Resonance Raman Spectroscopy of Soluble Guanylyl Cyclase Reveals Displacement of Distal and Proximal Heme Ligands by NO

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"Why NO?", asked Traylor and Sharma,[1] referring to the discovery that nitric oxide is a signaling molecule for an expanding repertoire of physiological events, including blood pressure regulation, immune response, and neurotransmission.[2] The target for the NO signal is soluble guanylyl cyclase (sGC), an intracellular enzyme catalyzing the conversion of guanosine 5′-triphosphate (GTP) to 3′,5′-cyclic guanosine monophosphate (cGMP), a regulatory molecule in cellular function.[3] Nitric oxide activates sGC,[4] greatly increasing both the rate of product formation and the affinity for the substrate.[4a] The molecular mechanism of the activation process is unknown, although the presence of a heme moiety in sGC is required.[4b] Traylor and Sharma[1] pointed to the extraordinary affinity of NO for Fe(II) heme ($K \approx 10^{15}$) and to its labilizing effect on proximal (trans) ligands. They conjectured that activation of sGC results upon displacement of an axial ligand,[5] an event that could trigger a conformational change in the enzyme, thereby inducing activation. We present spectral evidence for the displacement of axial heme ligands in sGC by NO, demonstrating, for the first time, that changes in the heme coordination environment are correlated with activation of the enzyme.

Resonance Raman (RR) spectra of ferrous sGC (Fe^{II}sGC) and its NO and CO adducts, Fe^{II}sGC(NO) and Fe^{II}sGC(CO), establish that NO displaces *both* proximal and distal ligands. High-frequency RR spectra of Fe^{II}sGC, Fe^{II}sGC(CO), and Fe^{II}sGC(NO) are shown in Figure 1.[6] The high-frequency region of the RR spectrum[7] of Fe^{II}sGC shows two ν_3 bands, indicative of a mixture of five- and six-coordinate heme species. The relative intensities of these bands depend on the laser power (Figure 1, inset); the six-coordinate species is photolyzed to the five-coordinate form at higher laser power.[8] The distal (sixth) ligand must be provided by the protein, since water is unlikely to form a low-spin adduct with Fe(II) heme. The likeliest candidate for both proximal and distal ligands is histidine, given the similarity of the high-frequency RR spectrum of Fe^{II}sGC to that of the model complex Fe^{II}PPIX(ImH)$_2$ (PPIX, protoporphyrin IX; ImH, imidazole).[9] The photolability of the distal ligand is unusual; protein ligands normally recombine on the picosecond time scale,[10a] so steady-state photodissociation in the Raman laser is normally undetectable. A precedent is available, however, in the alkaline form of Fe(II) cytochrome *c* peroxidase (CCP), which is also low-spin, six-coordinate, and

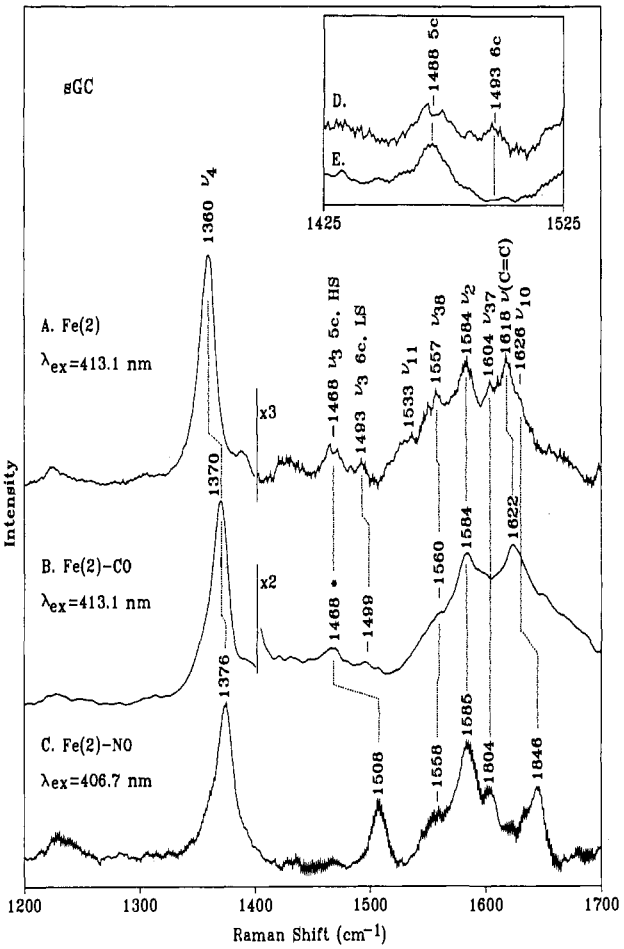

Figure 1. High-frequency resonance Raman spectra of Fe^{II}sGC (A), Fe^{II}sGC(CO) (B), and Fe^{II}sGC(NO) (C). The spectra were acquired as described in footnote 6. The labeling of the porphyrin bands conforms to the standard mode numbering scheme developed for nickel octaethylporphyrin and protoheme.[21] (A) Fe^{II}sGC, in 25 mM TEA-DTT, pH 7.8, 10 μM heme. (B) Fe^{II}sGC(CO), prepared by flushing sample A with CO. (C) Fe^{II}sGC(NO), prepared by flushing the tube containing the CO adduct with argon, introducing a small amount of NO, and photolyzing with a white lamp. The inset shows the effect of laser power on the intensities of the ν_3 band in Fe^{II}sGC. (D) The spectrum from A enlarged to show the ν_3 bands. (E) The same sample as D, recorded with the laser beam focused to a spot with a spherical lens.

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(5) Traylor and Sharma proposed that the binding of NO might free the proximal ligand to act as a catalytic base in the enzymatic reaction; however, the kinetics of activation are not consistent with this hypothesis. The enzyme is active in the absence of NO, and activation results in an increase in both K_m and V_{max}.[4a]

(6) Bovine lung soluble guanylyl cyclase was isolated as described elsewhere (Yu, A. E.; Hawkins, B. K.; Dawson, J. H.; Burstyn, J. N., manuscript in preparation). The enzyme was isolated in a heme-deficient form and reconstituted anerobically with Fe^{II}PPIX. Unbound Fe^{II}PPIX was removed by desalting on a Sephadex G-25 column. The spectra were recorded on samples of TEA (triethanolamine) buffer (25 mM), pH 7.8, 5 mM DTT (dithiothreitol), kept rigorously anaerobic. Resonance Raman spectra were acquired on a Spex 1877 triple spectragraph equipped with a cooled, intensified diode array detector (Princeton Instruments) from samples contained in a spinning NMR tube at room temperature. The incident laser light (20 mW) was focused onto the sample with a cylindrical lens, forming a 5-mm line source. Sloping base lines were corrected by numerical subtraction.

(7) The region 1350–1650 cm^{-1} contains porphyrin skeletal modes which are sensitive to the ligation mode and spin state of the heme. Spiro, T. G.; Li, X.-Y. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; Wiley: New York, 1988; Vol. 3, pp 1–38.

(8) The band position for the five-coordinate species in Fe^{II}sGC is at the same position as the analogous mode in the spectrum of Fe^{II}sGC(CO), which is also photolabile (see text). The band position for the six-coordinate form of Fe^{II}sGC is 6 cm^{-1} lower than that of the six-coordinate Fe^{II}sGC(CO), consistent with the known differences between six-coordinate low-spin hemes with and without a π-acid ligand like CO.

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Table 1. ν_3 and ν_{10} Frequencies (cm^{-1}) of Five- and Six-Coordinate Fe^{II}heme(NO) Adducts[a]

	ν_3	ν_{10}	ref
	5-c Fe^{II}–NO		
sGC	1508	1646	this work
Fe^{II}PPIX–NO	1508	1648	9
Hb + IHP		1644	16b
Mb (SDS/pH 9.2)	1512	1646	15
	6-c Fe^{II}–NO		
Mb	1501	1638	13
Hb	1500	1636	13
HRP	1501	1637	14

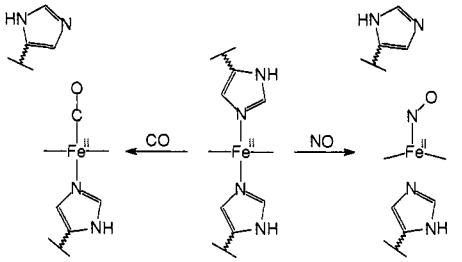
[a] Abbreviations: 5-c, five-coordinate; 6-c, six-coordinate; Mb, myoglobin; Hb, hemoglobin; HRP, horseradish peroxidase; PPIX, protoporphyrin IX.

photolabile.[10b] At high pH, a conformational change brings the distal histidine, which is 5.8 Å away from the iron in the acid form,[10c] into bonding contact with the iron. It was proposed that the iron–histidine bond in CCP is under tension due to conformational strain in the protein, and consequently the recombination rate following photolysis is lowered. Similar tension is implied for the iron–distal histidine bond in Fe(II)sGC and may be important for rapid binding of CO and NO, since prior dissociation of the distal ligand is required.

The low-frequency RR spectrum of the CO adduct of sGC (data not shown) exhibits a band at 495 cm^{-1} that is assigned to the stretching mode of the Fe–CO bond, ν(Fe–CO), on account of its 5-cm^{-1} downshift upon ^{13}CO substitution. The low frequency of the ν(Fe–CO) band in Fe^{II}sGC(CO) and the absence of an Fe–CO bending mode are both indicative of a linear CO ligand located in a hydrophobic pocket when the proximal ligand is histidine.[11] The Fe^{II}sGC(CO) adduct is photolabile, and the high-frequency RR spectrum (Figure 1B) contains two ν_3 bands at 1499 and 1468 cm^{-1}, corresponding to six-coordinate low-spin and five-coordinate high-spin hemes, respectively. The latter species arises from a small amount of photodissociation of the CO in the Raman laser beam.[12] All other Raman bands are at positions expected for a six-coordinate low-spin CO-ligated heme. Based on these spectral data, we conclude that the Fe^{II}sGC(CO) adduct is six-coordinate and that histidine is the likely proximal ligand.

The NO adduct of Fe^{II}sGC, Fe^{II}sGC(NO), shows exceptionally high frequencies for ν_3 and ν_{10}, characteristic of five-coordinate nitrosyl heme without any proximal ligand. The positions of these bands are coincident with those of the five-coordinate model complex, Fe^{II}PPIX(NO), whereas the corresponding frequencies for the NO adducts of myoglobin (Mb),[13] hemoglobin (Hb),[13] and horseradish peroxidase (HRP)[14] that are six-coordinate with proximal histidine ligands are 7–10 cm^{-1} lower (Table 1). Comparison of the spectral features of Fe^{II}sGC(NO) with those of known five-coordinate NO complexes of Mb[15] and Hb[16] further

Scheme 1



supports the formulation of Fe^{II}sGC(NO) as a five-coordinate nitrosyl heme (Table 1). We searched unsuccessfully for the ν(Fe–NO) band of Fe^{II}sGC(NO). This negative evidence is itself consistent with a five-coordinate structure,[17] since ν(Fe–NO) has not been observed for five-coordinate nitrosyl hemes.[13] On the other hand, the ν(Fe–NO) mode has been observed near 550 cm^{-1} for six-coordinate nitrosyl hemes.

These data suggest a mechanism for the activation of sGC by NO involving displacement of both proximal and distal histidine ligands (Scheme 1). Ligand displacement upon NO binding could trigger a conformational change leading to activation of the enzyme. This mechanism is consistent with the observation that PPIX, the metal-free cofactor, can activate sGC to the same extent as NO[18] since it is unable to bind either axial ligand. Activation of sGC by CO has been reported, although the extent of activation was significantly less (at least 10-fold) for CO than for NO.[19] We have never observed activation of sGC by CO,[20] although the displacement of the distal histidine ligand could provide a mechanism by which CO could activate the enzyme. In summary, the data presented here provide the first characterization of the coordination environment of the heme in sGC and suggest that proximal as well as distal ligand displacement by NO is intimately involved in the activation process.

Acknowledgment. This paper is dedicated to the memory of Teddy Traylor, whose inspiration and support will be greatly missed. This work was supported by a Grant-in-Aid from the American Heart Association (J.N.B.) and by NIH Grant GM33576 (T.G.S.). Fellowship support from the Department of Education (A.E.Y.) is gratefully acknowledged. We acknowledge the expert technical assistance of Dr. Robert M. Petrovich, Thomas D. Kim, and Elizabeth A. Dierks.

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(12) The extent of photodissociation is actually less than that suggested by the band intensities because the 1468-cm^{-1} band has a much higher enhancement factor.[10b] Photodissociation also produces band broadening in other Raman bands due to unresolved five-coordinate contributions.

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(17) The absence of a detectable ν(Fe–NO) band for five-coordinate nitrosyl hemes may reflect the shift of the Soret absorption band to higher energy upon loss of the proximal ligand. This shift away from the available krypton laser wavelengths decreases the resonance enhancement in the RR experiment. In the case of Fe^{II}sGC(NO), the Soret band is at 399 nm, while that of Fe^{II}sGC(CO) is at 426 nm.

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