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Pharmacological Application and Synthesis of Substituted porphyrins and their derivates

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ABSTRACT

The nature of the metal-proximal base bond of soluble guanylate cyclase from bovine lung was examined by EPR spectroscopy. When the ferrous enzyme was mixed with NO, a new species was transiently produced and rapidly converted to a five-coordinate ferrous NO complex. The new species exhibited the EPR signal of six-coordinate ferrous NO complex with a feature of histidine-ligated heme. The histidine ligation was further examined by using the cobalt protoporphyrin IX-substituted enzyme. The Co^{2+}-substituted enzyme exhibited EPR signals of a broad $g_{\perp;1}$ component and a $g_{\parallel;1}$ component with a poorly resolved triplet of ^{14}N superhyperfine splittings, which was indicative of the histidine ligation. These EPR features were analogous to those of α-subunits of Co^{2+}-hemoglobin in tense state, showing a tension on the iron-histidine bond of the enzyme. The binding of NO to the Co^{2+}-enzyme markedly stimulated the cGMP production by forming the five coordinate NO complex.

Keywords: guanylate cyclase, bovine lung, ligation, protoporphyrin

1. INTRODUCTION

Porphyrins are generated as the body buids hemoglobin, the compound that carries oxygen in blood cells. Porphyrin (from the greek word for purple) is made upfour ring compounds joined together, a structure conductive to holding a metal ion in its center. Iron in the heme porphyrin of hemoglobin magnesium in chlorophyll and cobalt in cynocobalmine, central metal and core nitrogen after modification play a vital role by hetro atoms such as sulfer oxygen, selenium, tellurium etc.

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porphyrins or tetra pyrrole macrocycles are involved in many important biological processes such as electron transfer, oxygen transport, light harvesting etc. But cental metal ion will not be transfer but that's also phatmacological area. Cobalt porphyrins are one of them, cobalt porphyrins are known to various endocrine activities, ligands which many attach to the cobalt atom of (I) include aromatic bases such as pyridine, imidazole or 2-methylmidazole in amounts which may be chloride, bromide carboxylate or hydroxide.

Co^{2+} Protoporphyrin IX Substitution-- Ignarro *et al.*[1] have reported a method to prepare the apoenzyme by lowering pH to 5.7. We attempted to prepare the

apoenzyme by this method, but the recovery of the Co^{2+}-substituted enzyme was very low at the final purification step. We tested the heme depletion as a function of pH and found that the heme in sGC was depleted by the DEAE cellulose chromatography under alkaline conditions. In brief, the supernatant fraction of homogenized tissue described above was adjusted to pH 8.5, and DEAE cellulose A-500 equilibrated with 50 mM TEA buffer at pH 8.5 containing protease inhibitors was poured to the supernatant. The enzyme was eluted by a linear gradient of 0-0.35 M NaCl. The fractions with cyclase activity that was assayed in the presence of protoporphyrin IX were further purified by GTP-agarose and Superdex 200-pg columns under the conditions described above. The apoenzyme was pooled and reconstituted with Co^{2+} protoporphyrin IX under anaerobic conditions. The remaining purification steps were the same as those used for the native enzyme purification. The enzyme Co^{2+} porphyrin-substituted by our method exhibited essentially the same optical and EPR spectral properties as the Co^{2+}-substituted enzyme, which was obtained by the method of Ignarro *et al.*[1].

2. Spectral Measurements

Absorption spectra were recorded with a Shimadzu MPS-2000 or a Perkin-Elmer Lamda 18 spectrophotometer at room and subzero temperatures. The temperature of the cuvette holder was controlled with thermomodule elements. The buffer systems used were 50 mM TEA buffer (pH 7.6) containing 5% glycerol and the same buffer containing 40% ethylene glycol for room and subzero temperature measurements, respectively. Other details were described in the figure legends.

EPR spectra were measured on a Varian E-12 X-band EPR spectrometer with 100-kHz field modulation. An Oxford flow cryostat (ESR-900) was used for liquid helium temperature measurements. The microwave frequency was calibrated with a microwave frequency counter (Takeda Riken, model TR 5212), and the magnetic field strength was determined by the nuclear magnetic resonance of water protons. Accuracy of g values was ±0.01 in the low magnetic field and ±0.005 in the high field. Other details were as described elsewhere[25].

NO complexes for EPR measurements were prepared in buffer containing 5% glycerol at −5 °C or in buffer containing 40% ethylene glycol at −24 °C as follows. The enzyme solution was transferred to a septum-capped EPR tube and flushed with oxygen-free argon gas for 10 min. Then NO gas previously washed with 1 N NaOH or an aliquot of SNAP solution was introduced to the tube with a gas-tight syringe. The formations of NO complexes in five- and six-coordinate states were ensured by directly measuring the optical spectrum of the sample in the EPR tube at −5 or −24 °C.

The ferric enzyme was prepared by adding a 2-fold excess of ferricyanide to the DTT-free ferrous enzyme, where DTT in the enzyme solution was removed by a Superdex 200HR (Amersham Pharmacia Biotech) HPLC column. For EPR measurements, the residual ferricyanide was freed of the solution by passing

through a Superdex 200HR HPLC column. The ferric enzyme was converted to N_3^- complexes by adding a desired amount of NaN_3. The EPR spectra of ferric N_3^- complex were measured at 5 or 15 K.

Co^{2+} Protoporphyrin IX-substituted Enzyme-- Apoenzyme used for the reconstitution with Co^{2+} protoporphyrin IX exhibited the basal and NO-stimulated activities of 38 and 165 nmol/min/mg of protein, respectively. The following titration experiments indicated that the apoenzyme preparation retained a correct binding site for protoheme IX. When the apoenzyme was titrated with protoheme under anaerobic conditions maintained by the addition of a slight excess of $Na_2S_2O_4$, the absorbance at 431 nm as well as the cyclase activity increased with an increased amount of protoheme, giving a clear inflection point. At the point the cyclase activity reached a plateau (data not shown), with the NO-stimulated activity of 2550 nmol/min/mg of protein. The basal activity of the reconstituted enzyme was 16 nmol/min/mg of protein. The resultant reconstituted enzyme exhibited an optical spectrum essentially identical to that of the native enzyme. Similar results were also obtained when titrated with Co^{2+} protoporphyrin IX. The Co^{2+}-substituted enzyme further purified by the method of the previous section exhibited retention times identical to those of the native enzyme when analyzed by a Superdex 200HR column and a Protein Pak G-DEAE HPLC column (data not shown). The results indicated that the Co^{2+}-enzyme had the same metal binding site as that of native enzyme, and had essentially identical molecular mass and protein surface charges to those of the native enzyme. The Co^{2+}-enzyme exhibited a specific activity of 8600 nmol/min/mg of protein in the presence of NO. Since an attempt to purify it to a homogenous state was unsuccessful, the comparisons of activity between cobalt- and iron-enzymes were done using a turnover number defined as µmol of cGMP/min/µmol of heme or cobalt porphyrin. The turnover number of the partially purified Co^{2+}-substituted enzyme was 5840 min^{-1} in the presence of NO, which was about 1.5-fold higher than that of native enzyme (3800 min^{-1}). The activation of the Co^{2+}-substituted enzyme by NO was about 50-fold, which was significantly low when compared with the native enzyme (270-fold). The lower activation of the Co^{2+}-substituted enzyme by NO was attributable to the high basal activity of 115 min^{-1}, which was about 8-fold higher than that of native enzyme (14 min^{-1}).

The partially purified Co^{2+}-substituted enzyme showed the Soret band at 405 nm and the visible band at 559 nm (Fig. 3*A*), which were nearly identical to those of Co^{2+}-substituted myoglobin[25]. The shoulder absorption around 430 nm marked by an *asterisk* was attributed to the contamination of the native iron-enzyme by the pyridine hemochromogen assay. The content was estimated to be less than 15%. The addition of NO to the Co^{2+}-enzyme slightly blue-shifted the Soret band to 399 nm and red-shifted the visible band to 569 nm (Fig. 3*B*). The spectral pattern of the NO complex was entirely different from that of Co^{2+}-myoglobin NO complex in a six-coordinate state, which

exhibited the Soret band at 421 nm and 539- and 577-nm bands in the visible region. These results suggested that the NO complex of Co^{2+}-substituted sGC was in a five-coordinate state. The coordination state was confirmed by a resonance Raman spectroscopy as described below.

The resonance Raman spectra of the Co^{2+}-substituted enzyme and the NO derivatives were summarized in Fig. 4, *A* and *B*. The Co^{2+}-enzyme exhibited the ν_4 and ν_3 Raman bands at 1371 cm^{-1} and at 1504 cm^{-1}, respectively, which closely agreed with those of Co^{2+}-myoglobin and -hemoglobin in a six-coordinate state[26]. The addition of ^{14}NO to the Co^{2+}-substituted enzyme shifted the ν_4 band to 1376 cm^{-1} from 1371 cm^{-1} with an appearance of the Raman band at 1682 cm^{-1}. The resonance Raman spectrum was markedly different from those of ^{14}NO complexes in a six-coordinate state[26]. The replacement of ^{14}NO by ^{15}NO eliminated the Raman band at 1682 cm^{-1} with a concomitant appearance of the 1648-cm^{-1} band and without detectable shift of other bands (Fig. 4*A*, *c* and *d*). In the low frequency region, we detected the shift of 523 cm^{-1} band upon the replacement of ^{14}NO with ^{15}NO (Fig. 4*B*, *b* and *inset*). These results indicated that the 1682- and 523-cm^{-1} bands were assigned to the NO stretching vibration ($\nu_{N\text{-}O}$) and Co-NO vibration ($\nu_{Co\text{-}NO}$), respectively. Both $\nu_{N\text{-}O}$ and $\nu_{Co\text{-}NO}$ values agreed with those of the corresponding vibration of five-coordinate NO complexes of Co^{2+} model porphyrins but not of six-coordinate NO complexes These findings indicated that the NO complex of Co^{2+}-substituted sGC was in a five-coordinate state.

The EPR spectrum of Co^{2+}-substituted enzyme was shown in Fig. 5*A*, with that of Co^{2+}-myoglobin for comparison. The Co^{2+}-substituted enzyme exhibited five-coordinate low spin signals at $g_\perp = 2.37$ and $g_\parallel = 2.04$ with poorly resolved eight-line hyperfine splitting due to ^{59}Co nucleus ($^{Co}A_\parallel = 7.4$ mT). The $g_\perp = 2.37$ component was significantly broad compared with that of Co^{2+}-myoglobin. The hyperfine splitting constant ($^{Co}A_\parallel = 7.4$ mT) agreed with that of other Co^{2+}-substituted hemoproteins with proximal histidine, suggesting the presence of a histidine residue as the proximal ligand in sGC. However, the triplet superhyperfine splitting due to the ^{14}N nucleus of the axial ligand was not well resolved in Fig. 5*A*. To gain firm evidence for the histidine ligation, EPR signals with 20-mT sweep width centered at 300 mT were accumulated to obtain a high quality spectrum. As shown in Fig. 5*B*, we could detect the three-line superhyperfine splitting due to the ^{14}N nucleus ($^{N}a = 1.7$ mT). Thus, the EPR signal of Co^{2+}-substituted sGC was characterized by a poorly resolved triplet splitting of the ^{14}N nucleus and the relatively broad $g_\perp$ component. These features resembled those of the α-subunit in Co^{2+}-hemoglobin tetramer in T-state rather than that of the β-subunit of Co^{2+}-hemoglobin in T-state[18], Co^{2+}-myoglobin, or Co^{2+}-horseradish peroxidase.[17]

Azide Complex-- As shown in Fig. 6*A*, the addition of N_3^- to the ferric enzyme

caused a decrease in the intensity of the Soret band and a remarkable increase in the intensity of the 635-nm band at room temperature, confirming the previous result. These spectral changes were unusual, because the N_3^- addition to other ferric hemoproteins such as metmyoglobin produced 420- and 540-nm low spin bands and reduced the 640-nm band intensity. The low spin bands in the metmyoglobin N_3^- complex were intensified by lowering the temperature, showing that the spin state was in a thermal spin equilibrium between low and high spin states. In contrast, the N_3^- complex of sGC did not display the spectral change by lowering the temperature to 77 K (data not shown). This indicated that the ferric heme of the N_3^--bound sGC was in a high spin state, not in a thermal spin equilibrium.

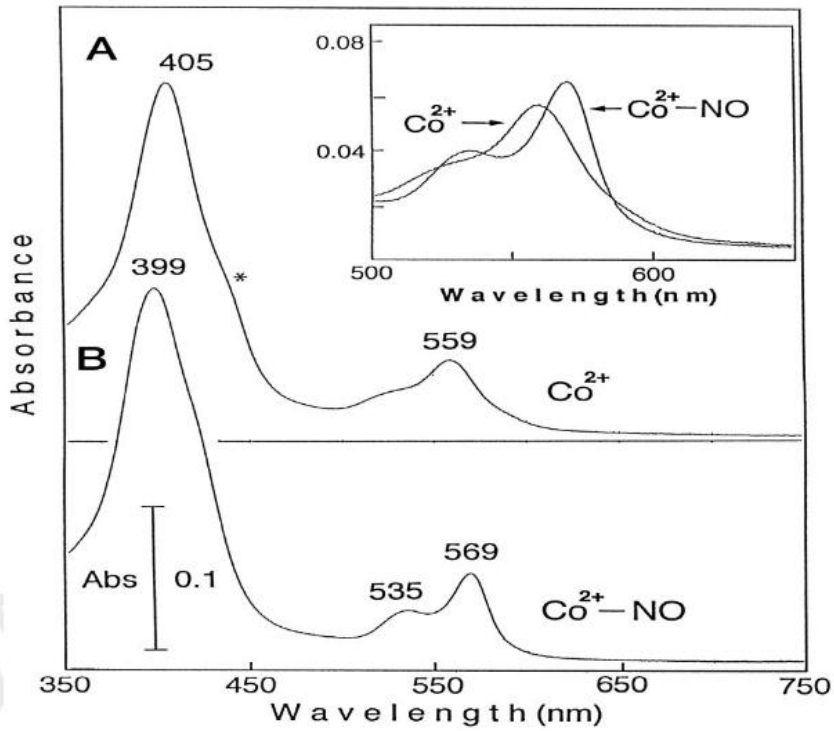

Fig. 3. Optical absorption spectra of Co^{2+} protoporphyrin IX-substituted sGC. *A*, optical spectrum of Co^{2+}-substituted enzyme under anaerobic conditions at 5 °C. *B*, optical spectrum of the NO complex of Co^{2+}-substituted enzyme at 5 °C. The NO complex was prepared by adding NO under anaerobic conditions. In these experiments, 50 mM TEA buffer, pH 7.6, containing 5% glycerol and 5 mM DTT was used.

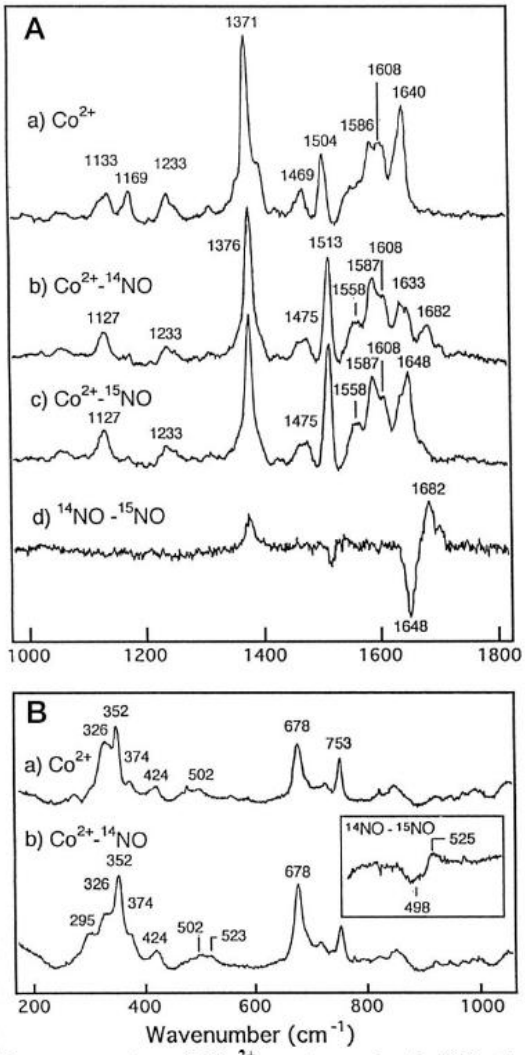

Fig. 4. Resonance Raman spectra of Co^{2+} protoporphyrin IX-substituted sGC. *A*, high frequency resonance Raman spectra of Co^{2+} porphyrin-substituted enzyme and its NO complexes. The spectra of the Co^{2+}-enzyme, Co^{2+}-^{14}NO complex, and Co^{2+}-^{15}NO complex are illustrated in *a*, *b*, and *c*, respectively. The difference spectrum between ^{14}NO and ^{15}NO complexes ($^{14}NO - {}^{15}NO$) is presented in *d*. *B*, low frequency resonance Raman spectra of Co^{2+} porphyrin-substituted enzyme (*trace a*) and the ^{14}NO complex (*trace b*). The difference spectrum between ^{14}NO and ^{15}NO is shown in the *inset*. These spectra were taken at 406.7 nm excitation wavelength. The buffer used in these experiments was 50 mM TEA buffer, pH 7.6, containing 5% glycerol and 5 mM DTT.

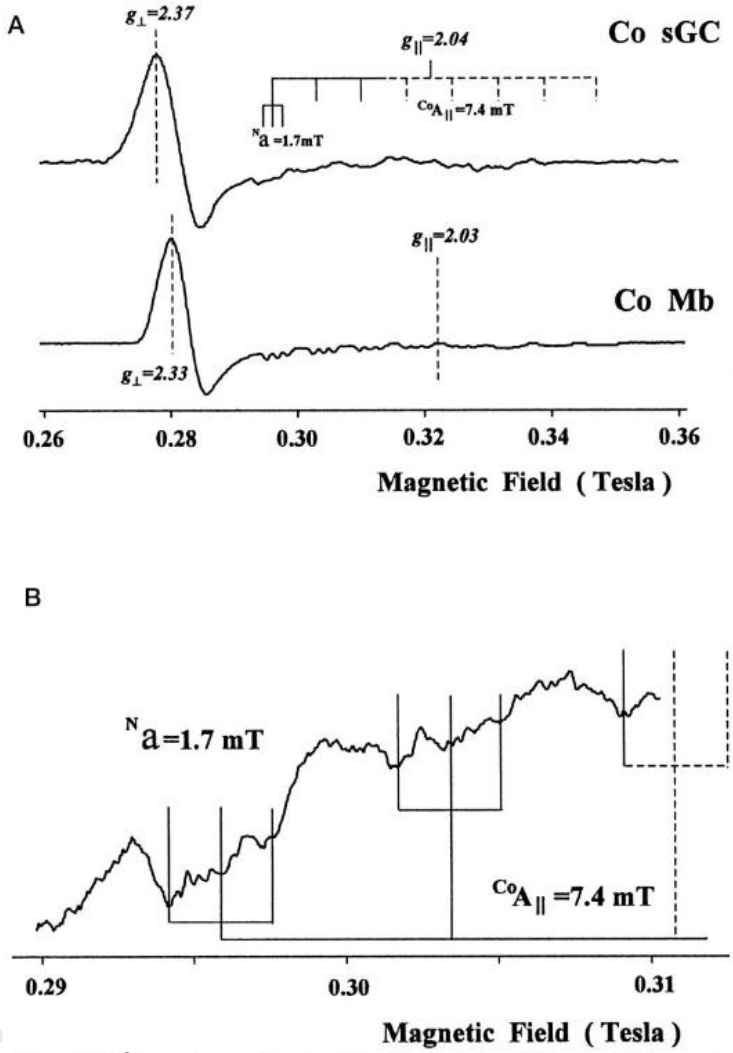

Fig. 5. EPR spectra of Co^{2+} protoporphyrin IX-substituted sGC. A, EPR spectrum of Co^{2+} protoporphyrin IX-substituted sGC (Co sGC) at 35 K (upper trace). The EPR spectrum of Co^{2+} protoporphyrin IX substituted myoglobin (Co Mb) is shown for comparison (lower trace). B, accumulated EPR spectrum of Co^{2+} protoporphyrin IX-substituted sGC between 0.29 and 0.31 mT. In this experiment, 40 scans were averaged. The buffer used in these experiments was 50 mM TEA buffer, pH 7.6, containing 10% glycerol and 5 mM DTT. ^{N}a and ^{Co}A denote coupling constants for hyperfine splitting by ^{14}N nucleus of proximal base and ^{59}Co nucleus, respectively.

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