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Linkage Isomerism

Distal Pocket Control of Nitrite Binding in Myoglobin**

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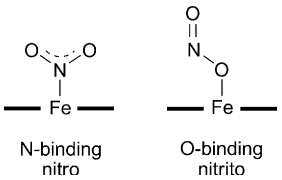
An important function of heme protein distal pockets is to orient substrates to allow selective reactions involving heme iron. The mammalian muscle protein myoglobin (Mb) has been utilized successfully as a model for distal pocket control of heme protein structure–function relationships.[1] Herein, we unambiguously demonstrate that the Mb active site pocket alters the preferred binding mode of a ligand from what is normally observed in its bioinorganic non-protein model.

The interaction of the nitrite anion (NO_2^-) with Mb has a long and fascinating history.[2] The curing of meat with nitrite, a century-old process, results primarily from the reaction of Mb with nitrite to generate the red-pink pigment derived from MbNO that is appealing in color to the meat purchaser.[3,4] The ability of Mb to reduce nitrite to bioactive nitric oxide, a vasodilator for blood pressure control, received renewed attention when it was shown that nitrite was protective against heart attacks in $Mb^{+/+}$ mice, but not in $Mb^{-/-}$ knockout mice, implicating this heme protein as a genuine nitrite reductase (NiR) protein under hypoxic conditions.[5] Although it had been assumed for decades that nitrite binds to Mb through the N-binding mode, the X-ray crystal structure of the ferric Mb–nitrite adduct revealed an unexpected O-binding mode (Mb(ONO)) that was, at the time, the sole example of nitrite O-binding to any heme protein.[6]



The occurrence of this rare nitrite O-binding mode led us to ask the following question: can we generate a nitrite N-binding mode for Mb to make it more akin to those observed in the few X-ray crystal structures determined for the nitrite adducts of genuine NiR enzymes, such as cyt cd_1 NiR,[7] cyt *c* NiR,[8,9] and other heme proteins?[10–13] Earlier attempts at achieving an N-bound nitrite structure in Mb failed, and these included chemical reduction[6] and photoreduction[14] of the ferric d^5 Mb(ONO) complex. The interaction of the nitrite N-atom with the heme Fe in Mb was demonstrated in the H64 V distal pocket mutant, but the 2.6 Å long Fe–NO_2 bond suggested only a very weak (electrostatic) interaction that correlated with a very slow nitrite reduction activity.[15]

The nitrite ligand binds to synthetic iron porphyrins almost exclusively through the N-binding mode.[16–18] Thus, we hypothesized that inserting a pre-formed heme-NO_2 complex into apoMb would retain the nitrite N-binding mode observed in the protein-free iron porphyrin nitrite compounds, and that the use of a reduced porphyrin (for example, a chlorin (Chl)) would maximize the chance of retaining the N-binding mode of nitrite because of the softer Fe center. Herein, we report the successful generation and crystallization of the first N-bonded nitrite Mb complex using the wild type protein and a chlorin macrocycle. We show that either the N-bonded or O-bonded structure can be achieved depending on the order of cofactor and nitrite addition to the protein. To the best of our knowledge, this is the first demonstration of NOx linkage isomerism in the same heme protein.

Addition of nitrite to the chlorin compound FeMPPaCl (MPPa = pyropheophorbide-a methyl ester; Supporting Information, Figure S1)[19] and insertion of the product into apoMb generated a MbChl–nitrite derivative (compound **A**); the UV/Vis spectrum of this species showed bands at 419 nm and 652 nm (Figure 1 a).[20–22] Compound **A** is stable in dilute solution for at least three days when stored at 4 °C, as judged by UV/Vis spectroscopy.

In contrast, addition of nitrite to a solution of aquomet chlorin-substituted Mb (MbChl(H_2O); $\lambda = 417$ and 656 nm)[23] resulted in the formation of a MbChl–nitrite species (compound **B**) with absorption bands at 422 and 662 nm (Figure 1 b), suggesting a different interaction of the nitrite ligand with the metal center. Compound **B** is also stable in dilute solution at 4 °C for at least three days, as judged by UV/Vis spectroscopy. Interestingly, UV/Vis spectral monitoring of the reaction of compound **A** with excess nitrite in solution reveals a rapid change in its spectrum to that of compound **B**.

We were successful in obtaining crystals of both compounds **A** and **B** suitable for an X-ray diffraction study. Crystals of compound **A**, obtained from the addition of preformed FeMPPa(NO_2) to apoMb, were obtained by the hanging-drop method (details are given in the Experimental Section and in the Supporting Information). The X-ray crystal structure of compound **A** identifies it as MbChl(NO_2), which contains the nitrite ligand in a predominantly N-bonded conformation (Figure 2).[24] The $2F_o-F_c$ electron density map and the F_o-F_c omit electron density map fit a final model in which the N atom of the nitrite ligand contacts the Fe center,

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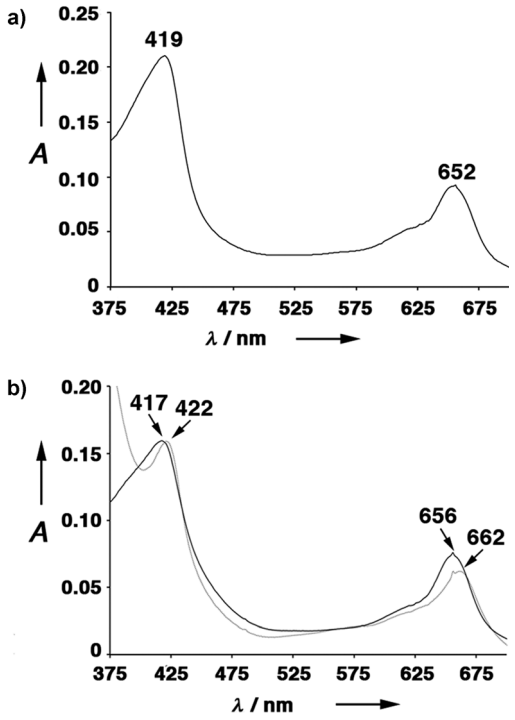

Figure 1. UV/Vis spectra of a) ferric hh MbChl–nitrite derivative compound **A** in the absence of excess nitrite, and b) ferric hh MbChl(H_2O) (solid line) and hh MbChl–nitrite derivative compound **B** (dashed line). Conditions: 30 mM Tris buffer, 1 mM EDTA, pH 7.4.

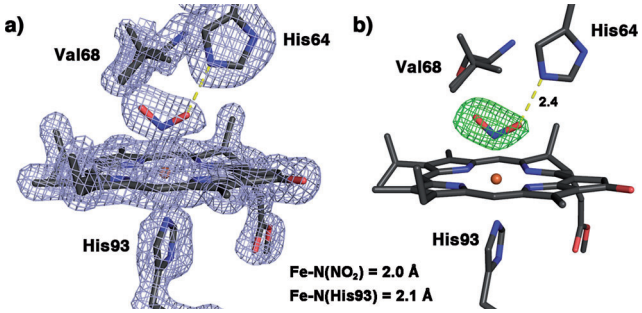

Figure 2. The heme site of the 1.65 Å resolution structure of MbChl-(NO_2) compound **A**. a) The $2F_o-F_c$ electron-density map (contoured at 1σ) and the final model; b) The F_o-F_c omit electron density map (contoured at 3σ) and the final model.

with an Fe–N(nitrite) bond distance of 2.0 Å. This distance is similar to that determined in the N-bound nitrite adducts of cyt *c* NiR (1.9 Å; 1.60 Å resolution),[8,9] nitrophorin 4 (2.0 Å; 1.40 Å resolution),[11] cyt cd_1 NiR (2.0 Å; 1.8 Å resolution),[7] and sulfite reductase hemoprotein (2.0 Å; 2.1 Å resolution).[10] The nitrite ligand plane essentially bisects a pair of porphyrin nitrogen atoms, and the nitrite ligand is stabilized in this binding mode by a strong hydrogen bond with the distal His64 ligand ($O\cdots N_{His} = 2.4$ Å; $Fe-N-O\cdots N_{His}$ torsion angle of 156°). The chlorin ester group is oriented in the direction of the proximal pocket and is hydrogen-bonded to the proximal Ser92 residue ((ester)O···O(Ser92) = 2.8 Å; Supporting Information, Figure S2). Importantly, the crystal structure of MbChl(NO_2) is the first demonstrated N-binding of nitrite to the wild-type (wt) protein of Mb.

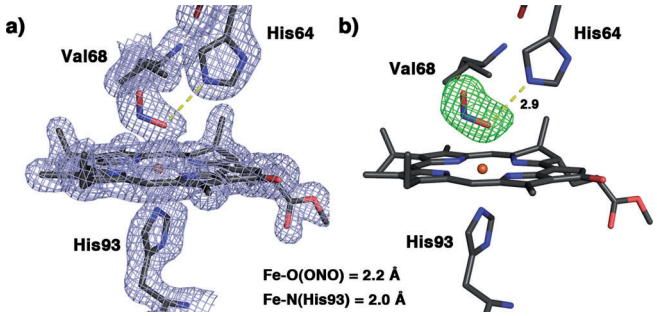

Figure 3. The heme site of the 1.65 Å resolution structure of MbChl-(ONO) compound **B**. a) The $2F_o-F_c$ electron density map (contoured at 1σ) and the final model; b) The F_o-F_c omit electron density map (contoured at 3σ) and the final model.

The crystal structure of compound **B** is shown in Figure 3. The $2F_o-F_c$ electron density map and the F_o-F_c omit electron density map fit a final model in which the nitrite ligand is predominantly O-bonded to the metal center in this MbChl-(ONO) complex.[25] There are several important points to note from the comparison of the two MbChl–nitrite structures: 1) these represent a remarkable nitrite linkage isomerism (N-bound vs O-bound) in the same protein; 2) the portion of the chlorin macrocycle near the protein exterior is shifted slightly upward in the MbChl(ONO) structure versus that in the MbChl(NO_2) structure, but with very little change in the positions of the amino acid residues that contact the macrocycle plane (Supporting Information, Figure S3 and S4); and 3) the chlorin ester group moves from a proximal position in MbChl(NO_2) (Figure 2) further towards the exterior of the protein in MbChl(ONO), losing the direct hydrogen-bonding interaction with Ser92 while forming a new hydrogen-bonding interaction with a H_2O molecule ((ester)O···OH_2 = 2.5 Å; Supporting Information, Figure S2). The O-binding of nitrite in the distal pocket of MbChl(ONO) is similar to that determined for wt Mb(ONO)[6] and for Hb(ONO) (Hb = hemoglobin);[26] these compounds were similarly prepared by the addition of nitrite to the holoproteins.

In conclusion, our results demonstrate that both N-binding and O-binding of nitrite are possible in the MbChl distal pocket. The strong hydrogen-bonding interaction between the N-bonded nitrite ligand and the distal His64 residue is consistent with proposed mehanisms for NiR enzymes that involve participation of H-donors in their active sites.[27] Our results also show that, although the N-binding mode of nitrite is preferred in bioinorganic (non-heme) model compounds and can be generated by the addition of FeMPPa(NO_2) to apoMb, the O-binding mode is preferred when nitrite is added to the holoprotein MbChl. This strongly suggests that the protein distal pocket residues are the prime directors of the nitrite binding mode in MbChl.

Experimental Section

Horse-heart Mb was purchased from Sigma, and Fe pyropheophorbide-a methyl ester chloride (FeMPPaCl) was purchased from Porphyrin Products (Utah).

Ferric MbChl(NO_2): A solution of FeMPPaCl (2 mg) and $NaNO_2$ (5 mg) in pyridine/H_2O (0.12 mL; 1:1 ratio) was vortexed to generate a solution of the chlorin nitrite product. The solution was added dropwise to a solution of apoMb (15 mL; 2–3 mM), the mixture stirred over ice for 1 h, and then dialyzed against Tris buffer (30 mM, pH 7.4, containing 1 mM EDTA) overnight. The product was purified by passing it through a G25 column, and concentrated to 0.6 mL. Crystals of the MbChl(NO_2) product were obtained by the hanging-drop method; the drops consisted of the purified protein solution (4 μL) with an equal volume of well solution (2.60 M $(NH_4)_2SO_4$, 100 mM TrisHCl, 1 mM EDTA, pH 7.4). Suitable crystals were harvested with cryoloops, passed through a cryoprotectant containing the well solution and 10% glycerol, then flash-frozen in liquid nitrogen prior to X-ray data collection.

Ferric MbChl(ONO): Excess $NaNO_2$ was added to a solution of ferric MbChl(H_2O) ($\lambda = 417$ and 656 nm)[23] in Tris buffer (30 mM, pH 7.4, containing 1 mM EDTA) to generate the MbChl(ONO) derivative ($\lambda = 422$ and 662 nm). The ferric MbChl(H_2O) compound is not stable,[23] and we were not able to obtain crystals for subsequent soaking with nitrite. We were successful in obtaining crystals of the MbChl(ONO) derivative by introducing nitrite during the preparation of MbChl(H_2O) to generate a MbChl–nitrite adduct that was then crystallized in the presence of excess nitrite. The resulting crystals were anaerobically soaked in a solution of the cryoprotectant (10 μL) and excess $NaNO_2$ for 1 day. The crystals were then harvested and stored as described above.

Crystal data: Ferric hh MbChl(NO_2): monoclinic, space group $P2_1$, $a = 34.3$, $b = 30.4$, $c = 64.1$ Å, $\beta = 105.0$, resolution range = 27.26–1.65 Å, unique reflections = 15230, completeness = 97.5(94.5)%, $I/\sigma(I) = 16.8(5.9)$, $R_{merge} = 0.038(0.143)$, $R(R_{free}) = 0.184(0.232)$. Ferric hh MbChl(ONO): monoclinic, space group $P2_1$, $a = 34.4$, $b = 30.5$, $c = 63.6$ Å, $\beta = 105.0$, resolution range = 26.45–1.65 Å, unique reflections = 15443, completeness = 98.7(95.8)%, $I/\sigma(I) = 17.8(5.3)$, $R_{merge} = 0.035(0.163)$, $R(R_{free}) = 0.193(0.218)$. The data in brackets refer to the highest resolution shells. Protein data bank (PDB) accession codes: 3V2Z (MbChl(NO_2)) and 3V2V (MbChl(ONO)). Details of the crystallography are given in the Supporting Information.

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