Rapid Reports

The Nitrite Anion Binds to Human Hemoglobin via the Uncommon *O*-Nitrito Mode[†]

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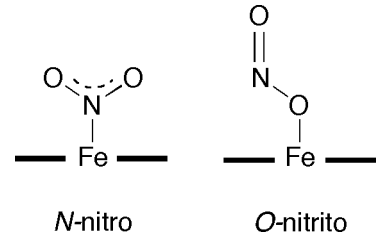
Received May 29, 2008; Revised Manuscript Received June 25, 2008

ABSTRACT: The nitrite anion is known to oxidize and degrade hemoglobin (Hb). Recent literature reports suggest a nitrite reductase activity for Hb, converting nitrite into nitric oxide. Surprisingly, no structural information about Hb−nitrite interactions has been reported. We have determined the crystal structure of the ferric Hb−nitrite complex at 1.80 Å resolution. The nitrite ligand adopts the uncommon *O*-nitrito binding mode. In addition, the nitrito conformations in the α and β subunits are different, reflecting subtle effects of the distal His in orienting the nitrite ligand in the *O*-nitrito binding mode.

The nitrite anion [NO_2^-; pK_a = 3.2 (*1*)] has been proposed to be a major storage pool of bioactive nitric oxide (NO) under hypoxic conditions in mammals (*2*). In this scenario, the heme proteins hemoglobin (Hb) and myoglobin (Mb) reduce nitrite to NO to supplement its production from the NO synthase pathway that requires dioxygen. It is interesting to note that Doyle and co-workers showed, almost three decades ago, that deoxyHb is capable of reducing nitrite to NO (*3*). Recently, growing interest in this formally nitrite reductase (NiR) reaction by Hb has spurred several groups to investigate in more detail the role of the heme in Hb in the binding and activation of nitrite (*4–6*). For example, Gladwin and co-workers (*7*) have reinvestigated the stoichiometry of this Hb-dependent NiR reaction and have confirmed that under strictly anaerobic conditions, the NO generated is trapped by unreacted deoxyHb to form HbNO. In partially aerated solutions, however, the NO is eventually released from HbNO through oxidation of the latter by an intermediate formed from the oxyHb−nitrite reaction (*6*). Interestingly, it has been demonstrated that infusion of nitrite into the forearm brachial artery results in an increase in blood flow in the forearm before and during exercise, implying a unique role of nitrite in vasodilation (*8*). Further, Friedman and workers have used sol−gel encapsulation techniques to show that the NiR reaction of Hb is faster in the R state than the T state (*9*). Despite the intense research activity in this area, however, no structural information regarding the nature of the interaction between the nitrite anion and the heme site of Hb exists.

There are two main types of nitrite binding to single metal centers that might be expected in heme proteins. The first is the N-bound nitro binding mode, and the second is the O-bound nitrito binding mode.



The crystal structures of the nitrite adducts of cytochrome cd_1 NiR from *Paracoccus pantotrophus* (*10*), cytochrome *c* NiR from *Wolinella succinogenes* (*11*), and sulfite reductase (SiR) heme protein from *Escherichia coli* (*12*) clearly reveal the *N*-nitro binding mode of nitrite. All three proteins have more than one distal pocket H-bonding interaction with the bound nitrite (cyt cd_1, His/His; cyt *c*, His/Arg; SiR, Lys/Lys/Arg). In 2006, we reported the first determination of the *O*-nitrito binding mode for binding of nitrite to a heme protein (*13*). We showed that the *O*-nitrito mode was present in the nitrite derivative of ferric horse heart Mb (hh Mb). We subsequently showed that the single distal His residue in this protein directs the nitrite ligand toward the *O*-nitrito binding mode in the manganese- and cobalt-substituted derivatives of hh Mb(ONO) (*14*). Although it has been previously assumed that the *N*-nitro binding mode is the active intermediate in heme-dependent NiR reactions, the *O*-nitrito binding mode as a viable intermediate was demonstrated by Silaghi-Dumitrescu for the cytochrome cd_1 NiR enzyme (*15*).

The Hb−nitrite interaction is a complex one. A suggested productive consequence of this interaction is the NiR reaction described above, where nitrite [∼0.3 μM in the red cell (*6*)] is reduced to NO. A negative consequence of the Hb−nitrite interaction is methemoglobinemia (*16–18*), where the generation of ferric Hb prevents the protein from carrying oxygen from the lungs to the muscle tissues. In addition, nitrite is known to bind to ferric Hb (*19*).

[†] This work was supported by National Institutes of Health Grant GM 064476 (to GBR-A).

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10.1021/bi801015c CCC: $40.75
Published on Web 07/17/2008

Table 1: Data Collection and Refinement Statistics[a]

space group	$P4_12_12$
a, *b*, *c* (Å)	53.54, 53.54, 191.77
wavelength (Å)	1.5418
resolution range (Å)	16.37−1.80
no. of observations	207394
no. of unique reflections	26808
completeness (%)	99.8 (98.3)
$I/\sigma(I)$	10.2 (2.9)
R_{merge} (%)[b]	8.2 (45.7)
R (%)[c]	20.6
R_{free} (%)[d]	23.7

[a] The data in parentheses are for the highest-resolution shell (1.86−1.80 Å). [b] $R_{merge} = \sum|I - \langle I\rangle|/\sum(I)$. [c] $R = \sum||F_o| - |F_c||/\sum|F_o|$. [d] R_{free} was calculated using 5% of the randomly selected diffraction data which were excluded from the refinement.

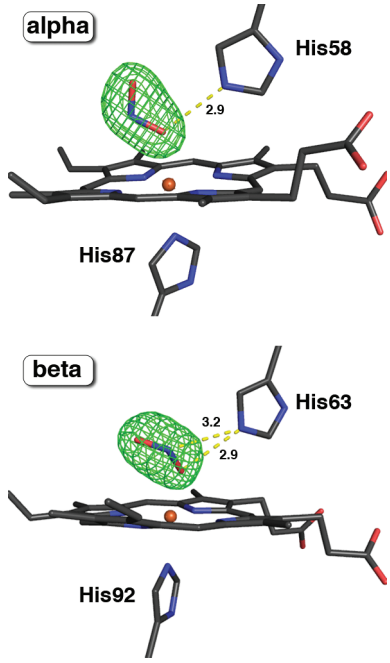

FIGURE 1: $F_o - F_c$ omit electron density maps (3.5σ) and final models of the heme environments in the 1.80 Å resolution crystal structure of ferric Hb(ONO). Hydrogen bonds between the nitrite ligand and the distal His residues are shown as yellow dotted lines, with distances in angstroms (PDB entry 3D7O).

In this work, we report the first crystal structure of a nitrite complex of adult Hb and show that the nitrite ligands bind to iron via the uncommon *O*-nitrito binding mode. Crystals of ferric Hb(ONO) were obtained by soaking R state aquometHb crystals at pH 6.8 with nitrite for 10 min at room temperature. A diamond-shaped crystal was selected for the determination of an X-ray crystal structure, and the structure was determined to 1.80 Å resolution (Table 1).

The heme environments of this ferric R state Hb(ONO) product are shown in Figure 1. There are two important observations in this Hb(ONO) structure. The first is the presence of the uncommon *O*-nitrito mode of binding of nitrite to the iron centers in both the α and β subunits. In the α subunit, the Fe−O distance and the Fe−N(His87) distance are both 2.0 Å. The nitrite O1 atom is within hydrogen bonding distance (2.9 Å) of the N^{ε} atom of the distal His58 residue, and the nitrite O2 atom is 3.3 Å from this N^{ε} atom. In the β subunit, the Fe−O distance is 1.9 Å and the Fe−N(His92) distance is 2.0 Å. The distal His N^{ε} atoms move ∼0.8 Å inward relative to their positions in R state HbCO (PDB entry 1IRD) to form H-bonds with the Fe−ONO fragments (Figure S1). The *O*-nitrito binding mode is clearly different from the *N*-nitro binding mode observed in the cytochrome cd_1 (*10*) and *c* (*11*) NiRs and in the sulfite reductase heme protein (*12*). It is interesting to note that the *N*-nitro binding mode prevails in all isolated iron porphyrin nitrite model compounds reported to date (*20*), the one exception being a disordered component of a picket fence model system (*21*). However, a similar *O*-nitrito binding was shown to exist in the monoheme derivative hh Mb(ONO) that contains a single hydrogen bonding residue in the distal pocket (*13*).

In our previous experimental and density functional theoretical (DFT) studies on an iron porphyrin model (nonprotein) system containing both NO and nitrite ligands (*22*), the *N*-nitro binding mode was calculated to be more stable than the *O*-nitrito binding mode by ∼4.3 kcal/mol. Further, a recent DFT calculation on a truncated Hb active site predicted a stable *N*-nitro binding mode of nitrite relative to the *O*-nitrito mode by ∼7 kcal/mol (*23*). Thus, it is likely that the presence of the single hydrogen-bonding His residues in the protein distal pockets in Hb (and Mb) directs a preferential and unexpected *O*-nitrito binding mode of nitrite in these latter ferric systems.

The second important observation in the Hb(ONO) structure shown in Figure 1 is the difference in nitrite conformations in the heme sites of the α and β subunits. In the α subunit, the Fe−O−N−O moiety is *trans* with a torsion angle of 174° (the O−N−O angle is 110°). We recently reported a similar *trans* *O*-nitrito binding conformation of nitrite in the Mb(ONO) compound (*13*). In the Hb β subunit, however, the Fe−O−N−O moiety is best modeled at this resolution as a distorted *cis*-like conformation with a torsion angle of −91° and an O−N−O angle of 113° (Figure S2). Further, the terminal nitrito O atom is directed away from the distal His63 residue, making its shortest contact with the distal Val67 (3.2 Å from a C_{γ} atom). Our recent DFT calculations on a nonprotein iron porphyrin nitrosyl nitrito complex revealed that the *trans* Fe−O−N−O conformation was only 3.3−3.5 kcal/mol higher in energy than the classic *cis* form (*22*). Related DFT calculations on a truncated Hb showed near-equivalent energies (within ∼0.5 kcal/mol) of the *cis* and *trans* conformations (*23*). It thus appears that the Hb distal pockets in the α and β subunits provide the appropriate environments for the stabilization of the two observed conformations in Figure 1.

Finally, we note that near-identical crystal structures of the ferric Hb(ONO) product with *O*-nitrito ligands were obtained both from the reaction of metHb with nitrite (this work) and from the reaction of deoxyHb with nitrite (2.0 Å resolution; data not shown). However, in our hands, the latter preparative method for crystallography frequently resulted in protein degradation and subsequent precipitation of product, consistent with the known nitrite-induced degradation of the Hb protein in the presence of oxygen.

The metHb reductase enzyme (*24*) can rereduce the metHb generated by the NiR activity back to the ferrous form; this prevents the accumulation of metHb. Any metHb present can bind unreacted nitrite to give Hb(ONO), although the exact nature of nitrite binding was unknown prior to this study. Further, under conditions of irreversible poisoning by

oxidants such as nitrite, the Hb protein degrades into Heinz bodies in the red cell (*25*).

Our crystal structure of Hb(ONO) provides the first definitive experimental insight into the nature of the Hb−nitrite interaction and provides an intellectual framework for a more complete understanding of this rather complex and physiological interaction between the nitrite anion and Hb.

ACKNOWLEDGMENT

We are grateful to Drs. Faik N. Musayev, Babak Andi, and Xiaodong Zhao for assistance with some aspects of the crystallography.

SUPPORTING INFORMATION AVAILABLE

Sample preparation, X-ray data collection, structure solution and refinement, and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI801015C