

Nitric Oxide Myoglobin: Crystal Structure and Analysis of Ligand Geometry

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ABSTRACT The structure of the ferrous nitric oxide form of native sperm whale myoglobin has been determined by X-ray crystallography to 1.7 Å resolution. The nitric oxide ligand is bent with respect to the heme plane: the Fe-N-O angle is 112°. This angle is smaller than those observed in model compounds and in lupin leghemoglobin. The exact angle appears to be influenced by the strength of the proximal bond and hydrogen bonding interactions between the distal histidine and the bound ligand. Specifically, the N_ε atom of histidine⁶⁴ is located 2.8 Å away from the nitrogen atom of the bound ligand, implying electrostatic stabilization of the FeNO complex. This interpretation is supported by mutagenesis studies. When histidine⁶⁴ is replaced with apolar amino acids, the rate of nitric oxide dissociation from myoglobin increases tenfold. *Proteins* 30:352–356, 1998. © 1998 Wiley-Liss, Inc.

Key words: myoglobin; nitric oxide; ligand binding; X-ray crystallography

INTRODUCTION

Nitric oxide functions as a messenger molecule in such diverse biological functions as neurotransmission, vascular relaxation, and inhibition of platelet aggregation.^{1–5} and references therein Heme proteins show an affinity for nitric oxide, which is much higher in the ferrous ($K_{\text{NO}} \approx 2 \times 10^{11} \text{ M}^{-1}$) than in the ferric oxidation state ($K_{\text{NO}} \approx 1 \times 10^4 \text{ M}^{-1}$). Nitric oxide can also act as either an oxidant or reductant in reactions with hemoproteins. Under anaerobic conditions a second molecule of nitric oxide reacts with ferric NO myoglobin to produce the nitric oxide complex of the ferrous protein.⁶ Nitric oxide also reacts rapidly with oxymyoglobin to produce metmyoglobin and nitrate.⁷

Studies of naturally occurring myoglobins and hemoglobins without a distal histidine^{8,9} have suggested that this residue plays a key role in regulating both the rate and equilibrium constants for nitric oxide binding. Mutagenesis studies have shown that the distal histidine in myoglobin inhibits bimolecular entry into the protein by stabilizing a water molecule in the deoxy heme pocket and lowers the dissociation rate constant by forming a hydrogen bond to the ligand (Eich, R.F., et al., unpublished data).^{10,11}

In contrast to the large number of dioxygen and carbon monoxide myoglobin and hemoglobin crystallographic studies, much less structural information is available for nitric oxide adducts. Several square pyramidal N₄-macrocyclic iron nitrosyl model complexes have been determined. Nitric oxide bound to ferrous tetraphenylporphyrin compounds assumes a bent (end-on) geometry, with Fe-N-O angles ranging from 131° to 149°.12–15 An angle of 144° was also reported for a pentacoordinate FeNO complex of a nonporphyrin N₄-macrocycle.¹⁶ The structure of horse nitric oxide hemoglobin has been determined to 2.8 Å,¹⁷ but the unrefined angle of 145° was based largely on one of the porphyrin model compounds. More recently, the structure of the nitric oxide complex of ferrous lupin leghemoglobin has been solved,¹⁸ and the Fe-N-O angle was reported to be 147°. The nitric oxide adduct of a similar heme protein, yeast cytochrome *c* peroxidase, was refined with a disordered ligand showing angles of 125° and 135° for the two Fe(III)NO conformers.¹⁹ Because of its central importance in both muscle physiology and its use as a heme protein prototype, we thought it would be important to determine the structure of the ferrous nitric oxide form of native sperm whale myoglobin.

MATERIALS AND METHODS

Crystallization and Manipulation

Native sperm whale myoglobin (Sigma, St. Louis, MO) was crystallized in a temperature-controlled environment (17°C) by the batch method. One part of a 60 mg/ml solution of protein in unbuffered deionized water was mixed with roughly three parts saturated aqueous ammonium sulfate. The resulting dark brownish-red aquometmyoglobin crystals were sealed into an airtight 4-ml vial containing 3 ml of a 100 mM phosphate, 300 mM sodium fluoride, 85%

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saturated ammonium sulfate solution. This solution was bubbled with nitrogen gas, and the crystals were allowed to soak for 48 hours, during which they took on a greenish cast. The gas space in the vial was again flushed with nitrogen and then charged with one atmosphere of nitric oxide twice, keeping an interval of 24 hours between charges to allow the nitric oxide to equilibrate with the dissolved nitrogen gas and to allow reduction to the ferrous MbNO complex.^{9,17} The vial was brought into an anaerobic glove box, and the now bright red crystals were mounted in sealed quartz capillary tubes before removal from the oxygen-free atmosphere. Our reduction procedure was adapted from that described by Deatherage and Moffat¹⁷ for the preparation of nitric oxide hemoglobin crystals.

Data Collection

A complete data set was collected from one single crystal at room temperature by using copper K α radiation from a Siemen's rotating anode operated at 50 mV/90 mA and a Rigaku R-axis IIC imaging plate system. The crystal was monoclinic, space group P2₁, as originally solved by Kendrew et al.²⁰ The unit cell dimensions were $a = 64.45 \text{ \AA}$, $b = 29.44 \text{ \AA}$, $c = 35.33 \text{ \AA}$, and $\beta = 106.3^\circ$. A total of 56,610 measured reflections from 120 images ($\Delta\omega = 1.5^\circ$) were reduced to 11,674 unique intensities with an R_{merge} of 4.4% (from 30 to 1.7 \AA , 80.1% complete) using the XDS²¹ software package (Table I).

Structure Determination and Refinement

The ferrous nitric oxide myoglobin structure was determined by using starting coordinates for cobalt oxymyoglobin²² (Brookhaven Protein Data Bank entry 1YOI) to calculate initial phases. The coordinates for the nitric oxide ligand were built from an electron density omit map. Solvent atoms were introduced in positive density peaks over four sigma. Cycles of conventional positional refinement against data from 5.0 to 1.7 \AA were carried out by using X-PLOR²³ alternated with manual fitting using the graphics software CHAIN. A final conjugate gradient least squares refinement was accomplished with SHELXL.²⁴ Engh and Huber²⁵ topology and parameters were used throughout the refinement process, but no restraints were placed on the iron or ligand to remove bias in ligand position. The crystallographic refinements converged to a final R of 16.6% (R_{free} of 25.2%) with root mean square bond deviations of 0.005 \AA (Table I). The atomic coordinates and structure factors (entry 1HJT) for ferrous nitric oxide sperm whale myoglobin have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

Data Analysis

The error in the positions of the atoms, and the angles between, are estimated by their standard

TABLE I. Selected Nitric Oxide Myoglobin Crystallographic Data (no sigma cutoff)

Overall (30.0 to 1.7 \AA)	
Total measured reflections	56,610
Number of unique reflections	11,674
Completeness for range (%)	80.1
Data redundancy	3.1
Merging R value (%)	4.4
Mean I/sigma (I)	32.7
Highest resolution shell (1.8 to 1.7 \AA)	
Completeness for range (%)	73.1
Data redundancy	2.5
Merging R value (%)	24.4
Mean I/sigma (I)	6.4
Space group	P2 ₁
Unit cell	$a = 64.45 \text{ \AA}$ $b = 29.44 \text{ \AA}$ $c = 35.33 \text{ \AA}$ $\beta = 106.3^\circ$
Refinement (5.0 to 1.7 \AA)	
Total unique reflections	10,947
Free R reflections	1,094
Completeness for range (%)	79.9
R value (working set) (%)	16.2
Free R value (test set) (%)	25.2
R value (working + test set) (%)	16.6
Root mean square deviations	
Bond lengths	0.005 \AA
Bond angles	1.593 $^\circ$
Dihedral angles	20.837 $^\circ$
Improper angles	1.421 $^\circ$

deviations based on a SHELXL full matrix least squares refinement. The method of Kabsch²⁶ was used to superpose atomic coordinates by overlaying the aromatic carbons and nitrogens of the heme. The graphics program RIBBONS,²⁷ as modified by Tod Romo, was used to display the structures.

RESULTS AND DISCUSSION

We prepared ferrous nitric oxide myoglobin crystals by the reductive nitrosylation of ferric metmyoglobin, introducing NO gas (after sodium fluoride) to crystals of the oxidized protein. The structure of the active site in nitric oxide myoglobin is shown in Figure 1. The classic "globin fold" of the protein remains unaffected by the bound nitric oxide, and the positions of the distal pocket residues are similar to those in dioxygen and carbon monoxide myoglobin structures. The most notable feature is the bent geometry of the iron-ligand complex, with an Fe-N-O angle of 112 $^\circ$, signifying that NO is acting as a one electron donor. This small angle, predicted to be 110 $^\circ$ in ferrous nitric oxide hemoglobin,^{28†} correlates with the presence of a short, strong axial bond opposite

[†]Reanalysis of the nitric oxide myoglobin room temperature single crystal electron paramagnetic resonance data also shows the Fe-N-O angle to be 110 $^\circ$ (unpublished work based on Hori, H., Ikeda-Saito, M., Yonetani, T. J. Biol. Chem. 256:7849–7855, 1981).

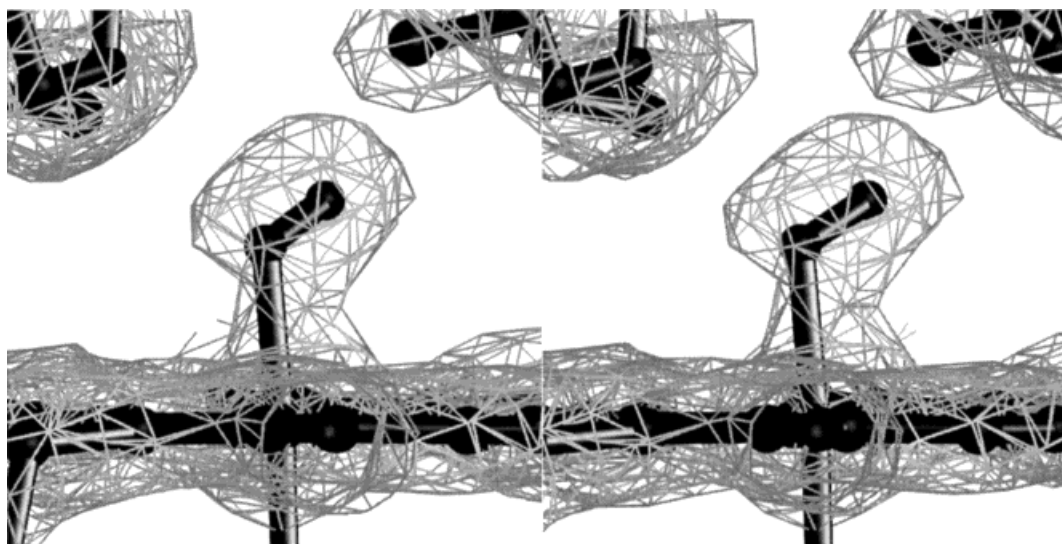


Fig. 1. Stereoview of the native sperm whale nitric oxide myoglobin structure. The view, perpendicular to the heme plane, is of the ligand bound in the active site with coordinates in black and electron density, contoured at 1.2 sigma, in silver.

TABLE II. Selected Data from Square Planar Iron-Nitric Oxide Crystal Structures

Compound [†]	Proximal-Fe	Fe-N	N-O	Fe-N-O	Fe out-of-heme-plane	Reference
Fe ^{II} (NO)N ₄ L	—	1.72 Å	1.17 Å	144°	0.39 Å	17
Fe ^{II} (NO)TPP	—	1.72	1.12	149	0.21	13
Fe ^{II} (NO)TpivPP [‡]	—	1.72	1.20, 1.26	143, 131	0.27	16
Fe ^{II} (NO)TPP(4-MePip)	2.46 Å	1.74	1.11	144	0.09	15
Fe ^{II} (NO)TPP(4-MePip) · CHCl ₃	2.33	1.72	1.14	139	0.08	15
Fe ^{II} (NO)TPP(1-Melm) [‡]	2.18	1.74	1.14, 1.12	138, 142	0.05	14
Horse hemoglobin(NO)	n.a.	1.74	1.1	145	0.07	18
Lupin leghemoglobin(NO)	2.22	1.72	1.22	147	-0.01	19
Sperm whale myoglobin(NO)	2.18 ± 0.03	1.89 ± 0.04	1.15	112 ± 5	0.00 ± 0.01	This work
Fe ^{III} (NO)TPP(OH ₂) · ClO ₄	2.00	1.65	1.15	174	<0.05	[§]
Fe ^{III} (NO)OEP · ClO ₄	—	1.64	1.11	177	0.29	[§]
Yeast cytochrome c peroxidase(NO) [‡]	2.04	1.82	n.a.	125, 135	n.a.	19

[†]N₄L, tetramethyldibenzotetraazacyclotetradecine; TPP, tetraphenylporphyrin; TpivPP, tetrakis(pivalamidophenyl)porphyrin; 4-MePip, 4-methylpiperidine; 1-Melm, 1-methylimidazole; OEP, octaethylporphyrin, n.a., not available.

[‡]Disorder in the nitric oxide oxygen.

[§]Scheidt, W.R., Lee, Y.J., Hatano, K. J. Am. Chem. Soc. 106:3191–3198, 1984.

that of the Fe-NO bond. An increase in the proximal bond length would predict a decrease in the Fe-NO bond length and an increase in the Fe-N-O angle due to increasing π -bonding between the metal and ligand.¹³ However, the histidine⁹³-iron bond does not lengthen in nitric oxide myoglobin compared with other liganded forms of the protein, and there is no displacement of the iron out of the heme plane toward the bound NO. The angle of the nitric oxide ligand may also be influenced by electrostatic interactions with the distal histidine. The N ϵ atom of histidine⁶⁴ is located 2.8 Å away from the nitrogen atom and 3.4 Å away from the oxygen atom of the bound ligand, implying favorable hydrogen bonding.

The Fe-N-O angle for nitric oxide myoglobin is significantly smaller than that observed in model

compounds and in lupin nitric oxide leghemoglobin (Table II). This difference could be attributed to both proximal and electrostatic effects. Unlike myoglobin, the bond between the proximal histidine and the iron in leghemoglobin is lengthened in the NO complex compared with that for the O₂ or CO forms of leghemoglobin. This lengthening suggests more back-bonding and would explain the larger Fe-N-O angle.¹⁸ However, the absolute values of the iron-histidine⁹³ bond length in lupin nitric oxide leghemoglobin and the corresponding bond lengths in the model compounds are similar to that observed in sperm whale nitric oxide myoglobin. In lupin leghemoglobin the distal histidine does not appear to influence the geometry of bound NO because the N ϵ atom of this residue is located 3.3 Å away from either the N or O

TABLE III. Parameters for Nitric Oxide Binding to Ferrous Sperm Whale Myoglobin (pH 7, 20°C)[†]

Protein	k'_{NO} ($\text{M}^{-1}\text{s}^{-1}$)	k_{NO} (s^{-1})	K_{NO} (M^{-1})
Native	21 ($\times 10^6$)	1.0 ($\times 10^{-4}$)	21 ($\times 10^{10}$)
Wild Type [‡]	22	0.98	22
His64Gln	43	1.1	39
His64Gly	220	8.0	28
His64Ala	150	12	13
His64Val	270	11	25
His64Phe	57	3.1	18
His64Leu	190	1.3	150

[†]These data were measured by Eich, R.F., et al., unpublished data and Ref. 11.

[‡]Recombinant myoglobin from synthetic gene with initiator methionine and residue 122 replaced with asparagine. All following mutants are derived from this wild-type protein.

atom. This lack of electrostatic interaction may also help explain why the angle in the lupin protein is closer to those observed in simple model compounds.

Crystal lattice effects may also contribute to the differences in the Fe-N-O angle between nitrosyl structures (Table II). We have determined a preliminary crystallographic structure of a mutant nitric oxide myoglobin (L29F) in the more loosely packed P6 space group and a larger Fe-N-O angle of 130° is observed (Brucker, E.A., Phillips, G.N., Jr., unpublished data). The Fe-C-O angle in the P6 crystal form of recombinant carbon monoxide myoglobin (169° in 2MGK)²⁹ is also significantly larger than that observed in the closely packed P2₁ crystal form of native carbon monoxide myoglobin (134° in 1VXF).³⁰ Infrared polarization measurements predict much larger (almost linear) iron-ligand angles than those found by X-ray crystallography; however, recent theoretical work has suggested that the IR transition dipole correlates with the metal d to ligand π^* backbonding rather than with the ligand diatomic bond.³¹ The extent of ligand angle distortion caused by crystal packing is still not clear.

The functional effects of the hydrogen bond between the distal histidine and bound nitric oxide can be seen by the changes in the rate and equilibrium constants for ligand binding when histidine⁶⁴ is mutated (Table III) (Eich, R.F., et al., unpublished data)¹¹ With the exception of the histidine⁶⁴ to leucine mutation,[‡] none of the substitutions cause a marked change in NO affinity. However, both the association and dissociation rates increase 10-fold when histidine⁶⁴ is replaced with apolar amino acids.

[‡]The leucine⁶⁴ substitution produces an unusually high nitric oxide affinity which is analogous to the abnormally high K_{CO} value observed for this mutant. The cause of the dramatic leucine effect has not yet been established.

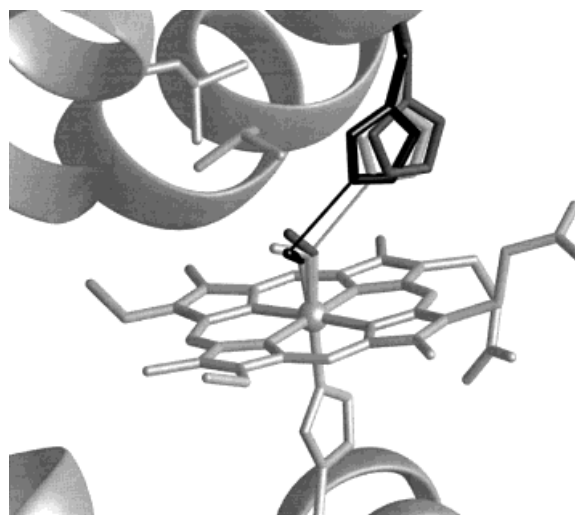


Fig. 2. Superposition of native sperm whale myoglobins. The nitric oxide structure (silver), highlighting the heme and residues leucine²⁹, distal histidine⁶⁴, valine⁶⁸, and proximal histidine⁹³, is overlaid with the molecular oxygen (1MBO, black) and carbon monoxide (1VXF, gray) ligand and distal histidine coordinates. Hydrogen bonds, interatomic distances under 3.0 Å, are represented by thin lines.

The association rate constants increase because an inhibitory water molecule is not present in the binding site of the deoxy forms of apolar mutants.³² The 10-fold increase in the dissociation rate constant is presumably due to the loss of hydrogen bonding to bound NO. The lack of effect on K_{NO} with apolar mutations at position 64 suggests that the free energy required to displace noncoordinated water from the distal pocket of deoxymyoglobin is roughly equal in magnitude to that released by formation of the hydrogen bond between the distal histidine and the bound nitric oxide.¹⁰ In contrast, the affinity of myoglobin for oxygen drops 100-fold with apolar substitutions at position 64.³³ These results suggest that the hydrogen bond between bound oxygen and the distal histidine is about 100 times stronger than the electrostatic interactions that hold water in the distal pocket of deoxy myoglobin and that stabilize bound NO.¹⁰

A comparison of the native sperm whale nitric oxide myoglobin coordinates with the structures of native sperm whale dioxygen and carbon monoxide myoglobin is shown in Figure 2. Nitric oxide (as a one electron donor) and molecular oxygen prefer a bent coordination geometry in organometallic compounds, whereas carbon monoxide prefers a more linear geometry. However, all three apolar diatomic gases appear to adopt similar geometrical positions in myoglobin.⁸ The main differences in these three liganded structures are the interactions between the

[§]The Fe-C-O angle in carbonmonoxymyoglobin does become more linear in the P6 crystallographic lattice (see above).

bound ligand and the distal histidine. The N_ε atom of histidine⁶⁴ is within hydrogen bonding distance to both atoms of molecular oxygen (2.8 Å and 3.0 Å) and to the N atom of nitric oxide (2.8 Å versus 3.4 Å to the oxygen atom). The interaction with bound carbon monoxide appears weaker with distances of 3.1 and 3.3 Å between N_ε and the C and O atoms, respectively. These distances are in agreement with the previously estimated electrostatic stabilization of the bound ligands by histidine⁶⁴: 1,000, 10, and threefold for O₂, NO, and CO, respectively (Eich, R.F., et al., unpublished data).^{10,11} These structural and functional correlations support the emerging theme of electrostatic effects as the dominant factor in regulating ligand binding to myoglobin and hemoglobin.

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