Structural Changes in the Heme Proximal Pocket Induced by Nitric Oxide Binding to Soluble Guanylate Cyclase†

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ABSTRACT: When expressed in *Escherichia coli*, the heme domain [β1(1−385)] of rat lung soluble guanylate cyclase (sGC) is isolated with a stoichiometric amount of bound heme [Zhao, Y., and Marletta, M. A. (1997) *Biochemistry 36*, 15959−15964]. Nitric oxide (NO) binding to the heme in β1(1−385) leads to cleavage of the Fe−His bond and formation of a five-coordinate NO−heme complex. Addition of imidazole to the five-coordinate NO complex shifts the Soret peak from 399 to 420 nm, which appears to result from the formation of a six-coordinate NO complex. Removal of the added imidazole by gel filtration results in formation of the five-coordinate NO complex once again. The EPR spectrum of the putative six-coordinate NO complex has nine distinct derivative-shaped lines (a triplet of triplets), which is the signature spectrum of a six-coordinate NO complex with two nitrogen atoms as the axial ligands. [^{15}N]Imidazole simplifies the six-coordinate NO complex EPR spectrum to six distinct derivative-shaped lines (a triplet of doublets), indicating that the other axial ligand in the six-coordinate NO complex is an imidazole molecule. These results show that NO binding to sGC not only leads to the cleavage of the Fe−His bond but also induces a conformational change which opens the heme proximal pocket large enough to accommodate an exogenous imidazole molecule. These observations have important implications for determining the NO activation mechanism of sGC.

Soluble guanylate cyclase (sGC)[1] plays an essential role in nitric oxide (NO) signaling by functioning as a NO receptor. NO has been shown to be involved in many biological processes, including neuronal signaling, vasodilatation, and the host response to infection (*2*, *3*). sGC catalyzes the generation of the second messenger cGMP from GTP (for reviews, see (refs 4 and 5). cGMP has been shown to regulate various cellular signal transduction pathways, including activation of cGMP-dependent protein kinases (*6*). In the presence of NO, sGC is activated up to 400-fold (*7*). The molecular details of both activation and deactivation of sGC are not fully understood. sGC is a heterodimeric hemoprotein composed of α1 and β1 subunits when it is isolated from lung tissue (*8*−*12*). The heme binding region and the catalytic site are localized in different regions of the protein. The heme binding pocket is formed from residues in the N-terminal region of the β1 subunit (*1*), while the catalytic site(s) is located in the C-terminal region of the α1 and β1 subunits (*13*). Coexpression of the C-terminal fragments of α1 and β1 [α1(367−691) and β1(306−619)] in COS cells was found to be sufficient for generating basal sGC activity, but not the NO-stimulated activity (13). We have previously demonstrated that the N-terminal fragment of the β1 subunit [β1(1−385)] when it is expressed in *Escherichia coli*, is isolated with 1 equiv of bound heme (*1*). The electronic absorption (*1*) and resonance Raman spectra (*14*) of β1(1−385) are almost identical to those of heterodimeric sGC, indicating that the catalytic domains of the β1 subunit and the α1 subunit are not required for the formation of the heme binding pocket. Therefore, β1(1−385) was used in this study to characterize the interaction between NO and the heme of sGC. When isolated from lung tissue (*12*, *15*, *16*) or recombinant baculovirus and/or 9 cells (P. E. Brandish and M. A. Marletta, unpublished results), sGC contains a ferrous high-spin, five-coordinate heme with a histidine residue as the only axial ligand as indicated by both electronic absorption and resonance Raman spectroscopic studies. We have further identified histidine 105 (H105) in the β1 subunit as the heme proximal ligand using site-directed mutagenesis and spectroscopic methods (*14*).

Activation of sGC by NO is mediated by the interaction of NO and the heme. Heme-deficient sGC retains basal sGC activity, but has lost its ability to respond to NO (*17*). NO readily forms a nitrosyl complex with the heme of sGC and shifts the Soret maximum from 431 to 399 nm (*12*). EPR (*18*), electronic absorption (*12*), and resonance Raman spectroscopic studies (*15*, *16*, *19*, *20*) have shown that the sGC−NO complex is five-coordinate with NO as the only

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[1] Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; CO, carbon monoxide; deoxyMb, ferrous myoglobin; DTT, dithiothreitol; EPR, electron paramagnetic resonance; GTP, guanosine 5′-triphosphate; Im, imidazole; 2-MeIm, 2-methylimidazole; 4-MeIm, 4-methylimidazole; *N*-MeIm, *N*-methylimidazole; NO, nitric oxide; sGC, soluble guanylate cyclase.

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axial ligand from the distal side. The bond between the proximal H105 and the heme iron is cleaved as a result of NO binding to the heme. Formation of the five-coordinate NO complex is necessary for the activation of sGC by NO (*21*). Although the exact mechanism of NO activation is not known, it is believed that NO binding to the heme in sGC triggers a conformational change in the protein that rearranges the active site of sGC and activates the enzyme. Here we present data that indicate that NO binding to the heme in sGC not only leads to the cleavage of the Fe−H105 bond but also opens up the heme proximal pocket to generate a cavity large enough to accommodate exogenous ligands such as imidazole, leading to the formation of a six-coordinate NO complex.

MATERIALS AND METHODS

Materials. sGC β1(1−385) was expressed in *E. coli* and purified as described previously (*1*). Nitric oxide (99.0%) and [^{15}N]nitric oxide (99%) were obtained from Matheson Gas Products (Chicago, IL). [^{15}N]Imidazole was from Isotec Inc. (Miamisburg, OH). PD-10 disposable desalting columns were from Pharmacia. Reacti-Vials and a Teflon septum were from Pierce. All other chemicals were purchased from Sigma unless otherwise stated.

Formation of a β1(1−385)−NO Complex. Just prior to use, NO was further purified by flushing it through a 50% (w/v) KOH solution. This NO was then used to flush a 2 mL Reacti-Vial sealed with a Teflon septum. Purified β1-(1−385) was made anaerobic in a cuvette sealed with a rubber septum by argon replacement using an oxygen-scavenged gas train. A 10 μL aliquot of NO gas was transferred from the Reacti-Vial using a Hamilton gastight syringe and injected directly into the solution containing β1-(1−385). The conversion of ferrous β1(1−385) (Soret peak at 431 nm) to nitrosyl β1(1−385) (Soret peak at 399 nm) was monitored spectroscopically.

Binding of Imidazole and Imidazole Derivatives to the β1-(1−385)−NO Complex. The β1(1−385)−NO complex was prepared as described above. An imidazole (3 M) stock solution was made anaerobic in a 2 mL Reacti-Vial by argon replacement as described above. Anaerobic additions of imidazole to the β1(1−385)−NO complex were made using a Hamilton gastight syringe. After each addition of imidazole, the electronic absorption spectrum was recorded. All spectra were recorded on a Cary 3E spectrophotometer equipped with a Neslab RET-100 temperature controller set at 10 °C. Difference spectra were generated by subtracting the initial the β1(1−385)−NO complex spectrum from those spectra of the β1(1−385)−NO complex in the presence of different concentrations of imidazole. The absorbance differences (Δ) at 420 and 394 nm were determined. The $\Delta\Delta A$ (Δ420 − Δ394) was plotted against the concentration of imidazole. The K_D of imidazole binding to the β1(1−385)−NO complex was obtained by fitting the plot ($\Delta\Delta$A vs imidazole concentration) using the equation $\Delta\Delta A = \Delta\Delta A_{max}[\mathrm{Im}]/(K_D + [\mathrm{Im}])$. The spectral K_D values of several imidazole derivatives binding to the β1(1−385)−NO complex were also determined using this same method.

Formation of a Six-Coordinate β1(1−385)−Imidazole Complex. Purified β1(1−385) was placed in a cuvette sealed with a rubber septum and was then made anaerobic as described above. Imidazole from an anaerobic stock solution was added directly to β1(1−385) using a Hamilton gastight syringe. The formation of a six-coordinate β1(1−385)−imidazole complex was monitored by a shift in the Soret peak to 414 nm.

Treatment of the Six-Coordinate β1(1−385)−Imidazole Complex with CO and NO. The six-coordinate β1(1−385)−imidazole complex was formed anaerobically by mixing β1-(1−385) and imidazole as described above. Carbon monoxide (CO) was then added directly to the headspace in the cuvette, and then the cuvette was placed on ice for 15 min, which led to a shift of the Soret peak to 423 nm. Treatment of the six-coordinate β1(1−385)−imidazole complex with NO anaerobically led to a shift in the Soret peak to 420 nm.

Conversion of the Six-Coordinate β1(1−385)−NO Complex to a Five-Coordinate NO Complex. A Pharmacia disposable PD-10 desalting column was used to remove the excess imidazole in the six-coordinate β1(1−385)−NO complex solution. The column was equilibrated with buffer [50 mM Hepes (pH 7.4), 5 mM DTT, and 100 mM NaCl]. The sample (0.5 mL) was loaded onto the PD-10 column and washed with 2 mL of buffer, and then was eluted with 1 mL of buffer. Conversion of the six-coordinate NO complex to a five-coordinate NO complex was monitored by the shift of the Soret peak from 420 to 399 nm.

EPR Characterization of the β1(1−385)−NO Complexes. EPR spectra were obtained using a Bruker ESP300E X-band spectrometer equipped with a standard TE102 rectangular cavity and an Oxford instruments ESR-9 helium flow cryostat. Experimental conditions were as follows: temperature, 25 K; microwave frequency, 9.5 GHz; microwave power, 5 mW; modulation frequency, 100 kHz; modulation amplitude, 2.012 G; and time constant, 41 ms. NO complexes were prepared as follows. Purified β1(1−385) (500 μM) in 50 mM Hepes (pH 7.4), 5 mM DTT, 100 mM NaCl, and 10% glycerol was made anaerobic in a 1 mL Reacti-Vial using an oxygen-scavenged gas train as described above. NO (100 μL) was removed from a 2 mL Reacti-Vial anaerobically using a Hamilton gastight syringe and injected into the Reacti-Vial containing the β1(1−385) solution. The solution containing the β1(1−385)−NO complex was then transferred anaerobically to an EPR tube with a rubber septum using a Hamilton gastight syringe. To form the putative six-coordinate NO complex with imidazole, imidazole was added anaerobically to the EPR tube containing the β1(1−385)−NO complex.

RESULTS

Formation of a Five-Coordinate β1(1−385)−NO Complex. The EPR spectrum of the β1(1−385)−NO complex is shown in Figure 1. The hyperfine splitting results in a spectrum with three prominent lines (Figure 1, spectrum A), a signature pattern of five-coordinate ferrous heme−[^{14}N]-NO complexes (*18*, *22*). The β1(1−385)−NO complex EPR spectrum is simplified to two prominent lines when [^{15}N]-NO is used (Figure 1, spectrum B), indicating that the hyperfine splitting results from the interaction between the unpaired electron spin and the nucleus of the nitrogen atom in NO. These results indicate that NO forms a five-coordinate complex with β1(1−385).

Binding of Imidazole to the β1(1−385)−NO Complex. When imidazole was added to the five-coordinate β1(1−

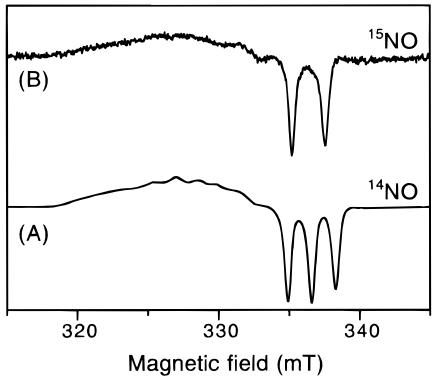


FIGURE 1: EPR spectra of sGC β1(1−385)−NO complexes at 25 K: (A) β1(1−385)−[^{14}N]NO and (B) β1(1−385)−[^{15}N]NO. The experimental conditions are specified in Materials and Methods.

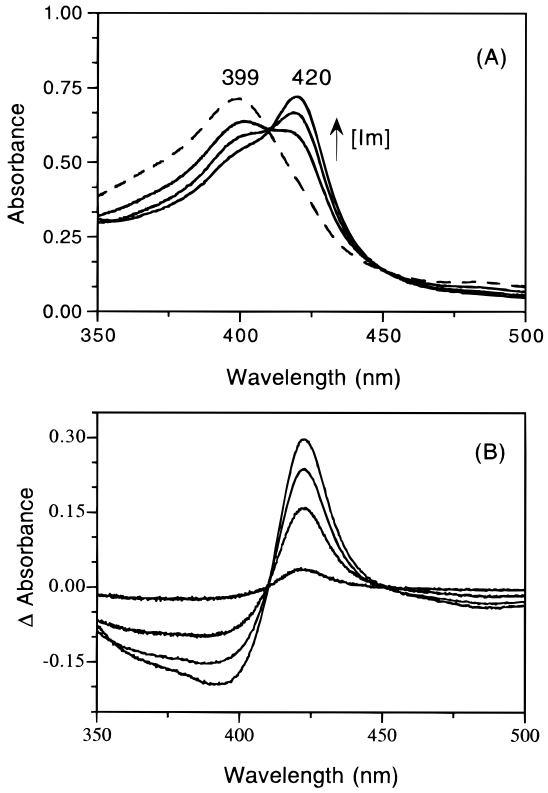
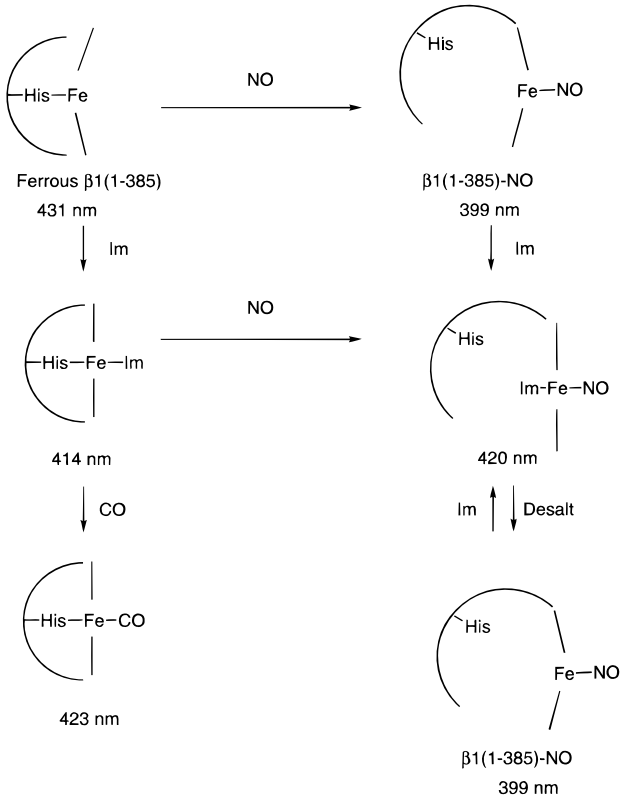


FIGURE 2: (A) Binding of imidazole to the five-coordinate β1(1−385)−NO complex. Electronic absorption spectra of β1(1−385)−NO complexes in the absence of imidazole (− − −) and in the presence of increasing amounts of imidazole (—). (B) Electronic absorption difference spectrum for binding of imidazole to the five-coordinate β1(1−385)−NO complex. The Soret peak shift from 399 to 420 nm resulted in the peak at 422 nm and trough at 394 nm in the difference spectra. The initial concentration of the β1-(1−385)−NO complex was 6.6 μM. The imidazole concentrations were 238, 403, and 700 mM for the three spectra, respectively.

385)−NO complex, the Soret peak was shifted from 399 to 420 nm with an isosbestic point at 410 nm (Figure 2A). The difference spectra are shown in Figure 2B. The conversion of the five-coordinate β1(1−385)−NO complex to the complex with Soret maximum at 420 nm is dependent on the concentration of imidazole (Figure 2). The 420 nm Soret peak appears to result from a six-coordinate NO complex since NO was found to be still associated with the heme in the presence of imidazole (see below). Furthermore, six-coordinate NO complexes typically have a Soret maximum at 420 nm (*23*).

Scheme 1: Summary of Imidazole Binding to Ferrous β1(1−385) and Ferrous β1(1−385)−NO[a]



[a] The six-coordinate imidazole/NO complex (λ_{max} = 420 nm) is formed in the presence of excess NO. Desalting of the complex removes this excess NO as well as the bound and excess imidazole.

Equilibrium between the Five-Coordinate β1(1−385)−NO Complex and the Six-Coordinate NO Complex. As described above, addition of imidazole converted the β1-(1−385)−NO complex to what appeared to be a six-coordinate NO complex (Soret maximum at 420 nm). The putative six-coordinate NO complex (Soret maximum at 420 nm) can be converted back to a complex that appears to be a five-coordinate NO complex simply by desalting to remove the excess imidazole (Scheme 1). The regenerated β1(1−385)−NO complex has the same electronic absorption spectrum as that of the original five-coordinate β1(1−385)−NO, which has a Soret peak at 399 nm, a split α/β band and a shoulder at 485 nm (data not shown), indicating that the regenerated β1(1−385)−NO complex is also five-coordinate. When imidazole was added back to the regenerated five-coordinate NO complex, it formed the putative six-coordinate NO complex once again with a Soret peak at 420 nm (Scheme 1). When imidazole was added to the ferrous β1-(1−385) in the absence of NO, the Soret peak shifted from 431 to 414 nm which appears to be a six-coordinate complex with imidazole bound to the heme from the distal side (Figure 3B). CO was able to replace imidazole to form a six-coordinate complex which has a characteristic sharp Soret peak at 423 nm and a split α/β band (Figure 3C). The CO complex spectrum shown in Figure 3C is identical to those of the β1(1−385)−CO complex (*1*) and of native heterodimeric sGC−CO (*12*), showing that the heme iron in the β1(1−385)−imidazole complex is still ferrous. When NO was added to the β1(1−385)−imidazole complex, the Soret maximum shifted from 414 to 420 nm which indicated

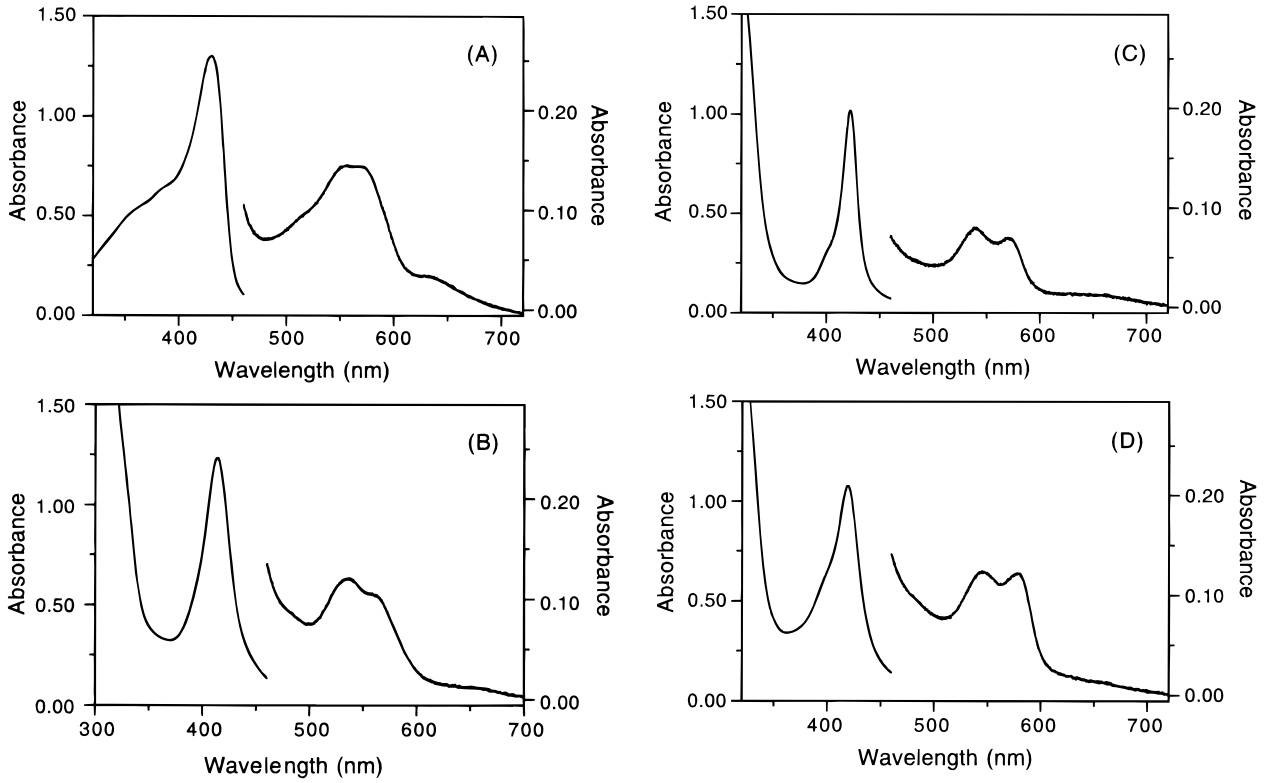


FIGURE 3: Electronic absorption spectra of β1(1−385) (10.5 μM) and the imidazole, CO, and NO complexes: (A) β1(1−385), (B) β1-(1−385) and imidazole, (C) β1(1−385) and imidazole, and then add CO, (D) β1(1−385) and imidazole, and then add NO. The left scale refers to the Soret region, and the right scale refers to the α/β region.

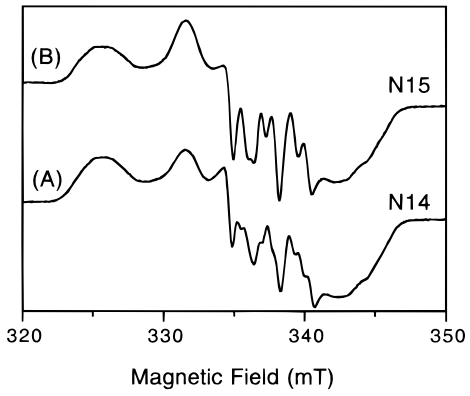


FIGURE 4: EPR spectra of the six-coordinate β1(1−385)−NO complexes (A) in the presence of [^{14}N]imidazole and (B) in the presence of [^{15}N]imidazole. Experimental details are described in Materials and Methods.

that NO replaced the imidazole to form a six-coordinate NO complex (Figure 3D).

EPR Characterization of the Six-Coordinate NO Complex. In the presence of imidazole, the EPR spectrum of the β1-(1−385)−NO complex was converted from a spectrum with three prominent lines (Figure 1, spectrum A) to a spectrum showing nine distinct derivative-shaped lines (Figure 4A), which is the well-documented signature pattern of a six-coordinate NO complex with a nitrogenous ligand as the second axial ligand (a triplet of triplets) (*24*). This EPR spectrum not only provides convincing evidence for the formation of a six-coordinate NO complex in the presence of imidazole but also indicates that the sixth ligand has an *I* of 1; therefore, it probably is a nitrogen atom from either histidine residue or imidazole). When [^{15}N]imidazole was used, the EPR spectrum (Figure 4B) was simplified to six distinct derivative-shaped lines (a triplet of doublets). This result unambiguously indicates that the sixth ligand is an imidazole molecule, and not a histidine residue from the protein.

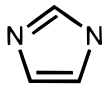
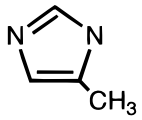
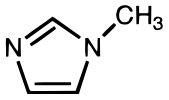
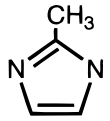
Formation of Six-Coordinate NO Complexes with Imidazole Derivatives. Both 4-methylimidazole (4-MeIm) and *N*-methylimidazole (*N*-MeIm) form six-coordinate NO complexes with the β1(1−385)−NO complex. However, 2-methylimidazole (2-MeIm) apparently does not bind, because there were no changes in the electronic absorption spectrum up to the highest concentration examined (2.5 M). K_D values for binding imidazole and imidazole derivatives to the β1-(1−385)−NO complex are shown in Table 1.

DISCUSSION

We recently reported that NO forms a ferrous nitrosyl complex with β1(1−385) (*1*). The electronic absorption spectrum of the β1(1−385)−NO complex is identical to that of the heterodimeric sGC−NO complex which is a five-coordinate complex with a Soret maximum at 399 nm (*12*). On the basis of the electronic absorption spectrum, the β1-(1−385)−NO complex also appeared to be a five-coordinate complex (*1*).

EPR is widely used to measure the coordination state of ferrous heme−NO complexes, since the ferrous heme−NO complex is EPR active with an *S* of $^1/_2$. Five-coordinate and six-coordinate ferrous−NO complexes with histidine as the proximal ligand have unique hyperfine and superhyperfine splitting patterns (*24*). Many five-coordinate and six-coordinate ferrous-NO complexes of hemoproteins and model compounds have been reported (*22*, *25*, *26*). Five-coordinate

Table 1: Binding Imidazole and Imidazole Derivatives to the Five-Coordinate β1(1−385)−NO Complex[a]

Compounds	K_D (mM)
 Imidazole	877 ± 78
 4-methylimidazole	700 ± 160
 N-methylimidazole	1206 ± 186
 2-methylimidazole	–

[a] The ΔΔA (Δ420 − Δ394) was plotted against the concentration of imidazole. The K_D of imidazole to β1(1−385)−NO complex was obtained by fitting the plot of ΔΔ*A* vs imidazole concentration using the equation $\Delta\Delta A = \Delta\Delta A_{max}[Im]/(K_D + [Im])$. The K_D for other imidazole derivatives was obtained using the same method.

ferrous heme−[^{14}N]NO complexes give a spectrum with three prominent lines which can be simplified to two prominent lines when [^{15}N]NO is used. The hyperfine splitting results from the interaction between the unpaired electron spin and the nuclear spin of nitrogen ($I = 1$ for ^{14}N, $I = {}^1/_2$ for ^{15}N). The EPR spectra of six-coordinate ferrous heme−[^{14}N]NO complexes have been shown to have nine distinct derivative-shaped lines due to the interaction between the unpaired electron and two nitrogen atoms (one from [^{14}N]NO and the other one from the ^{14}N of the proximal histidine). The three-line EPR spectrum of the β1(1−385)−[^{14}N]NO complex (Figure 1) unequivocally indicated that the β1(1−385)−NO complex is five-coordinate. Cleavage of the Fe−His bond upon NO binding to the heme of sGC may result from the weak Fe−His bond, as revealed by resonance Raman spectroscopic studies (*15*). These results further support our conclusion that the heme environment of β1(1−385) is very similar, if not identical, to that of heterodimeric sGC (*1*).

Addition of imidazole to the five-coordinate β1(1−385)−NO complex shifted the Soret maximum from 399 to 420 nm with an isosbestic point at 410 nm (Figure 2). We assigned this new complex as a six-coordinate β1(1−385)−NO complex on the basis of the following two observations. First, addition of imidazole to the five-coordinate β1(1−385)−NO complex did not result in the replacement of NO by imidazole since the putative six-coordinate β1(1−385)−NO complex could be converted back to the five-coordinate β1(1−385)−NO complex by simply removing the excess imidazole (Scheme 1). This regenerated β1(1−385)−NO complex had an electronic absorption spectrum identical to that of the original five-coordinate β1(1−385)−NO complex (data not shown), which has a Soret peak at 399 nm, a split α/β band, and a shoulder at 485 nm (*1*). Furthermore, if imidazole replaced NO as a ligand, the Soret peak would be expected to be shifted from 399 to 414 nm because the six-coordinate His−Im or Im−Im heme complex has a Soret maximum at 414 nm (Scheme 1). Second, the EPR study of the putative six-coordinate β1(1−385)−NO complex revealed a spectrum with nine distinct derivative-shaped lines under natural abundance isotope conditions, which is characteristic of a six-coordinate ferrous heme−NO complex with a nitrogen atom coordinated to the heme iron from the proximal side. The EPR spectrum of the putative six-coordinate β1(1−385)−NO complex exhibits rhombic symmetry ($g_1 = 2.082$, $g_2 = 2.01$, and $g_3 = 1.98$). The coupling constant associated with g_2 and the nitrogen of [^{14}N]NO is 22 ± 1 G, while the coupling constant for g_2 and the ^{14}N of the proximal Im is 7 ± 1 G. This spectrum is very similar to those of many well-characterized six-coordinate NO−hemoprotein complexes in terms of spectral shape, *g* values, and coupling constants (*24*, *27*). Added imidazole did not displace NO from the distal side of the heme. Gel filtration removed imidazole from the six-coordinate NO complex, but not NO. These results indicate that the affinity of NO for the sGC heme is very high. The only reported K_d for NO binding to the sGC heme is estimated to be less than 250 nM (*21*). High-affinity binding of NO to sGC may become an obstacle to deactivating the NO-activated sGC. Clearly, NO binding needs to be reversible if simple dissociation accounts for deactivation of the enzyme. At this point, the deactivation mechanism is not obvious.

Addition of imidazole to the five-coordinate β1(1−385)−NO complex leads to the formation of a six-coordinate β1-(1−385)−NO complex as discussed above. For the six-coordinate NO complex, one axial ligand is NO. What is the other axial ligand? As indicated by the EPR spectrum, the atom coordinated directly with the heme iron from the proximal side is a nitrogen atom (Figure 4). There are two candidates for the sixth ligand: a histidine residue from sGC or the added imidazole. The added imidazole may simply have caused a conformational change that drove the proximal histidine back to reform the Fe−H105 bond; alternatively, the proximal histidine may form a hydrogen bond with other amino acid residues in sGC when the bond between the proximal histidine and the heme iron is cleaved by NO binding to the heme. The added imidazole could disrupt this hydrogen bond, thereby freeing the proximal histidine to re-form a bond to the heme iron. The other possibility is that the added imidazole diffuses into the heme proximal pocket and itself forms an Fe−Im bond. To distinguish between these possibilities, [^{15}N]imidazole was used to form the six-coordinate β1(1−385)−NO complex. If the added imidazole coordinates directly to the heme, a simplified EPR spectrum would be expected because of the lower spin multiplicity of ^{15}N compared to that of ^{14}N. [^{15}N]Imidazole did indeed change the EPR spectrum from a triplet of triplets

to a triplet of doublets (Figure 4), which is expected when imidazole is coordinated directly to the heme (*24*). This result provides direct evidence that the sixth ligand is an imidazole molecule, and not a histidine residue from the protein. Formation of the six-coordinate NO complex with imidazole and NO as axial ligands is not just a property of this truncated β1 subunit fragment since addition of imidazole to the five-coordinate heterodimeric sGC−NO complex also leads to a shift of the Soret peak from 399 to 420 nm (data not shown).

It is not clear whether imidazole could replace the proximal histidine in the absence of NO. For such a complex to form, the bond to the proximal histidine which occupies the heme binding pocket and is coordinated to the heme iron has to be broken, and then the histidine residue would have to move from its original position so that an imidazole molecule could enter the heme binding pocket to form an Fe−Im bond from the proximal side. While unlikely, we cannot rule out this possibility.

Formation of a six-coordinate β1(1−385)−NO complex with an added imidazole molecule as the proximal ligand indicates that NO binding to the heme in sGC not only leads to the cleavage of the Fe−H105 bond but also opens the heme proximal pocket, creating a space large enough to accommodate an imidazole molecule. It is believed that the heme Fe is pulled out of the porphyrin plane toward the heme proximal side when the heme is five-coordinate, high-spin, ferrous. In deoxyMb, for example, the Fe(II) has moved 0.55 Å out of the heme plane toward the proximal side (*28*). When NO binds to the heme in sGC, the Fe−His bond is cleaved and it is speculated that, at the same time, the Fe is pulled out of the plane toward the distal side of the heme. When oxygen binds to the deoxyMb, Fe(II) is pulled toward the heme plane (0.22 Å out of the plane on the proximal side compared to 0.55 Å) (*29*). This movement of Fe out of the plane by NO may generate some space on the proximal side; however, it probably is not large enough to accommodate an imidazole molecule. Additional movement must occur in order for there to be enough space. Either the original proximal histidine or the heme plane has moved from their original place so that enough space is generated for an imidazole molecule to diffuse into the heme proximal pocket and form an Fe−Im bond.

What is the driving force that opens the heme proximal pocket when NO binds to the heme in sGC? It has been shown that the Fe−His bond is weaker in sGC than that of most hemoproteins (*14, 15*). Actually, the Fe−H105 stretch of sGC, observed at 204 cm^{-1}, is the lowest value observed for hemoproteins with a histidine as the proximal ligand (*15*). The very low frequency observed for the Fe−His stretching was attributed to the absence of a hydrogen bond between the His N^{δ} and other amino acid residues or H_2O (*15*). On the basis of the results presented here, another factor may contribute to the observed weak Fe−His bond. The sGC peptide chain may pull the proximal histidine away from the heme plane and prevent it from forming a strong Fe−His bond. If this is the case, formation of the Fe−His bond will generate a strain in the peptide chain. When NO binds to the heme in sGC from the distal side and breaks the Fe−His bond, the proximal histidine moves to release this strain, thus opening the heme proximal pocket.

sGC is activated by NO binding to its heme cofactor, although the activation mechanism is not fully understood. NO binding to sGC leads to the release of the proximal histidine and porphyrin structural changes that accompany the movement of the Fe out of the plane toward the distal side. It is proposed that the porphyrin structural change triggers a conformational change, which results in sGC activation (*30*). It is not clear whether the sGC activation is triggered by the release of the proximal histidine, by the porphyrin structure change, or by both. The function of the proximal histidine in NO activation is not clear. Our results presented in this paper indicate that the cleavage of the Fe−His bond leads to a conformational change that opens the proximal pocket. It is possible that the cleavage of the Fe−His bond is responsible for the NO activation and that the porphyrin structural change is not directly involved in activation. Recently, we found that when the proximal H105 in β1(1−385) was mutated to a glycine residue, heme binding to this mutant was restored by adding imidazole to the cell culture media and purification buffers (*14*). Such a mutant in heterodimeric sGC is under construction and will help in defining the function of the proximal histidine in NO activation. The β1(1−385)−NO H105G has the same electronic absorption spectrum (*14*) and resonance Raman spectrum (J. P. M. Schelvis, Y. Zhao, M. A. Marletta, and G. T. Babcock, manuscript in preparation) as wild type β1-(1−385) and heterodimeric sGC NO complexes. Since the proximal ligand is not covalently attached to the protein of the β1(1−385) H105G mutant, this mutation in heterodimeric sGC will be important for dissecting the contributions of proximal histidine and porphyrin structural changes to the NO activation.

When added to the five-coordinate β1(1−385)−NO complex, all methyl-substituted imidazoles except 2-methylimidazole (2-MeIm) form six-coordinate β1(1−385)−NO complexes and shift the Soret peak from 399 to 420 nm. As shown in Table 1, the affinity for the five-coordinate β1-(1−385)−NO complex decreases in the order 4-MeIm > Im > *N*-MeIm ≫ 2-MeIm. The same trend was observed for the affinity of methyl imidazoles for the nitrosyl complex of the myoglobin H93G heme ligand mutant (*31*). The extremely low affinity of 2-MeIm for the five-coordinate β1-(1−385)−NO complex is likely due to the steric interaction between the methyl group in 2-MeIm and the heme plane. Like the case of the myoglobin H93G mutant (*31*), basicities and the ability for hydrogen bond formation could be used to explain the different affinities of these three imidazole derivatives (4-MeIm, *N*-MeIm, and Im) for the five-coordinate β1(1−385)−NO complex.

In summary, NO binding to the heme in sGC not only leads to the cleavage of the Fe−His bond and the formation of a five-coordinate NO complex but also induces a conformational change that rearranges the structure of the heme proximal pocket. Understanding the structural changes in the heme proximal pocket and the iron porphyrin induced by NO binding to the heme is important for elucidating the NO activation mechanism.

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REFERENCES

1. Zhao, Y., and Marletta, M. A. (1997) *Biochemistry 36*, 15959−15964.
2. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev. 43*, 109−142.
3. Bredt, D. S., and Snyder, S. H. (1994) *Annu. Rev. Biochem. 63*, 175−195.
4. Garbers, D. L., and Lowe, D. G. (1994) *J. Biol. Chem. 269*, 30741−30744.
5. Wong, S. K.-F., and Garbers, D. L. (1992) *J. Clin. Invest. 90*, 299−305.
6. Lincoln, T. M., Komalavilas, P., Boerth, N. J., MacMillan-Crow, L. A., and Cornwell, T. L. (1995) *Adv. Pharmacol. 34*, 305−322.
7. Stone, J. R., and Marletta, M. A. (1995) *Biochemistry 34*, 14668−14674.
8. Garbers, D. L. (1979) *J. Biol. Chem. 254*, 240−243.
9. Gerzer, R., Böhme, E., Hofmann, F., and Schultz, G. (1981) *FEBS Lett. 132*, 71−74.
10. Kamisaki, 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.
11. Mulsch, A., and Gerzer, R. (1991) *Methods Enzymol. 195*, 377−383.
12. Stone, J. R., and Marletta, M. A. (1994) *Biochemistry 33*, 5636−5640.
13. Wedel, B., Harteneck, C., Foerster, J., Friebe, A., Schultz, G., and Koesling, D. (1995) *J. Biol. Chem. 270*, 24871−24875.
14. Zhao, Y., Schevis, J., Babcock, G. T., and Marletta, M. A. (1998) *Biochemistry 37*, 4502−4509.
15. Deinum, G., Stone, J. R., Babcock, G. T., and Marletta, M. A. (1996) *Biochemistry 35*, 1540−1547.
16. Tomita, T., Ogura, T., Tsuyama, S., Imai, Y., and Kitagawa, T. (1997) *Biochemistry 36*, 10155−10160.
17. Ignarro, L. J., Degnan, J. N., Baricos, W. H., Kadowitz, P. J., and Wolin, M. S. (1982) *Biochim. Biophys. Acta 718*, 49−59.
18. Stone, J. R., Sands, R. H., Dunham, W. R., and Marletta, M. A. (1995) *Biochem. Biophys. Res. Commun. 207*, 572−577.
19. Yu, A. E., Hu, S., Spiro, T. G., and Burstyn, J. N. (1994) *J. Am. Chem. Soc. 116*, 4117−4118.
20. Fan, B., Gupta, G., Danziger, R. S., Friedman, J. M., and Rousseau, D. L. (1998) *Biochemistry 37*, 1178−1184.
21. Stone, J. R., and Marletta, M. A. (1996) *Biochemistry 35*, 1093−1099.
22. Kon, H. (1975) *Biochim. Biophys. Acta 379*, 103−113.
23. Yoshimura, T., and Ozaki, T. (1984) *Arch. Biochem. Biophys. 229*, 126−135.
24. Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S., and Reed, G. H. (1972) *J. Biol. Chem. 247*, 2447−2455.
25. Kon, H., and Kataoka, N. (1969) *Biochemistry 8*, 4757−4762.
26. Taketa, F., Antholine, W. E., Mauk, A. G., and Libnoch, J. A. (1975) *Biochemistry 14*, 3229−3233.
27. Takahashi, S., Wang, J., Rousseau, D. L., Ishikawa, K., Yoshida, T., Host, J. R., and Ikeda-Saito, M. (1994) *J. Biol. Chem. 269*, 1010−1014.
28. Takano, T. (1977) *J. Mol. Biol. 110*, 569−584.
29. Phillips, S. E. V. (1980) *J. Mol. Biol. 142*, 531−554.
30. Ignarro, L. J., Ballot, B., and Wood, K. S. (1984) *J. Biol. Chem. 259*, 6201−6207.
31. Decatur, S. M., Franzen, S., DePillis, G. D., Dyer, R. B., Woodruff, W. H., and Boxer, S. G. (1996) *Biochemistry 35*, 4939−4944.

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