YC-1 Facilitates Release of the Proximal His Residue in the NO and CO Complexes of Soluble Guanylate Cyclase*

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The benzylindazole compound YC-1 has been shown to activate soluble guanylate cyclase by increasing the sensitivity toward NO and CO. Here we report the action of YC-1 on the coordination of CO- and NO-hemes in the enzyme and correlate the events with the activation of enzyme catalysis. A single YC-1-binding site on the heterodimeric enzyme was identified by equilibrium dialysis. To explore the affect of YC-1 on the NO-heme coordination, the six-coordinate NO complex of the enzyme was stabilized by dibromodeuteroheme substitution. Using the dibromodeuteroheme enzyme, YC-1 converted the six-coordinate NO-heme to a five-coordinate NO-heme with a characteristic EPR signal that differed from that in the absence of YC-1. These results revealed that YC-1 facilitated cleavage of the proximal His-iron bond and caused geometrical distortion of the five-coordinate NO-heme. Resonance Raman studies demonstrated the presence of two iron-CO stretch modes at 488 and 521 cm\(^{-1}\) specific to the YC-1-bound CO complex of the native enzyme. Together with the infrared C-O stretching measurements, we assigned the 488 cm\(^{-1}\) band to the iron-CO stretch of a five-coordinate CO-heme and the 521 cm\(^{-1}\) band to the iron-CO stretch of a five-coordinate NO-heme. These results indicate that YC-1 stimulates enzyme activity by weakening or cleaving the proximal His-iron bond in the CO complex as well as the NO complex.

Soluble guanylate cyclase (sGC),\(^1\) a proteoheme-containing hemoprotein, is a well characterized NO receptor involved in cell-cell signal transduction pathways associated with neural communication and vasodilation (1–7). sGC purified from the rat and bovine lung are heterodimeric proteins composed of \(\alpha\)- and \(\beta\)-subunits (8–10) and catalyze the conversion of GTP to cyclic 3',5'-guanosine monophosphate (cGMP) (8, 11–14). The enzyme is activated by as much as 200-fold upon NO binding to the heme prosthetic group (11–14). The enzyme contains a stoichiometric amount of heme bound to histidine 105 of the N-terminal region of the \(\beta\)-subunit through a weak His-iron bond (15–17). The C-terminal regions of the two subunits, which share sequence homology to the catalytic site of adenylate cyclases, are thought to comprise the catalytic domain. The weak proximal His-iron bond plays a crucial role in the ability of the enzyme to form an enzymatically active five-coordinate NO-heme. NO initially binds to the heme to form an inactive six-coordinate NO complex, which is then converted to an active five-coordinate NO complex leading to cleavage of the weak His-iron bond, thereby resulting in a 200-fold increase in activity above the basal level (18, 19). Although the formation of NO-heme is known to occur in two steps as described above, details for the activation of the catalytic domain coupled with NO binding remain elusive.

There has been much interest concerning the possible physiological role of CO in the activation of sGC, but its role as a signaling molecule remains uncertain because of its poor sGC-stimulating properties. Wu et al. (20) reported that a benzylindazole compound YC-1 (3-(5'-hydroxymethyl-3'-furyl)-1-benzylindazole; structure shown in Scheme 1) is a NO-independent activator of platelet sGC. Subsequent work indicated that YC-1 stimulates in vitro cyclase activity of the CO-bound enzyme to a level comparable with that of NO activation (21). Significant stimulation of the ferrous and the ferrous NO enzymes by YC-1 was also noted (21, 22). Despite the important observation of YC-1 sensitization of the enzyme toward NO and CO, no firm structural information concerning the binding of this molecule to the enzyme is available. For instance, some reports indicate that YC-1 binds to the N-terminal region of the \(\beta\)-subunit (22), whereas work using a newly discovered antiplatelet reagent suggests that the YC-1-binding site is located on the \(\alpha\)-subunit (23).

The effects of YC-1 on the CO-heme coordination of the enzyme have been examined by the resonance Raman method. The results indicated that although YC-1 altered the CO-heme coordination, the CO-heme of the YC-1-bound enzyme was in a six-coordinate state with a proximal ligand \(\text{trans} \) to CO (22). Therefore, the YC-1-dependent activation of the CO bound enzyme did not apparently couple to the cleavage of the proximal His-iron bond. However, analyses of CO recombination kinetics suggest that the proximal His-iron bond may weaken or be replaced by a different base upon YC-1 binding (24).

Although there is no evidence that an endogenous YC-1-like molecule plays a physiological role in regulating sGC activity, an investigation of its interaction with the enzyme will contribute to understanding mechanisms of regulation of the catalytic activity. In this study we attempted to solve the ambiguities

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§ The abbreviations used are: sGC, soluble guanylate cyclase; cGMP, cyclic 3',5'-guanosine monophosphate; EPR, electron paramagnetic resonance; SNAP, S-nitroso-N-acetylpenitino-3-trihenamlamine; SNAP, S-nitroso-N-acetylpenitino-3-trihenamlamine; HPLC, high performance liquid chromatography; T, tesla; MOPS, 3-(N-morpholino)propanesulfonic acid; DMF, dimethylformamide.
regarding the YC-1-dependent stimulation of the NO and CO complexes of sGC. To obtain clear evidence of YC-1-dependent changes in the NO coordination, we have prepared the stable six-coordinate NO-heme by dibromodeuteroheme substitution. The NO complex of the reconstituted enzyme contained a significant amount of the six-coordinate NO-heme when the NO complex was prepared at pH 8.3. Binding of YC-1 resulted in a complete loss of the six-coordinate NO-heme with the concomitant formation of a five-coordinate NO-heme. This clearly demonstrates that YC-1 binding facilitates the cleavage of the proximal His-iron bond. YC-1-dependent scission of the proximal His-iron bond was confirmed by vibrational spectroscopic studies on the native CO-bound enzyme. This is a first observation for the formation of a five-coordinate CO-heme and provides a molecular mechanism for the YC-1-dependent CO sensing function of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Red, Blue, and Yellow Agarose Resins—**Agarose resins with dye ligands were prepared by an epoxy coupling method (25). Packed Sepharose 4B (100 g; Amersham Biosciences) was suspended in 150 ml of 1 N NaOH solution containing 500 mg of NaBH₄ and 8 ml of 1.4-butane-diol-diglycidylether (Sigma-Aldrich). The gel was gently shaken for 5 h at 30 °C and was collected using a glass filter funnel and then washed with 3 liters of H₂O. The packed gel was suspended in 150 ml of 0.5 M sodium carbonate buffer, pH 12, and reacted with 1.2 g of Cibacron Brilliant Red 3B-A (Sigma-Aldrich), Cibacron Blue F3G-A (Fluka), or Cibacron Brilliant Yellow 3G-P (Sigma-Aldrich) for 15 h at 4 °C with gentle shaking. The resultant derivatized gels were suspended in 200 ml of sodium carbonate solution containing 0.2 M glycyne and gently agitation overnight at 30 °C to completely block any residual epoxy groups.

**Enzyme Purification—**Fresh bovine lung (5 kg) was minced and homogenized using a Waring blender in 12 liters of 50 m M TEA buffer, pH 7.6, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM EDTA, and 55 mM β-mercaptoethanol (buffer A). Protease inhibitors and β-mercaptoethanol were included in all the buffers throughout the purification unless stated otherwise. The homogenate was clarified by centrifugation at 13,500 × g for 20 min, and the supernatant was then mixed with 1.4 kg of DEAE cellulose A-500 (Seikagaku Kogyo, Tokyo, Japan) equilibrated with buffer A. The slurry was stirred for 1 h at 4 °C and collected by sedimentation. Subsequently, the resin was washed two times with buffer A and poured into a column. The enzyme was eluted with a 3.5-liter linear NaCl gradient of 0–3.5 mM in buffer A. The active fractions were concentrated and washed with 20 mM MOPS buffer, pH 7.6, using a Minicon concentrator (Millipore) and then was applied to a Red Sepharose column (5 × 50 cm) equilibrated with the MOPS buffer described above. The enzyme was eluted by a linear gradient of 0–4 mM NaCl. The pooled active enzyme, which was equilibrated with 20 mM MOPS buffer, pH 7.6, was adsorbed to a Yellow agarose column, and the protein was eluted by a linear gradient of 0–0.7 M ATP. The concentrated active fractions were further purified on a Superdex 200-10/30 column (2.6 × 60 cm; Amersham Biosciences) followed by a Source Q15 HPLC column (1.6 × 10 cm; Amersham Biosciences). sGC was purified to apparent homogeneity using a ceramic hydroxylapatite HPLC column (Bio-Rad), where the elution was carried out by increasing the phosphate concentration from 0 to 0.45 M at pH 7.6 in the absence of EDTA. The overall yield was about 20%. The resultant homogenous enzyme preparation was stored in liquid nitrogen until use.

**Dibromodeuteroheme IX-substituted Enzyme—**The apoenzyme was obtained by a previously described method (18) with the following modifications. The DEAE cellulose column fractions of crude homogenate treated at pH 8.5 were equilibrated with 20 mM MOPS buffer, pH 7.6, and applied to a Blue Sepharose column. When protein was eluted with a linear gradient of 0–0.4 mM NaCl, the cyclase activity was recovered as two peaks, one containing the holoenzyme and the other containing the apoenzyme. The apoenzyme was reconstituted with dibromodeuteroheme in 40 mM TEA buffer, pH 7.5, containing the protease inhibitor mixture described earlier supplemented with pepstatin A, leupeptin, and E64, under anaerobic conditions at 20 °C. The remaining purification steps were the same as those used for the native enzyme purification.

**Equilibrium Dialysis—**In equilibrium dialysis, 6% (v/v) DMF was added to the buffer to maintain the required concentration of the poorly water-soluble YC-1. A five-cell rotating equilibrium dialyzer (Spectrum) was used for equilibrium dialysis experiments. Chambers (250 μl) were separated by dialysis membrane with a cut-off of 14 kDa. One chamber was filled with the ferrous enzyme and the opposite chamber contained the desired amount of YC-1. In some cases, one chamber was filled with the ferrous enzyme and the desired amount of YC-1, and the opposite one contained the buffer solution alone. After introducing the sample (180 μl) into each chamber, the dialysis cells were rotated at a constant rate at 27 °C. The reaction achieved equilibrium within 5 h under these conditions. After dialysis for 6 h at 27 °C under constant rotation, the samples in each chamber were removed by a gas-tight syringe for quantitative analyses of YC-1. An aliquot of the enzyme solution was used to determine the heme concentration and to check the integrity of the enzyme. After dialysis, ~85% of the enzyme was recovered as the active form with the same optical spectra and SDS-PAGE profile as the native enzyme. The samples removed from both chambers were diluted with a 2-fold volume of DMF to prevent the adsorption of YC-1 on the inner surface of sample cup. The concentration of YC-1 was determined by HPLC using a C18 column at a constant flow rate of 1 ml/min of 50% (v/v) methanol. The amount of enzyme-bound YC-1 was calculated from the difference in the concentration between the two chambers with and without enzyme.

**Activity Measurements—**The enzyme activity was measured as described previously (18). In brief, the assays were conducted in 50 mM HEPES buffer, pH 7.5, supplemented with 5 mM MgCl₂, 1 mM GTP in a final volume of 235 μl, and 10 μl of the enzyme was added to the mixture. The reaction was started by the addition of 5 μl of SNAP (2 mM) and was conducted for 10 min at 37 °C. After terminating the reaction by addition of 10 μl of 30% (v/v) acetic acid, cGMP was determined by HPLC as described previously (18). For the inhibition by CO, the assay mixture was saturated with CO gas prior to the addition of the enzyme.

**EctoPhosphorase—**Reducing SDS-PAGE was carried out using an 8% acrylamide running gel. The protein was visualized using either Coomassie Brilliant Blue or silver stain (Daiichi Chemical Co., Tokyo, Japan).
protein consisting of the enzyme was a heterodimeric enzyme under anaerobic conditions.

Reagents—GTP and cGMP were obtained from Wako Pure Chemical Inc. (Tokyo, Japan). Research grade NO and CO were obtained from Takachiho Chemical Co. (Tokyo, Japan). YC-1 and SNAP were purchased from ALEXIS (San Diego, CA). Dibromodeuteroheme was prepared according to the method of Seybert et al. (26). Other chemicals, purchased from Nacalai Tesque Co. (Kyoto, Japan), were of the highest commercial grade and were used without further purification.

RESULTS

Properties of Native and Dibromodeuteroheme-substituted Enzymes—The homogenous native enzyme exhibited a NO-dependent activity of 27–30 μmol/min/mg protein at 37 °C in the presence of Mg^{2+}. This activity corresponded to a turnover of about 3,800 min^{-1} (μmol of GMP/min/μmol of heme). SDS-PAGE analyses indicated that the enzyme was a heterodimeric protein consisting of the α-subunit of 78 kDa and the β-subunit of 70 kDa. The enzyme contained 0.95 protoheme IX/heterodimer, in which the protein and heme were determined by a modified biuret method (27) and by the pyridine hemochromogen method (28), respectively. The optical spectra of the ferrous and the ferrous NO enzymes were identical to previous results (18).

The dibromodeuteroheme-substituted enzyme had a subunit structure identical to that of the native enzyme (data not shown). The reconstituted enzyme in the ferrous state exhibited the Soret band at 426 nm and the visible absorption at 553 nm, indicative of a five-coordinate high spin state (Fig. 1). The addition of NO yielded the NO complexes with an intense Soret band at 426 nm and the visible absorption at 553 nm, indicative of a five-coordinate high spin state (Fig. 1). The enzyme under anaerobic conditions.

Effects of pH and YC-1 on Cyclase Activities—When guanylate cyclase activities were measured at pH 7.5, YC-1 enhanced the activity of the native NO-bound enzyme by about 1.1-fold (Fig. 3), confirming previous results (21, 22). The level of the stimulation by YC-1 is significantly higher at pH 8.3 (1.24-fold) than at pH 7.5. Similar experiments performed using the dibromodeuteroheme-substituted enzyme provide a clear mechanism of action for YC-1 (see Fig. 3). In this case, YC-1 stimulates the cyclase activity by 1.28-fold at pH 7.5 and by 2-fold at pH 8.3, suggesting that the NO complex of dibromodeuteroheme-substituted enzyme is largely made up of the catalytically inactive six-coordinate NO-heme. The activities of the reconstituted enzyme at both pH 7.5 and 8.3 are about 60% of those of the native enzyme even in the presence of YC-1. The reasons for heme-dependent changes in the activity are unknown.

Optical Spectral Characterization of Ferrous NO Complexes—Fig. 4 shows the optical absorption spectra of the NO complex of the native and substituted enzymes. As demonstrated in Fig. 4, the NO complex of native enzyme at pH 8.3 comprises a small amount of six-coordinate NO-heme detected as a shoulder around 420 nm (18). The disappearance of the shoulder at 420 nm and the enhanced 399-nm band after binding of YC-1 indicates conversion of the six-coordinate NO-heme to the five-coordinate NO-heme (Fig. 4A, spectrum c). As shown in Fig. 4B, the NO complex of dibromodeuteroheme-substituted enzyme exhibited two Soret bands at 393 and 410 nm. The 410-nm band was assigned as the six-coordinate NO complex of the substituted enzyme, as established by EPR spectroscopy described below. The effects of pH and YC-1 binding on the optical spectra of the NO complexes were consistent with the activity measurements, in which the inactive six-coordinate NO-heme accumulated at pH 8.3. To determine the coordination states of the NO-hemes, both NO-ligated forms of the native and the reconstituted enzymes were rapidly frozen and then analyzed by EPR spectroscopy.
At pH 8.3, a weak EPR signal at The spectral features agreed with the previous reports (18, 30). A new five-coordinate NO-heme was characterized by three new five-coordinate NO-heme signal with rhombic symmetry. The 14N16O complex of the native enzyme in the absence of YC-1 exhibits characteristic EPR signals of axially symmetric five-coordinate NO-heme (g₁ = 2.069 and g₂ = 2.009) with a triplet 14NO hyperfine splitting (Fig. 5). The spectral features agreed with the previous reports (18, 30). At pH 8.3, a weak EPR signal at g = 1.976 was caused by the six-coordinate NO-heme in the absence of addition to EPR at pH 8.3, containing 4% (v/v) DMF. When desired, 120 μM YC-1 was added to the mixture. The reaction was started by the addition of SNAP and conducted at 37 °C for 10 min. The enzyme activity was expressed as turnover number (μmol of cGMP formed/min/μmol of heme). In the inset, the structure of dibromodeuteroheme is illustrated. Di-BR sGC in the figure donates dibromodeuteroheme-reconstituted sGC.

EPR Characterization of Five- and Six-coordinate NO-hemes—The 14N16O complex of the native enzyme in the absence of YC-1 exhibits characteristic EPR signals of axially symmetric five-coordinate NO-heme (g₁ = 2.069 and g₂ = 2.009) with a triplet 14NO hyperfine splitting (Fig. 5). The spectral features agreed with the previous reports (18, 30). At pH 8.3, a weak EPR signal at g = 1.976 caused by the six-coordinate NO-heme in the absence of addition to EPR at pH 8.3, containing 4% (v/v) DMF. When desired, 120 μM YC-1 was added to the mixture. The reaction was started by the addition of SNAP and conducted at 37 °C for 10 min. The enzyme activity was expressed as turnover number (μmol of cGMP formed/min/μmol of heme). In the inset, the structure of dibromodeuteroheme is illustrated. Di-BR sGC in the figure donates dibromodeuteroheme-reconstituted sGC.

InfraRed and Resonance Raman Spectra—YC-1 stimulated the cyclase activity of the CO-bound enzyme from 0.8 to 27 μmol/min/mg protein, as reported previously (21, 22). The YC-1-induced changes in the iron-CO stretching vibration (νFe-CO) have been reported (22), but precise analyses of the C-O stretching vibration (νC=O) and of the effects of YC-1 on the νFe-CO were not carried out (22, 31). The infrared spectra of the 12C16O complex under various conditions are summarized in Fig. 6. We found a sharp band at 1987 cm⁻¹ and a broad band centered at 1968 cm⁻¹ both at 15 and 25 °C in the absence of YC-1 (Fig. 6, traces A and B). The bands at 1987 and 1968 cm⁻¹ shifted to 1943 and 1924 cm⁻¹ upon 13C16O substitution, respectively, indicating that they can be assigned to the νFe-CO mode (data not shown). At 15 °C, binding of YC-1 slightly diminished the 1987-cm⁻¹ band and resulted in a small enhancement of the 1972-cm⁻¹ band (Fig. 6, trace C). Raising the temperature up to 25 or 32 °C markedly intensified the band at 1972 cm⁻¹ with the appearance of a shoulder at 1965 cm⁻¹ (Fig. 6, traces D and E). The temperature-dependent changes were reversible. The shoulder at 1965 cm⁻¹ is also seen in the difference spectrum (Fig. 6, inset). No additional CO stretch band was detectable even when the concentration of sGC and YC-1 was increased (Fig. 6, trace F). These data demonstrate that there are two types of CO complex in the presence of YC-1, one with a 1972-cm⁻¹ band and the other with a 1965-cm⁻¹ band. The effect of YC-1 on the formation of the two CO complexes is more apparent at elevated temperatures, suggesting an increase in affinity of YC-1 at a higher temperature.

To determine the identity of these two CO species, we searched the νFe-CO mode by resonance Raman spectroscopy. The νFe-CO and the iron-carbon-oxygen bending vibration (νFe-C-O) of the CO complex in the absence of YC-1 have been reported to be 472 and 562 cm⁻¹, respectively (17). The corresponding Raman bands in our experiments were at 475 and 565 cm⁻¹.
YC-1 Binding to Guanylate Cyclase

Fig. 5. EPR spectra of the $^{14}$N$^1$O complex of native and dibromodeuteroheme-reconstituted sGC. Trace a, EPR spectrum of the $^{14}$N$^1$O complex of native sGC in the absence of YC-1 at pH 7.5; trace b, native sGC in the absence of YC-1 at pH 8.3; trace c, native sGC in the presence of YC-1 at pH 8.3; trace d, dibromodeuteroheme-reconstituted sGC in the presence of YC-1 at pH 8.3; trace e, dibromodeuteroheme-reconstituted sGC in the presence of YC-1 at pH 8.3; trace f, native sGC in the presence of 3 mM Ca$^{2+}$ and 2.5 mM GTP. EPR spectra were taken at 77 K and by 100 kHz field modulation with 0.5 millitesla width. The microwave power was 5 mW. The enzyme concentrations were 35 µM as with heme. In these experiments, 40 scans were averaged. Other experimental conditions are described in the legend of Fig. 4. Di-Br sGC in the figure denotes dibromodeuteroheme-reconstituted sGC.

512, and 509 cm$^{-1}$ by $^{12}$C$^{15}$O, $^{13}$C$^{16}$O, and $^{13}$C$^{18}$O respectively (Fig. 7, traces b–e). This monotonous frequency shift as the mass of CO increases assigns this band to the $v_{PCO}$ mode. Furthermore, this Raman band is not observed in the spectrum of the CO complex in the absence of YC-1 (Fig. 7, trace a) and is intensified by increasing the YC-1 concentration (Fig. 7, inset). These reveal that YC-1 generates another CO adduct distinct from the major CO adduct. After this, we designate this CO adduct with YC-1 as the minor CO adduct. The shoulder at 1965 cm$^{-1}$ is a candidate for the $v_{C-O}$ mode of the minor CO adduct, because there are no other YC-1-sensitive infrared bands in the CO stretching region. Comparison of the $v_{C-O}$ infrared band with the $v_{PCO}$ Raman band under similar conditions enables us to assign the $v_{C-O}$ mode of the minor CO adduct. The noticeable difference in the shape between the $v_{C-O}$ and the $v_{PCO}$ modes (Figs. 7, inset, and 6, trace F) is because the former mode has a shoulder at 1965 cm$^{-1}$, whereas the latter exhibits a band at 521 cm$^{-1}$. Excluding the difference, the $v_{C-O}$ mode can be superposed on the $v_{PCO}$ mode, indicating that the 1965-cm$^{-1}$ band should be assigned to the $v_{C-O}$ mode of the minor CO adduct. Given the mode assignments, the major CO adduct is characterized by the $v_{PCO}$ and $v_{C-O}$ modes at 488 and 1972 cm$^{-1}$, respectively, and the minor one is characterized by the $v_{PCO}$ at 521 cm$^{-1}$ and the $v_{C-O}$ at 1965 cm$^{-1}$.
YC-1 Binding to Guanylate Cyclase

The presence of YC-1 (100 mM NaCl, 5% (v/v) glycerol and 4% (v/v) DMF. All of the samples contained 6 (v/v) DMF including those without YC-1. The resonance curve between

\[ \text{Fe-CO} \] and the iron-carbon-oxygen bending vibration at ambient temperature.

Our recent findings and those of others show that the binding of NO to the ferrous sGC initially yields a short-lived six-coordinate NO complex that decays to the five-coordinate NO complex with a rhombic EPR signal is specific to GTP among purine nucleotides, because neither Mg\(^{2+}\) nor Ca\(^{2+}\)-ATP causes detectable changes in the NO-heme unit of sGC-NO in the presence of Ca\(^{2+}\)-GTP (Fig. 5, trace f). The formation of the five-coordinate NO complex with a rhombic EPR signal is specific to GTP among purine nucleotides, because neither Mg\(^{2+}\)-ATP nor Ca\(^{2+}\)-ATP causes detectable changes in the NO-heme unit. This result is puzzling because GTP-binding sites are known to bind exogenous sigma-donor ligands such as oxygen. Therefore, it is feasible that the introduction of electron withdrawing substituents strengthens the internal proximal His-iron bond primarily through \( \sigma \)-bonding interaction of the imidazole nitrogen-iron linkage. Indeed, heme derivatives with strong electron-withdrawing substituents are known to bind imidazole with a higher affinity.

Delocalization of the unpaired electron is known to be highly sensitive to the geometry of the iron-nitrogen-oxygen unit. By using the reconstituted enzyme, we solved the question of how YC-1 stimulates the NO-bound enzyme. Optical and EPR spectroscopic studies indicate that YC-1 facilitates the NO-induced dissociation of the proximal His ligand and yields a new five-coordinate NO-heme with a rhombic EPR signal. The EPR signal of the new NO-heme species with YC-1 closely resembles those of P-420-NO complexes or of some heme-model NO complexes (42–46). In the ferrous NO-heme adducts, bound NO has been known to adopt a bent iron-nitrogen-oxygen geometry. The increase in the \( g \) value anisotropy described for the NO-bound adduct of sGC with YC-1 closely resembles those of P-420-NO complexes or of some heme-model NO complexes (42–46). In the ferrous NO-heme adducts, bound NO has been known to adopt a bent iron-nitrogen-oxygen geometry (47). The increase in the \( g \) value anisotropy described for the NO-bound form of the five-coordinate NO complex of sGC indicates an increase in the delocalization of the unpaired electron residing on the p\( \pi^* \) of NO to iron d\( \pi \)-orbital (48). Delocalization of the unpaired electron is known to be highly sensitive to the geometry of the iron-nitrogen-oxygen unit (49, 50). On the basis of these considerations, we propose that YC-1 binding increases the iron-nitrogen-oxygen bond angle, thereby increasing the d\( \pi \)-p\( \pi^* \) overlap. The five-coordinate NO-heme with a rhombic EPR signal was also observed for sGC-NO in the presence of Ca\(^{2+}\)-GTP (Fig. 5, trace f). The formation of the five-coordinate NO complex with a rhombic EPR signal is specific to GTP among purine nucleotides, because neither Mg\(^{2+}\)-ATP nor Ca\(^{2+}\)-ATP causes detectable changes in the NO-heme unit. This result is puzzling because GTP-binding sites are located on the C-terminal catalytic domains of both \( \alpha \)- and \( \beta \)-subunits, whereas the YC-1-binding site is thought to be at the N-terminal side of the \( \beta \)-subunit. However, GTP may bind to a site other than the catalytic site and thereby serve as an effector molecule regulating heme reactivity.

**FIG. 7. Effects of YC-1 on resonance Raman frequencies of the iron-CO stretch and the iron-carbon-oxygen bending vibrations.** Trace \( a \), the resonance Raman spectrum of the ferrous \(^{12}\)C\(^{16}\)O sGC in the absence of YC-1. Trace \( b \), spectrum of the ferrous \(^{12}\)C\(^{16}\)O sGC in the presence of YC-1 (100 \( \mu \)M). Trace \( c \), ferrous \(^{13}\)C\(^{16}\)O sGC in the presence of YC-1 (100 \( \mu \)M). Trace \( d \), ferrous \(^{12}\)C\(^{18}\)O sGC in the presence of YC-1 (100 \( \mu \)M). In the inset, the iron-CO stretching vibration of the ferrous \(^{12}\)C\(^{16}\)O or \(^{13}\)C\(^{16}\)O sGC in the absence and presence of YC-1 (350 \( \mu \)M) is illustrated. The buffer used is 50 mM TEA buffer, pH 7.5, containing 50 mM NaCl, 5\% (v/v) glycerol and 4\% (v/v) DMF. All of the samples contained 6 (v/v) DMF including those without YC-1. The resonance Raman spectra were collected using an excitation wavelength of 413.1 nm at ambient temperature.

The difference in the CO coordination between the minor and major CO adducts can be assessed by the well defined correlation curve between \( \nu_{\text{CO}} \) and \( \nu_{\text{Fe-CO}} \) (32–36). As shown in Fig. 8, the data points of the YC-1-free CO complex and the major CO adduct correspond to the correlation curve but tend toward data points for a five-coordinate CO-heme (33, 37, 38). This provides the first clear evidence for the cleavage of the proximal His-iron bond in the CO complex induced by YC-1 and accounts for the YC-1-dependent CO responsiveness of the enzyme.

**FIG. 8. Plot of \( \nu_{\text{Fe-CO}} \) versus \( \nu_{\text{C-O}} \) frequencies for the CO adducts of sGC and for selected hemes and hemoproteins.** The open circles represent data of six-coordinate CO adducts taken from the data cited in Refs. 32, 33, and 35. The closed circles are for the CO-sGC without YC-1 and the major CO adduct of sGC with YC-1. The open squares represent five-coordinate CO adducts (33, 36, 37), and the closed square is for the minor CO adduct of sGC in the presence of YC-1.

The lower electron density on the heme-iron increases the affinity for exogenous sigma-donor ligands and decreases the affinity for exogenous sigma-acceptor ligands such as oxygen. Therefore, it is feasible that the introduction of electron withdrawing substituents strengthens the internal proximal His-iron bond primarily through sigma-bonding interaction of the imidazole nitrogen-iron linkage. Indeed, heme derivatives with strong electron-withdrawing substituents are known to bind imidazole with a higher affinity.
Denninger et al. (22) have reported that the heterodimeric sGC and the enzymatically inactive truncated β-subunit formed a six-coordinate CO-heme but not a five-coordinate CO-heme in the presence of YC-1. The 521-cm⁻¹ Raman band, which was assigned to the νFe-CO of five-coordinate CO-heme in this work, was obvious in their Raman spectra and was downshifted by 13CO₂ replacement (Fig. 4 in Ref. 22). Nevertheless, they have not remarked on this Raman band being isotope-sensitive. Their finding that the Raman band is absent in the spectra of the truncated β-subunit implies that the formation of the five-coordinate CO complex is specific to the heterodimeric sGC. The detection of a five-coordinate CO-heme presented in this study represents the first example in a native hemoprotein. The spectroscopic characteristics match those of the five-coordinate CO-heme reported for some mutant hemoproteins including the CooA (CO-sensing transcriptional activator) variant with a H77Y substitution and the proximal base mutant of bacterial heme oxygenase (37, 38).

The major CO adduct of the YC-1-bound CO complex of sGC was characterized by the νFe-CO at 488 cm⁻¹, the δFe-C-O at 589 cm⁻¹, and the νC=O at 1727 cm⁻¹. To our knowledge, the δFe-C-O frequency at 488 cm⁻¹ of the major CO adduct is anomalously high for six-coordinate CO-hemes with proximal neutral imidazole, because the δFe-C-O frequencies of those CO-hemes are within a limited range of 577–579 cm⁻¹. Moreover, the 5-cm⁻¹ isotope shift of the δFe-C-O in the major CO adduct by changing from 12CO to 13CO is small. The δFe-C-O frequency is similar to that of CO-ligated horseradish and cytochrome c peroxidases at low pH (585–587 cm⁻¹), but in these cases the νFe-CO frequency is about 535 cm⁻¹. Thus, the separation between νFe-CO and δFe-C-O in the major CO adduct of sGC is exceptionally large.

This characteristic might be explained by increased bending in the iron-CO unit (34). Whatever the cause of this anomalous behavior, it is clear that the major CO adduct binds a neutral ligand at the position trans to CO (Fig. 8). In a six-coordinate CO-heme with a neutral proximal imidazole, the heme-iron has been known to tightly bind imidazole by a CO-ligand effect (51). Whatever the cause of this anomalous behavior, this result appears to be in contrast to a resonance Raman experimental result, in which the binding site of YC-1 is on the β-subunit (22). However, the azido group of the BAY analogue is spatially separated from the pyrazolopypyridine core by a benzoyl spacer. Therefore, the pyrazolopypyridine core may in fact bind to a site on the β-subunit so that the reactive azido group is in close contact with those distant cysteine residues of the α-subunit. These considerations suggest that the binding site of YC-1 may be located at the dimer interface on the β-subunit as shown for the binding site of forskolin, a potent activator of adenylate cyclase (54).

The results from this study are consistent with a view that YC-1 stimulates the CO- and NO-bound sGC by weakening or cleaving the proximal His-iron bond. The reason why ferrous sGC was stimulated by YC-1 is not clear, because YC-1 does not cause a detectable shift in the νFe-His Raman frequency (22). We therefore infer that the YC-1-dependent stimulation of the ferrous sGC exclusively occurs through a heme-independent mechanism (55).

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REFERENCES

1. Furchgott, R. F., and Zawadzki, J. V. (1980) Nature 288, 373–376
2. Ignarro, L. J., and Kadowitz, P. J. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 171–191
3. Waldman, S. A., and Murad, F. (1987) Pharmacol. Rev. 39, 163–196
4. Garthwaite, J., Charles, S. L., and Chess-Williams, R. (1988) Nature 336, 383–388
5. Bread, D. S., and Snyder, S. H. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9030–9033
6. Moncada, S., and Higgs, E. A. (1991) Eur. J. Clin. Invest. 21, 361–374
7. Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V., and Snyder, S. H. (1993) Science 259, 381–384
8. Stone, J. R., and Marletta, M. A. (1994) Biochemistry 33, 5636–5640
9. Kamisaka, Y., Saheki, S., Nakane, M., Palmieri, J. A., Kuno, T., Chang, B. Y., Waldman, S. A., and Murad, F. (1986) J. Biol. Chem. 261, 7236–7241
10. Humbert, P., Niroumand, F., Fischer, G., Mayer, B., Koesling, D., Hinsch, K.-D., Gauspohl, H., Frank, R., Schultz, G., and Böhme, E. (1990) Eur. J. Biochem. 190, 273–278
11. Ignarro, L. J., Wood, K. S., and Welin, M. S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2670–2673
12. Wolin, M. S., Wood, K. S., and Ignarro, L. J. (1982) J. Biol. Chem. 257, 13132–13132
13. Gerzer, R., Hofmann, F., and Schultz, G. (1981) Eur. J. Biochem. 116, 479–486
14. Ignarro, L. J., and Ballist, B., and Wood, K. S. (1984) J. Biol. Chem. 259, 6201–6207
15. Wedel, B., Humbert, P., Harteneck, C., Fuerster, J., Malkewitz, J., Böhme, E., Schultz, G., and Koesling, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2592–2596
16. Zhao, Y., and Marletta, M. A. (1997) Biochemistry 36, 15959–15964
17. Denizot, G., Stone, J. R., Babcock, G. T., and Marletta, M. A. (1996) Biochemistry 35, 1540–1547
18. Makino, R., Matsuda, H., Oyabashi, E., Shiro, Y., Inzuka, T., and Hori, H. (1999) J. Biol. Chem. 274, 7174–7173
19. Zhao, Y., Brandish, P. E., Balou, D. P., and Marletta, M. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14753–14758
20. Wu, C. C., Ko, F. N., Lee, F. Y., and Teng, C. M. (1995) Br. J. Pharmacol. 116, 1973–1978
21. Friebe, A., Schultz, G., and Koesling, D. (1996) EMBO J. 15, 6863–6868
22. Denninger, J. W., Schelvis, J. P. M., Brandise, P. E., Zhao, Y., Babcock, G. T., and Marletta, M. A. (2000) Biochemistry 39, 4191–4198
23. Stasch, J.-P., Becker, E. M., Alonso-Alia, C., Apeler, H., Dembskowy, B., Feuer, A., Gerzer, R., Minuth, T., Perzhon, E., Pleij, U., Schroder, H., Schroeder, W., Stahl, E., Steinke, W., Straub, A., and Schramm, M. (2001) Nature 410, 212–215
24. Khartitonov, V. G., Sharma, V. S., Madge, D., and Koesling, D. (1999) Biochemistry 38, 10699–10706
25. Sundberg, L., and Forath, J. (1974) J. Chromatogr. 90, 87–98
26. Seybert, D., Moffat, K., Gibson, Q. H., and Chang, C. K. (1977) J. Biol. Chem. 252, 4226–4231
27. Vatanasi, T. (1961) J. Biol. Chem. 236, 1680–1688
28. Paul, K. G., Theorell, H., and Akesson, A. (1953) Acta Chem. Scand. 7, 1284–1287
29. Satchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 680–672
30. Stone, J. R., Sands, R. H., Dunham, W. R., and Marletta, M. A. (1995) Biochem. Biophys. Res. Commun. 207, 572–577
31. Kim, S. Y., Denizot, G., Gardner, M. T., Marletta, M. A., and Babcock, G. T. (1996) J. Am. Chem. Soc. 118, 6760–6770
32. Tsukabi, M., Hiwatashi, A., and Ichikawa, Y. (1987) Biochemistry 26, 4535–4540
