymostatin). After sonication, the lysate was centrifuged at 100 000*g* for 1 h and the supernatant fraction was loaded on a 20 mL column with DEAE-FF Sepharose (Amersham). After washing the column with 90 mL of buffer, the enzyme was eluted with a linear 0−100% gradient of buffer A (25 mM TEA pH 7.5, 10% glycerol and 500 mM NaCl). Yellow fractions containing sGC activity were loaded on a 30 mL column with His-Bind resin (Novagen) and washed with buffer A containing 50 mM imidazole. His-tagged sGC heterodimer was eluted with 150 mM imidazole in 25 mM TEA buffer, pH 7.4, with 10% glycerol. Yellow fractions containing sGC were pooled, supplied with 2 mM GSH, and loaded on a 5 mL HiTrap DEAE-FF Sepharose column (Amersham Pharmacia Biotech). The column was washed with 25 mM TEA, pH 7.5, 10% glycerol, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, and 2 mM GSH and eluted with the same buffer containing 250 mM NaCl. Alternatively, to prepare the enzyme in D_2O buffer for the FTIR measurements, the 5 mL HiTrap column was washed and eluted with buffers prepared with D_2O. The enzyme obtained at this stage (>95% purity) was used for experiments. The purity of the enzyme was tested by Coomassie staining of the preparation displayed on SDS−PAGE as shown in Figure 1A. Heme spectra were measured, and the heme concentration in the preparation was estimated on the basis of $\epsilon_{431} = 130\ mM^{-1}\ cm^{-1}$.[18] Previously performed pyridine−hemochromagen measurements[17] of heme content in similar sGC preparations showed a good correlation with the spectral measurement and was consistent with 0.9 heme moiety per sGC heterodimer. The purified enzyme was then concentrated on Amicon 30 concentrator (Millipore) to a concentration of 30 μM heme for Raman resonance studies or 130−150 μM heme for IR spectroscopy.

Anaerobic sGC sample was prepared by passing a stream of argon for at least 10 min over 200 μL of enzyme sealed in a gastight vial. To prepare the gaseous ligand-bound enzymes, a stream of pure NO or CO gas was passed over the anaerobic sGC until a change in coloration of the enzymes was evident (typically ~3−5 s). To measure the IR or RR spectra of these enzymes, the samples were transferred anaerobically with a gastight syringe.

Assay of sGC Activity. Enzyme activity was assayed by formation of [^{32}P]cGMP from α[^{32}P]GTP at 37 °C as described previously.[17] The final concentration of dimethyl sulfoxide (DMSO), which is used as a

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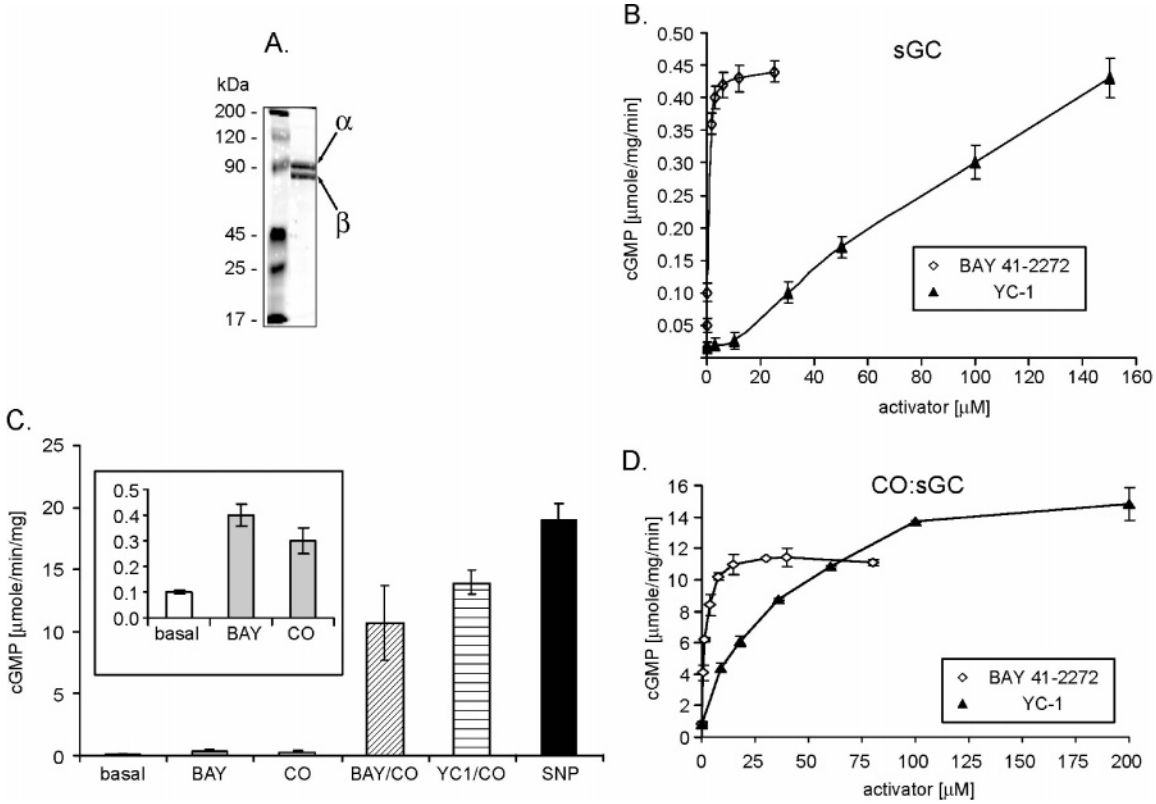

Figure 1. Activation of sGC by gaseous and allosteric activators. sGC enzyme purified to homogeneity was tested for activity under various stimulation. (A) Coomassie blue staining of 3 μg of purified sGC (right lane) and molecular weight marker (left lane). (B) Activation of sGC enzyme by various concentrations of YC-1 (triangles) or BAY-41-2272 (diamonds). Data of three independent measurements performed in triplicates are presented as the mean $\pm$ SD. (C) Comparison of sGC activity at 37 °C tested either aerobically, in the presence of 100 μM sodium nitroprusside (SNP), 5 μM BAY-41-2272, or without additives (basal), or anaerobically, with CO-saturated buffers with and without 5 μM BAY-41-2272 or 100 μM YC-1. Data are presented as the mean $\pm$ SD of three independent measurements performed in triplicate for basal, CO, CO/YC-1, and SNP assays and of five independent measurements performed in triplicate for CO/BAY-41-2272 combination. (D) Activation of CO−sGC enzyme at various concentrations of allosteric activators YC-1 (triangles) and BAY-41-2272 (diamonds). Data from one representative experiment ($n = 3$) performed in triplicates are presented as mean $\pm$ SD.

vehicle for BAY-41-2272 and YC-1 stock solutions, was not higher than 0.1% for BAY-41-2272 and 2% for YC-1 assays. These concentrations of DMSO do not affect the sGC activity. Activity of CO-treated enzyme was measured in gastight vials using CO-equilibrated reaction buffer.

FTIR Difference Spectroscopy. The FTIR spectra were measured on a Nicolet Magna 870 infrared spectrophotometer, using an FTIR cell with CaF_2 windows and a 75-μm spacer. Spectra were collected at 4 cm^{-1} spectral resolution. The sample holder (Aldrich, Milwaukee, WI) was modified to allow liquid from a constant-temperature bath to circulate around the holder, ensuring that the protein solutions were kept at a constant temperature of 15 °C. To measure the effects of temperature on the CO vibrational modes, the temperature of the sample holder was varied between 15 and 35 °C. The spectra were recorded in blocks of 250 scans, and two such spectra were added to obtain the final spectrum. Before adding the blocks, the spectra were subtracted from one another to ensure that no differences were observed between the spectra. The frequencies reported for the CO stretching vibrational mode were determined by curve fitting the band with an assumed Gaussian function.

Resonance Raman Measurements. The RR spectra were obtained with a Spex 1269 spectrometer (with slit widths set at 20−50 μm) equipped with a Princeton Instruments ICCD-576 UV-enhanced detector. The excitation lines employed were the 406.7 nm line from a Spectra-Physics Model 100-K3 krypton ion laser and the 442 nm line from a Liconix Model 4240NB helium−cadmium laser. The laser power at the sample was ~3 mW, using a cylindrical lens to form a line image that reduces effective photon flux at the sample to avoid photolysis;[19] before spectral acquisition, the RR spectrum in the ν_4 region was monitored to confirm lack of photolysis. The spectra were calibrated with indene (high frequency) or fenchone (low frequency) and processed with Grams 32 software (Thermo Electron Corporation). A 442 or 413 nm Notch filter (Kaiser Optical) was used to remove the Rayleigh scattered line during the spectral measurement. The NMR tube was spun and a 180° backscattering geometry was employed. During measurements the spinning NMR tubes were positioned in a low-temperature Dewar cell of in-house design, which allows the spinning tube to be immersed in a bath of circulating cold water whose temperature was measured by a thermometer; note that although the laser excitation beam traverses the circulating bath before striking the sample tube, Raman signals attributable to the water from the bath were too weak to be observed and did not interfere with the measurements.

Results and Discussion

Activation of sGC Enzyme: YC-1 vs BAY-41-2272. Figure 1B compares the activity of sGC−CO complex in the presence of two effectors YC-1 and BAY-41-2272, showing that it is not possible to achieve saturation with YC-1 within the solubility range of the compound (up to 200 μM) in aqueous solutions. However, the newly described allosteric regulator, BAY-41-2272, achieves half-maximal activation at the relatively low concentration of 0.5−1.5 μM. Note, however, that even at maximal activation (>5 μM BAY-41-2272) the activity of sGC enzyme is only a small fraction of sGC activity in the presence

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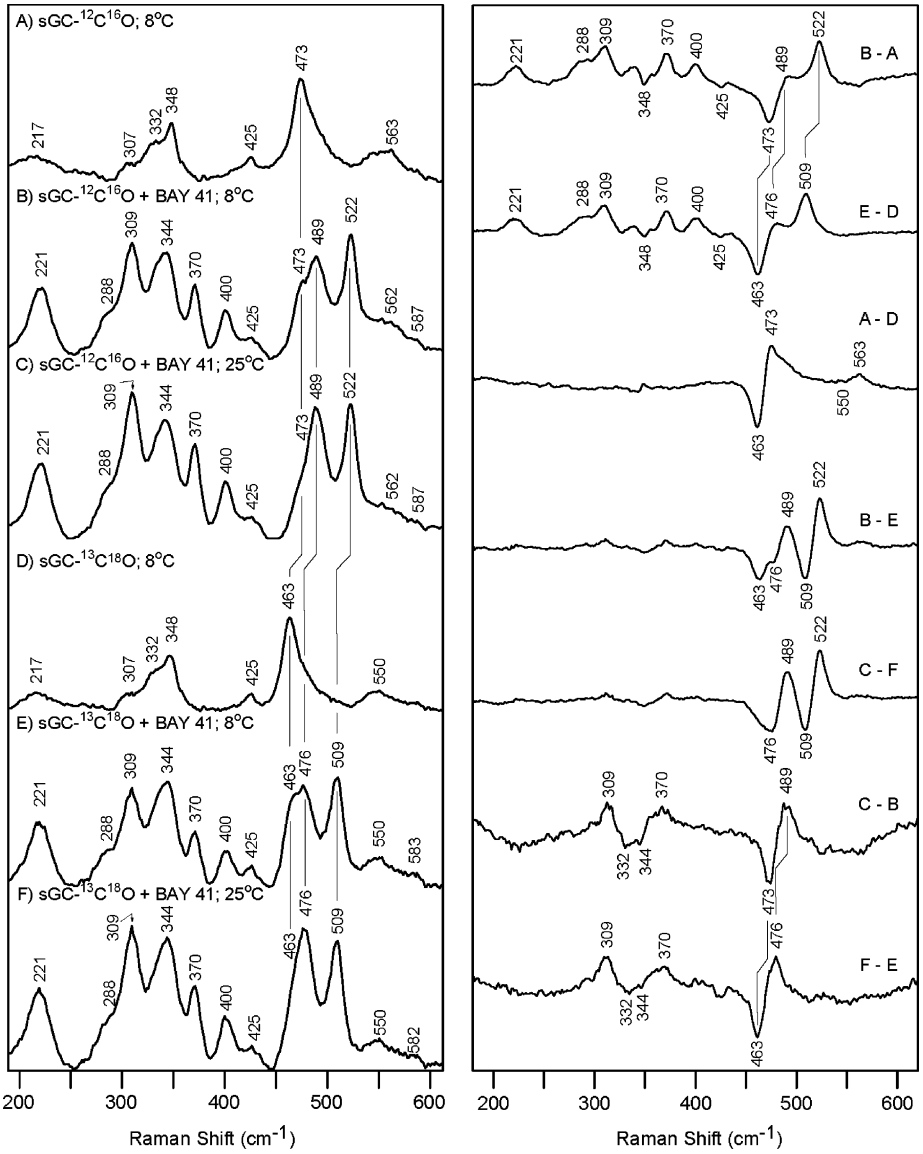

Figure 2. Effects of BAY-41-2272 on resonance Raman frequencies of sGC. sGC (30 μM) in 50 mM TEA, pH 7.4, 2.5 mM $MgCl_2$, and 4 mM glutathione was exposed to CO and used for measurements. (Left panel) Low-frequency spectra of sGC enzyme exposed to $^{12}C^{16}O$ (traces A−C) or $^{13}C^{18}O$ (traces D−F) gas in the absence or presence (traces B, C, E, F) of 150 μM BAY-41-2272. The measurements were recorded at 8 °C (traces A, B, D, E) or 25 °C (traces C and F). (Right panel) Difference spectra of presented traces.

of NO (data shown in Figure 1 C). Combined BAY-41-2272/CO treatment is necessary to induce activity comparable to NO activation (Figure 1C). Although both YC-1 and BAY-41-2272 compounds stimulate the CO−sGC enzyme to similar levels (70% and 50% of the NO-stimulated activity), only low concentrations of BAY-41-2272 are required to produce the maximal effect (Figure 1D).

Fe−CO and CO Stretching Vibrations of sGC−CO. Following two early RR studies[20,21] that reported results for sGC samples that apparently contained fractions of a 6-coordinate, low-spin form of the protein in addition to a 5-coordinate high-spin form, Babcock and co-workers showed that a preparation containing a pure 5-coordinate, high-spin protein exhibited an iron−histidine stretching frequency, ν(Fe−N_{his}), at 205 cm^{-1} and yielded two forms of 6-coordinate, low-spin CO adducts upon exposure to CO; the dominant form exhibited a ν(Fe−CO) stretching mode at 473 cm^{-1}, while a minor component was observed at 487 cm^{-1}.[22] Very recently, Kitagawa and co-workers have confirmed the existence of the major and minor forms of the CO adduct.[16]

The present study was undertaken to determine the nature of the CO−heme adduct in the high-output form of sGC by analysis of the RR and FTIR spectra of purified human enzyme after treatment with CO and BAY-41-2272 activator. Specifically, attention is focused on changes in the ν(Fe−CO) and ν(C−O) modes after the addition of BAY-41-2272. In the absence of BAY-41-2272, the dominant ν(Fe−CO) vibration was found to occur at 473 cm^{-1}, along with a weak high-frequency shoulder near 489 cm^{-1} (Figure 2, trace A), which agrees well with the previously reported values.[15,16,22,23] This assignment

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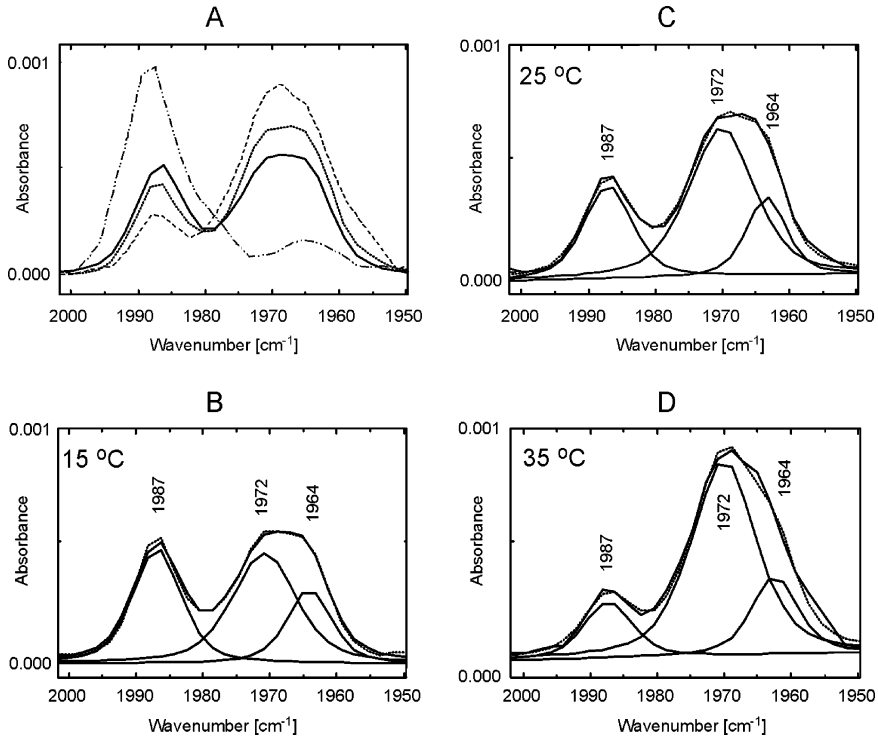

Figure 3. (A) FTIR spectra of the CO stretching vibrations of sGC in the presence of CO (dashed dotted line) and CO:BAY at 15 °C (solid line), 25 °C (small dashes) and 35 °C (large dashes). The CO stretching vibrations of CO−sGC:BAY has been deconvoluted into three bands at 1987, 1972, and 1964 cm^{-1}, using a Gaussian fitting function, at (B) 15 °C, (C) 25 °C, and (D) 35 °C.

was also supported by the isotopic shift to 463 cm^{-1}, with a high frequency shoulder near 476 cm^{-1}, when the heavier $^{13}C^{18}O$ ligand was used (Figure 2, traces D and A−D). It is also noted that in the FTIR spectrum, shown as the dotted−dash line (- • • -) line in panel A of Figure 3, a weaker ν(C−O) shoulder is observed at about 1979 cm^{-1} in addition to the intense band seen at 1987 cm^{-1}; i.e., the dominant form exhibits the ν(Fe−CO)/ν(C−O) modes at 473/1987 cm^{-1}, while the minor form shows these at 489/1979 cm^{-1}.

We wish to note here that some discrepancies exist in the number and frequencies of the ν(C−O) modes reported for CO−sGC (with no effectors added) in previous reports. In the present work, (in the absence of BAY-41-2272) a very weak feature occurring near 1964 cm^{-1} is seen occasionally (one spectrum out of four). In two previous works from one laboratory, a feature was seen near 1965 cm^{-1} (in the RR spectrum) in one report[14] but was not observed in the IR spectrum in an earlier report.[24] In more recent work, a relatively strong band can be seen at 1968 cm^{-1} in the reported IR spectra,[15] but its presence is difficult to understand, given the small (or nonexistent) intensities reported in other works. Furthermore, in a very recent work that mainly focused on RR results, no strong evidence was obtained for the existence of a ν(C−O) band near 1965 cm^{-1} in the absence of added effectors.[16] Thus, in the absence of any additional evidence, the presence of a band near1964−1968 cm^{-1} (in the absence of effectors) is assumed to arise from a spectral artifact.

Addition of BAY-41-2272 induces apparent structural changes that result in observed changes in the RR and FTIR spectra. In contrast to the previously reported RR studies with YC-1, where only a very weak feature appeared at 522 cm^{-1},[14,15] addition of BAY-41-2272 resulted in the appearance of a relatively strong feature at 522 cm^{-1}, a significant decrease in the intensity of the 473 cm^{-1} band, and an apparent (vide infra) small increase in the intensity of the 489 cm^{-1} feature (Figure 2, traces B and B−A). When $^{13}C^{18}O$ was used, addition of BAY-41-2272 diminished the 463 cm^{-1} ν(Fe−CO) band and produced the new band at 509 cm^{-1}, along with only a slight increase in intensity in the region near 476 cm^{-1} (Figure 2, traces E and E−D). This isotopic shift also confirmed the assignments of the newly observed band as a ν(Fe−CO) vibrational mode.

Although the frequencies of the bands observed in the presence of BAY-41-2272 are almost identical to the frequencies of the bands observed in the presence of YC-1,[15] the intensities of the bands are significantly different. While studies with YC-1 showed a major ν(Fe−CO) band at 488 cm^{-1} and a minor band at 521 cm^{-1},[14,15] in our studies this latter band (522 cm^{-1}) is of much greater intensity (see difference traces, B−A (or E−D), in Figure 2). It is also noted that in the FTIR spectra, upon addition of BAY-41-2272, the intensity of the 1987 cm^{-1} band decreases substantially and a new broad band appears with a maximum near 1970 cm^{-1} (see panel B of Figure 3). As is shown in panel B of Figure 3, the broad envelope centered near 1970 cm^{-1} is apparently comprised of two bands exhibiting frequency maxima at 1972 and 1964 cm^{-1}; as explained below, the relative contributions of these two components apparently change with temperature, an observation consistent with changes seen in the RR data acquired over a narrower temperature range.

In summary of the spectroscopic studies probing the effects of BAY-41-2272 addition on the vibrational modes of the FeCO fragment, in the absence of the BAY compound, the CO-bound sGC protein has a dominant form exhibiting ν(Fe−C) and ν-

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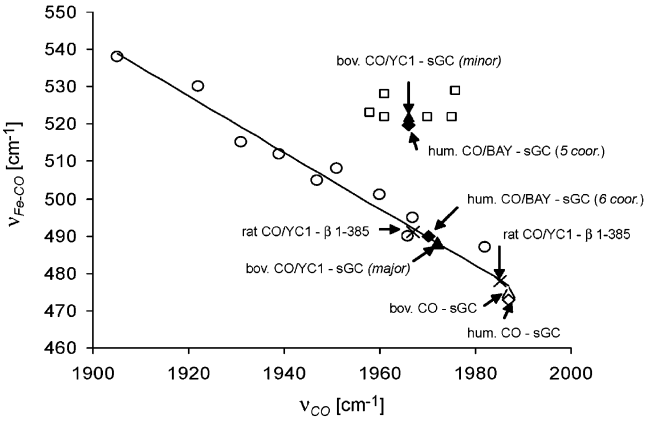

Figure 4. Correlation plot of ν(CO) versus ν(Fe−CO) for CO adducts of sGC and other selected hemes and hemoproteins. The opened circles are the data of 6-coordinated CO adducts;[31] the opened squares are the data for the 5-coordinated CO adducts.[31−33] Data points for the CO complex with various sGC enzymes: (x) CO adduct of rat recombinant β1−385 fragment with and without YC-1;[14] (△) bovine CO−sGC;[15] (▲) major (6-coordinated) and minor (5-coordinated) CO adduct of bovine sGC in the presence of YC-1;[15] (◇) CO-adduct of recombinant human sGC (this report); (◆) CO adducts (5- and 6-coordinate forms) of recombinant human sGC in the presence of BAY-41-2272 (this report).

(C−O) modes at 473/1987 cm^{-1} and a minor form with 489/1979 cm^{-1} bands. Both of these forms have vibrational signatures characteristic of 6-coordinate CO complexes with a neutral imidazole in the trans position; note that both pairs of frequencies fit well (Figure 4) with the line plotted for many imidazole-ligated 6-coordinate CO complexes. In the presence of BAY-41-2272, three forms of CO complex are present. One form exhibits frequencies (473/1987 cm^{-1}) identical to the form that exists in the absence of BAY-41-2272; this form dominates at lower temperatures (i.e., trace B in Figure 3). The second 6-coordinate species observed in the presence of BAY-41-2272 is slightly different from the minor component observed in the absence of the BAY compound inasmuch as the ν(C−O) now appears at 1972 cm^{-1}, rather than 1979 cm^{-1}, although any corresponding shift in the ν(Fe−CO) mode is apparently too small to reliably detect (given the coexistence of the 473 cm^{-1} feature). Finally, of most importance, the addition of BAY-41-2272 induces formation of a species that exhibits the 522/1964 cm^{-1} set of vibrational bands; these two frequencies are characteristic of a 5-coordinate CO adduct, as can be seen by the positions of these points in the plot in Figure 4.

Interesting temperature effects are observed for the CO−sGC adduct in the presence of BAY-41-2272, as can be seen by careful inspection of Figures 2 and 3. Upon comparing traces B and C (and C−B difference trace) in Figure 2, an increase in temperature from 8 to 25 °C causes a redistribution of the component forms, with the intensity of the 489 cm^{-1} band increasing at the expense of the 473 cm^{-1} form; it is emphasized here that the decrease of the 473 cm^{-1} component does not lead to a further increase in the intensity of the 522 cm^{-1} component. Thus, the temperature increase leads only to formation of more of the second type of 6-coordinate heme Fe−CO species. This behavior is confirmed by the comparable differences observed for the $^{13}C^{18}O$ adducts shown in trace F−E.

Similarly, in the FTIR spectra shown in Figure 3, changes in the intensities of the ν(C−O) bands were also observed as the temperature was varied between 15 and 35 °C; i.e., the intensity of the band at 1987 cm^{-1} decreases with increasing temperature, while the broad band located at lower wavenumbers simultaneously increases. The shape of the envelope of bands near 1965−1975 cm^{-1} suggests that it is composed of two closely located bands, deconvolution indicating the presence of one band at 1972 cm^{-1} and another at 1964 cm^{-1} (panels B, C, and D) in Figure 3. While it is not reasonable to assume that the relative RR intensities reflect fractional populations, owing to selective enhancement issues, it is expected that the relative FTIR intensities could provide more reliable estimates. However, to extract accurate relative intensities from these overlapped IR spectral bands, higher quality data will be required. In fact, future efforts will be focused on conducting careful studies of both the FTIR spectral properties and the temperature dependence of the enzymatic activity in order to provide estimates of the relative activities of the various forms present.

Activation of Low-Frequency Heme Deformation Modes. Of special interest in this work is the observation of new heme bands associated with the activated complex; i.e., several low-frequency heme bands appear at 400, 370, 309, 288, and 221 cm^{-1} simultaneously with the appearance of the 489 and 522 cm^{-1} forms (see difference trace B−A in Figure 2). These are shown to be bands associated with the heme group and not the Fe−CO fragment by the fact that they occur at virtually the same frequencies in traces B−A and E−D of Figure 2 (where $^{13}C^{18}O$ was used for traces D and E). While definitive assignments of these modes will require documentation of shifts observed for protein containing specifically deuterated hemes, as has been accomplished for several other heme proteins,[25−27] tentative assignments are made here on the basis of results of those earlier works. The 400 and 370 cm^{-1} bands are almost certainly attributable to the modes usually assigned to "δ(vinyl)" and "δ(propionate)" deformation modes, respectively; though more recent work employing hemes bearing deuterated methyl substituents documents relatively large shifts in these modes, implying that they are more properly described as "pyrrole deformation" modes that contain contributions from substituent bending motions.[26] The appearance of the feature near 309 cm^{-1} is most reasonably attributed to activation of the γ_7 out-of-plane mode associated with movement of the methine carbons, an assignment that would be confirmed by significant ($\approx$7 cm^{-1}) shifts upon methane deuteration,[25−28] while the feature appearing near 288 cm^{-1} is tentatively assigned to the in-plane ν_{52} mode. While it is tempting to associate the appearance of a feature near 221 cm^{-1} with the ν(Fe−N_{his}) of a (presumably) photodissociated species, simultaneous measurements of the ν_4 region did not reveal substantial intensity near 1360 cm^{-1}, as would be the case if significant amounts of a 5-coordinate, high-spin species were being formed. Furthermore, very recent work from another laboratory, which appeared during the revision of the current work, confirms the appearance of a mode near this region for SGC bound with YC-1, GTP, and CO and, additionally, makes the point that photolytic transients of these CO adducts, generated with high peak power pulsed lasers, reveal the ν-

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($Fe-N_{his}$) at 204 cm^{-1}; i.e., the photolyzed transient has the same frequency as the native form.[29,30]

It is also noted that the 221 and 400 cm^{-1} bands are associated mainly with the 5-coordinate species, as can be verified by noting that they are not present in trace C−B; i.e., as the 473 cm^{-1} form converts to the 489 cm^{-1} form, these bands are not seen to increase (they track the behavior of the 522 cm^{-1} form). The 309 and 370 cm^{-1} bands may be present in the 5-coordinate form, but are definitely present in the 6-coordinated 489 cm^{-1} form, since they increase along with the 489 cm^{-1} band as the temperature is increased from 8 to 25 °C. The present work provides the first clear evidence for the activation of these low-frequency heme deformation modes (e.g., at 221 and 400 cm^{-1}) upon formation of the 5-coordinate form; in previous works employing YC-1, only very small amounts of the 5-coordinate form were produced. Future work will address the issue of the correspondence of these low-frequency modes of this 5-coordinate CO adduct with the 5-coordinate NO form.

Biological Implications. The present study employs powerful spectroscopic methods to document the coordination states and structures of the heme-signaling site of sGC. The results show that the relative activities of sGC preparations bound with different effectors are not correlated with populations of given coordination states. Thus, while YC-1 binding activates the CO-bound form of sGC to an extent comparable to the 5-coordinate NO activated enzyme, RR studies document little[15] or no[14] formation of a 5-coordinate CO−sGC. In fact, while the authors of the former paper clearly document the minimal formation of a 5-coordinate form, they did not actually state the implied conclusion that the major form in the presence of YC-1, a 6-coordinate form, must then be an *active* form; i.e., while NO binding activates the catalytic site by forming a 5-coordinate heme site, YC-1 activates the catalytic site by forming a 6-coordinate heme site that is apparently slightly different than the original 6-coordinate CO−sGC protein. In the present work, employing the BAY-41-2272 effector, which activates the CO-bound sGC to an extent comparable to the 5-coordinate NO-bound form (i.e., ∼50%), it is demonstrated that binding of the effector generates *both* a 5-coordinate form and a 6-coordinate form. It seems likely that both of these forms possess active catalytic sites, though their activities relative to each other and to the 6-coordinate YC-1-bound form or 5-coordinate NO-bound form are not known at this time. On the basis of the fact that the relative populations of the 5-coordinate and 6-coordinate forms of the BAY-41-2272-bound sGC are affected by temperature, and are readily monitored by vibrational spectroscopy as shown here, it will be possible to determine the relative activities of the two forms in future studies that simultaneously monitor the temperature dependence of the activity and the spectroscopically documented relative populations.

Summary

The CO−sGC complex exists as a mixture of major and minor 6-coordinate forms having FeCO fragments with slightly different vibrational frequencies. Binding of BAY-41-2272 at 15 °C results in the generation of two additional forms, one of which is a 5-coodinate form. The remaining CO/BAY-bound sGC consists of the initial 6-coordinate form and a slightly different 6-coordinate form. As the temperature is increased from 15 to 35 ° C, the fraction of 5-coordinate form does not change, but the first 6-coordinate form [having ν(Fe−CO) and ν(C−O) frequencies of 473 and 1987 cm^{-1}] converts to the second 6-coordinate form (having corresponding vibrational frequencies of 489 and 1972 cm^{-1}). The relative activities of these spectroscopically documented forms will be determined in future studies wherein the temperature dependence of the enzymatic activity will be analyzed in terms of the carefully determined fractional populations.

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