Published in final edited form as:

Inorg Chem. 2013 March 4; 52(5): 2277–2279. doi:10.1021/ic302685h.

Porphyrin-Substituted H-NOX Proteins as High Relaxivity MRI Contrast Agents

Michael B. Winter^{1,3}, Piper J. Klemm¹, Christine M. Phillips-Piro^{1,3}, Kenneth N. Raymond¹, and Michael A. Marletta^{1,2,3,4,*}

¹Department of Chemistry, University of California, Berkeley, CA 94720 3220

²Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720 3220

³California Institute for Quantitative Biosciences, University of California, Berkeley, CA 94720 3220

⁴Division of Physical Biosciences, Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720 3220

Abstract

Heme proteins are exquisitely tuned to carry out diverse biological functions while employing the identical heme cofactor. Although heme protein properties are often altered through modification of the protein scaffold, protein function can be greatly expanded and diversified through replacement of the native heme with an unnatural porphyrin of interest. Thus, porphyrin substitution in proteins affords new opportunities to rationally tailor heme protein chemical properties for new biological applications. Here, a highly thermal-stable Heme Nitric oxide/Oxygen binding (H-NOX) protein is evaluated as a magnetic resonance imaging (MRI) contrast agent. T_1 and T_2 relaxivities measured for the H-NOX protein containing its native heme are compared to the protein substituted with unnatural Mn(II)/(III) and Gd(III) porphyrins. H-NOX proteins are found to provide unique porphyrin coordination environments and have enhanced relaxivities compared to commercial small molecule agents. Porphyrin substitution is a promising strategy to encapsulate MRI-active metals in heme protein scaffolds for future imaging applications.

Magnetic resonance imaging (MRI) has emerged as a powerful tool to profile mammalian physiology. MRI is widely used in medicine, for example, to distinguish between normal and disease tissue in patients in order to improve diagnosis and therapy. MRI has numerous advantages over other imaging strategies – it is noninvasive, able to differentiate between soft tissue, and has high lateral and depth resolution ($\sim 100\ \mu\text{m}$ to $\sim 1\ \text{mm}$).¹ These features allow for the construction of detailed images that provide a dynamic picture of changing physiological conditions.

MRI signal is derived from the relaxation of water molecule protons and is greatly enhanced through the implementation of contrast agents, which are currently used in 45 percent of MRI scans.² Contrast agents containing paramagnetic metals [e.g., Fe(III), Mn(II), Mn(III), and Gd(III)] increase the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of nearby water molecule protons via dipole interactions.³ A parameter termed relaxivity ($r_{n=1,2}$) is used to quantify the concentration dependence of contrast agents on proton relaxation rates

*Corresponding Authors: marletta@scripps.edu.

Supporting Information. Supplemental protein characterization. X-ray crystal structure data collection and refinement statistics. This material is available free of charge via the Internet at <http://pubs.acs.org>.

($\text{mM}^{-1}\text{s}^{-1}$) and can be enhanced by increasing the number of inner-sphere water molecules (q), increasing inner-sphere water exchange rates ($1/\tau_M$), and slowing the molecular tumbling rate ($1/\tau_R$).⁴ Numerous chemical strategies have been implemented to optimize these parameters for improved MRI signal.⁵⁻⁷ In spite of the diverse frameworks developed, demetallation of contrast agents under aqueous, biological conditions has been an ongoing challenge.⁸ Currently, approved contrast agents are Gd(III) based, but leaching of Gd(III) has been linked to severe health problems, such as nephrogenic systemic fibrosis.¹ Additionally, Gd(III) from hospital wastewater has been detected in the environment.⁹ The high toxicity of Gd(III) combined with increasing global demand for this rare earth element has renewed interest in MRI contrast agents employing alternate metals and/or macromolecular scaffolds.¹⁰⁻¹³

To address these challenges, we have evaluated porphyrin substitution in a native heme protein scaffold as an alternative strategy to generate high relaxivity MRI contrast agents. The Heme Nitric oxide/Oxygen binding (H-NOX) domain from *Thermoanaerobacter tengcongensis* (*Tt*H-NOX) has been previously shown to be a robust platform for heme protein based biological tools.^{14,15} *Tt*H-NOX is stable under extreme temperatures ($>70^\circ\text{C}$), highly tolerant of site-directed mutagenesis, and can be readily expressed with genetically-encoded tags.^{14,15} These features could be exploited to improve protein biocompatibility (e.g., through derivatization), enhance relaxivity, and facilitate targeted delivery in biology.

To evaluate *Tt*H-NOX as an MRI contrast agent, the relaxivity of the protein containing its native heme cofactor was first compared to myoglobin (Mb). Iron(III) complexes display lower ionic relaxivities than lanthanides.^{1,4} However, the decreased cost and lower environmental impact of Fe(III) make it a noteworthy alternative. Relaxivities determined for high-spin, ferric aqua Mb (1.6 and $2.9\text{ mM}^{-1}\text{s}^{-1}$ for T_1 and T_2 , respectively) were similar to previously reported values.¹⁶ The analogous *Tt*H-NOX high-spin ferric complex¹⁷ displayed significantly improved T_1 and T_2 relaxivities of 5.9 and $14.5\text{ mM}^{-1}\text{s}^{-1}$, respectively (Figure 1 and Table 1). *Tt*H-NOX is a larger protein (22 kDa vs. 17 kDa) with a more extended conformation, which could increase relaxivity by slowing solution tumbling. Comparison of UV-visible spectra for ferric *Tt*H-NOX and Mb (Figure 1 and Table S1) also suggests that the histidyl-ligated heme groups are in different chemical environments and/or conformations. Indeed, previously published crystal structures have shown that the heme group is highly distorted in *Tt*H-NOX¹⁸ and that the proteins have unique heme pocket architectures.^{18,19} Therefore, it is possible that distinct structural features in the *Tt*H-NOX scaffold could enhance relaxivity by modulating heme electronic properties, increasing inner-sphere water exchange, and/or improving solvent accessibility to the heme pocket.

Due to the apparent higher relaxivity of the *Tt*H-NOX scaffold, its native heme cofactor was substituted with an unnatural Mn porphyrin to further improve MRI signal. High spin Mn(II)/(III) has a higher ionic relaxivity than high spin Fe(III). However, small molecule Mn complexes have seen only limited use because of aqueous demetallation.¹ Mn(III) protoporphyrin IX was incorporated into *Tt*H-NOX [Mn(III) *Tt*H-NOX] using an expression-based strategy employing the RP523 strain of *Escherichia coli*. The RP523 strain cannot biosynthesize heme (limiting heme contamination) and does not require heme transporter coexpression²⁰ unlike conventional expression hosts.²¹ UV-visible spectral characterization of purified Mn *Tt*H-NOX indicated that the protein was isolated with a bound Mn(III) porphyrin (Figure 2a and Table S1).²⁰ Specific Mn porphyrin substitution was confirmed by nanoelectrospray ionization (nanoESI) mass spectrometry (MS) followed by tandem mass spectrometry (MS/MS) of the intact porphyrin-protein complex (Figure S1). Importantly, Mn(III) *Tt*H-NOX was found to be stable under biological conditions. As with

the ferric protein, Mn(III) *Tt*H-NOX exhibited no detectible porphyrin loss or demetallation in plasma (at least 24 h at 37 °C) (Figure S2).

Both Mn(II) and Mn(III) *Tt*H-NOX are predicted to be paramagnetic. Therefore, T_1 and T_2 relaxivities were measured for the protein in each oxidation state. Purified Mn(III) *Tt*H-NOX was reduced under anaerobic conditions with dithionite and found to autoxidize rapidly upon exposure to air ($t_{1/2} = \sim 10$ min at ambient temperature) (Figure 2a). Therefore, the Mn(II) protein was characterized in the absence of O₂. Mn(II) *Tt*H-NOX displayed T_1 and T_2 relaxivities of 3.6 and 3.8 mM⁻¹s⁻¹, respectively, whereas Mn(III) *Tt*H-NOX exhibited much higher respective relaxivities of 12.0 and 16.8 mM⁻¹s⁻¹ (Figure 2b and Table 1). The T_1 relaxivity of Mn(III) *Tt*H-NOX is similar to previously described Mn-TPP derivatives.²² However, the relaxivity is higher than that of a recently reported P450 protein substituted with Mn protoporphyrin IX (Mn-BM3h),²¹ other Mn-containing proteins,^{23,24} as well as the commercial MRI contrast agent MnDPDP ($S = 5/2$) (Table 1).²⁵

To provide molecular-level insight into the high relaxivity of Mn(III) *Tt*H-NOX, single crystals were obtained of the Mn-substituted protein under aerobic conditions. X-ray absorption spectra of the crystals collected at the Mn and Fe K-edges confirmed specific Mn incorporation and lack of Fe contamination (Figure S3). A crystal structure was solved of Mn(III) *Tt*H-NOX at 2.1 Å resolution by molecular replacement using the previously reported structure of heme-bound *Tt*H-NOX (PDB ID 1U55) as the search model (Figure 3 and Table S2).¹⁸ The crystal structure was obtained in the C₂ space group with two molecules in the asymmetric unit (herein referred to as chains A and B).

The Mn *Tt*H-NOX structure was found to overlay with the heme containing protein to a high degree overall (rmsd values range from 1.14-2.03 Å) (Figure S4). Nearly full-occupancy (>98%) porphyrin binding was also observed in the heme pocket for both protein molecules (Figure S5). In chain B, an ordered water molecule coordinates the Mn atom and forms a hydrogen bond with a conserved distal pocket tyrosine residue (Y140) (Figure 3) analogous to the Fe(III) protein.¹⁸ In addition, the conserved proximal histidine ligand (H102) binds the metal at the remaining coordination site (Figure 3). In chain A, however, the proximal histidine is in alternate bound and unbound rotamers, and no ordered water is evident at the Mn center (Figure S6). Together, these structural data suggest greater ligand binding dynamics in Mn(III) *Tt*H-NOX compared to the Fe(III) protein. It is possible that the weakened histidine affinity in Mn(III) *Tt*H-NOX for the oxophilic metal could open an additional water coordination site and/or improve water accessibility. Thus, built-in conformational flexibility in the *Tt*H-NOX scaffold may serve as an unanticipated mechanism to enhance agent relaxivity.

To further improve the relaxivity of *Tt*H-NOX, Gd(III) mesoporphyrin IX was incorporated into the protein scaffold via reconstitution of the apo protein,²⁶ and the resulting Gd protein complex was found to be stable in aqueous buffer. T_1 and T_2 relaxivities recorded for Gd(III) *Tt*H-NOX were significantly higher than Mn(II), Mn(III), and Fe(III) *Tt*H-NOX. Indeed, the T_1 and T_2 relaxivities of Gd(III) *Tt*H-NOX are ~10-fold higher than current commercial Gd(III)-based agents when measured under equivalent conditions (Table 1).

Porphyrin-substituted H-NOX proteins are a promising proof-of-concept class of contrast agents for MRI technology. H-NOX scaffolds form stable metal complexes under aqueous conditions and provide unique porphyrin coordination environments. This new class of agents could be further modified through genetic or chemical means to enhance MRI signal and biocompatibility. In addition, alternate metalloporphyrin analogues could be incorporated in the heme pocket to further improve agent properties. Together, these

strategies afford new opportunities to rationally design tunable, heme protein frameworks for future biological imaging applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH GM070671 (M.A.M.) and HL069832 (K.N.R.). We would like to thank the QB3/Chemistry MS Facility at UC Berkeley (in particular A. Iavarone) and scientists at beamline 8.3.1 (ALS). We would also like to thank Dr. E. S. Underbakke and Dr. A. Nierth for experimental assistance, and Prof. E. E. Weinert for providing purified heme-bound *Tt*H-NOX. Finally, we would like to thank members of the Marletta and Raymond labs for helpful discussions.

ABBREVIATIONS

H-NOX	Heme Nitric oxide/Oxygen binding
Tt	<i>Thermoanaerobacter tengcongensis</i>
Mb	myoglobin
MS	mass spectrometry

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Figure 1. (A) Steady-state absorbance spectra of ferric aqua Mb (—) and TtH-NOX (—). (B) Relaxivities of Mb and TtH-NOX at 37 °C and 60 MHz.

Figure 2.

(A) Time-resolved UV-visible spectra of Mn(II) to Mn(III) *TtH*-NOX oxidation in air at room temperature. (B) Relaxivities of Mn(II) and Mn(III) *TtH*-NOX at 37 °C and 60 MHz in the absence of O₂ [Mn(II)] and under aerobic conditions [Mn(III)].

Figure 3. X-ray crystal structure of Mn(III) *TtH*-NOX at 2.1 Å resolution (PDB ID 4IT2). (A) Cartoon representation of monomeric Mn(III) *TtH*-NOX (chain B). (B) Zoom-in view of Mn(III) protoporphyrin IX bound in the heme pocket. $2F_o - F_c$ electron density for the porphyrin, water ligand, and several heme pocket residues is shown.

Table 1

Relaxivities of protein complexes and select commercial MRI contrast agents.

Complex	r_1 (mM ⁻¹ s ⁻¹)	r_2 (mM ⁻¹ s ⁻¹)	q value	Ref. ^a
Fe(III) Mb	1.60 ± 0.01	2.9 ± 0.1	1	
Fe(III) <i>Tf</i> H-NOX	5.90 ± 0.04	14.5 ± 0.4	1	
Mn(II) <i>Tf</i> H-NOX	3.7 ± 0.1	3.8 ± 0.2	N/D	
Mn(III) <i>Tf</i> H-NOX	12.0 ± 0.2	16.8 ± 0.5	1	
Gd(III) <i>Tf</i> H-NOX	28.7 ± 5.0	39.9 ± 4.9	N/D	
Mn-BM3h (WT)	2.6 ± 0.1 ^b	N/D	N/D	21
MnDPDP	2.8 ^c	N/D	N/D	25
Gd-DTPA	3.3 ± 0.3	3.9 ± 0.3	1	1
Gd-DOTA	3.0 ± 0.3	3.5 ± 0.3	1	1
Gd-HP-DO3A	2.9 ± 0.3	3.4 ± 0.3	1	1

N/D = not determined.

^aThis work unless noted.^b200 MHz and 23 °C.^c10 MHz and 25 °C. All other complexes are at 60 MHz and 37 °C.