Heme Proteins

DOI: 10.1002/ange.200904799

Determinants of Ligand Affinity and Heme Reactivity in H-NOX Domains**

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Investigations into the mechanisms by which heme proteins control ligand affinity and reactivity have been studied for decades, with the globins serving as model systems for histidyl-ligated proteins.[1] The discovery of a novel family of heme proteins, Heme Nitric oxide/OXygen (H-NOX) binding domains,[2–4] has provided an opportunity to investigate if factors previously found to control ligand affinity and reactivity in the globins can be generalized between protein folds or if there are additional determinants.

The H-NOX family includes the heme domain of soluble guanylate cyclase (sGC), the mammalian NO receptor,[5] as well as heme proteins from bacteria, such as *Thermoanaerobacter tengcongensis* (*Tt*).[3] H-NOX domains are a potentially illuminating choice for further investigations into ligand affinity because sGC does not bind O_2, even under ambient conditions, whereas other members of the family, such as *Tt* H-NOX, bind O_2 with very high affinity ($K_d = 90$ nM).[3,6] A distinctive feature of the wild-type (WT) *Tt* H-NOX crystal structure was a distal-pocket hydrogen-bonding network, in which a key H bond from Y140 to the bound O_2 was observed, as well as H bonds from N74 and W9 to Y140 (Figure 1).[4] The absence of this hydrogen-bonding triad in H-NOX domains that do not bind O_2 highlights hydrogen bonding as a major component of O_2 binding. A distal H bond to O_2 has been noted to be important for the globins. However, the introduction of a tyrosine residue into sGC does not result in O_2 binding,[7] which suggests that there are additional determinants of ligand affinity in H-NOX domains.

To further investigate how the H-NOX fold regulates ligand binding, and how this regulation is similar or dissimilar to that of the globins, site-directed mutagenesis was performed on *Tt* H-NOX. It has been found that the introduction of phenylalanine mutations into the distal pocket of myoglobin (Mb), at positions such as L29 and V68, dramatically alters the oxygen affinity by decreasing the size of off-heme ligand-binding sites and reducing ligand "trapping" (Table 1).[8–10] In addition to altering O_2 affinity, reaction of ferrous-oxy proteins with NO, or NO dioxygenation (Scheme 1, Table 1), in Mb was decreased by the addition of distal pocket bulk due to the blocking off-heme binding sites, preventing NO from readily residing in the heme pocket near the bound O_2.[8,11] To probe the effects of distal-pocket bulk on the O_2 affinity and reactivity toward NO of H-NOX proteins, as compared to the globins, isoleucine 75 and leucine 144 were mutated to phenylalanine, since these residues are both in the back of the distal pocket (Figure 1) in positions analogous to those of L29 and V68 in Mb.

The I75F, L144F, and I75F/L144F mutants were spectrally similar to WT *Tt* H-NOX (see Figure S1 in the Supporting Information); however, kinetic characterization revealed dramatic changes upon the introduction of distal-pocket bulk. The I75F mutation resulted in an approximately nine-fold increase in the O_2 off-rate ($k_{off} = 11.19\ s^{-1}$), whereas the L144F mutation resulted in an approximately 13-fold increase in the O_2 off-rate ($k_{off} = 16.06\ s^{-1}$; Table 1). The two mutations had a synergistic effect in the I75F/L144F double mutant, which had an O_2 off-rate of $45.7\ s^{-1}$. Thus, a 37-fold increase was observed in the O_2 off-rate upon the introduction of only two mutations, neither of which involved a direct change in the hydrogen-bonding network. Typically, such large changes in O_2 off-rates occur when the primary hydrogen-bond donor to O_2 is altered or removed.[9]

There were also significant changes in the O_2 on-rate upon introduction of the mutations (Table 1). These changes in combination with the changes in the O_2 off-rate resulted in large increases in the dissociation constant (K_d) for O_2. WT *Tt* H-NOX has a K_d value for O_2 of approximately 90 nM, whereas the I75F mutant was found to have a K_d value of 497 nM. The increase in the K_d value was even more dramatic for the L144F ($K_d = 2.36$ μM) and I75F/L144F mutants ($K_d = 11.15$ μM). Thus, 6-, 27-, and 126-fold increases in the K_d value relative to that of the WT protein were observed for the I75F, L144F, and I75F/L144F mutations, respectively. To date, these changes in O_2 affinity are the largest reported for *Tt* H-NOX mutants that stably bind O_2. Similar mutations in Mb actually resulted in significantly decreased O_2-dissociation rates and

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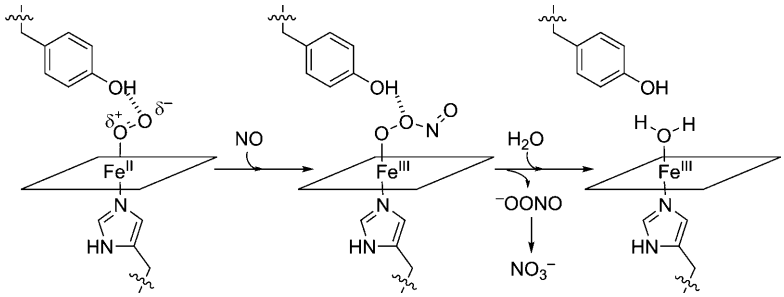
[**] Funding for this research was provided by the National Institutes of Health National Heart, Lung, and Blood Institute Award F32L090174 (E.E.W.), NIH grant GM 070671 (M.A.M.), and a grant from the Rogers Family Foundation (M.A.M.). We are grateful to Dr. Jay Winkler and the Beckman Institute Laser Resource Center at the California Institute of Technology for assistance with on-rate measurements, and members of the Marletta laboratory for critical reading of this manuscript. H-NOX = heme nitric oxide/oxygen.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200904799.



Scheme 1. NO dioxygenation reaction.

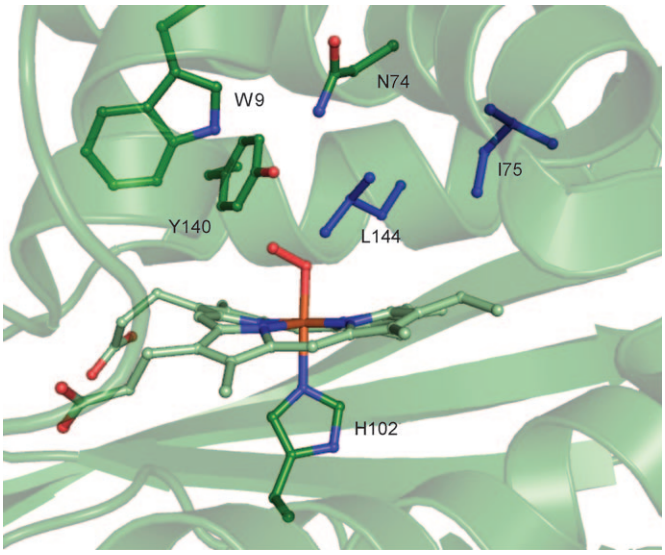


Figure 1. Structure of the WT *Tt* H-NOX heme pocket.[4] The heme group and key residues are shown in green. L144 and I75 are highlighted in blue.

binding constants that corresponded to very tight binding.[8] Thus, the introduction of distal-pocket bulk in *Tt* appears to result in different changes to the protein structure than those that occur in Mb.

To investigate the cause of the changes in O_2 affinity upon the introduction of distal-pocket bulk, we solved the structure of *Tt* I75F/L144F (PDB ID 3IQB). In Mb, the mutation of distal-pocket residues to phenylalanine resulted in the increased bulk filling cavities in the heme pocket.[11] However, the introduction of phenylalanine residues into *Tt* H-NOX led to rearrangement of the heme pocket; a more elongated protein structure resulted (with an approximately 2.5 Å increase in the distance from α helix F to α helix B; Figure 2A). The I75F/L144F heme group was found to be much flatter than the extremely distorted heme group observed in WT *Tt* H-NOX (root-mean-square deviation from planarity: 0.20 and 0.45 Å, respectively; Figure 2B) and tilted within the pocket to accommodate the increased bulk (Figure 3A). The addition of the two phenylalanine residues also altered the conformation of F78, which resulted in a van der Waals contact with α helix A and a translation of that helix away from the heme group (Figure 3B). The combined changes in heme positioning and the protein scaffold resulted in a longer Fe^{II}–Y140 distance in I75F/L144F (6.4 Å) relative to that in the WT (5.1 Å; Figure 2B).

Besides the changes in distal-pocket hydrogen bonding, there were also differences in the proximal pocket of I75F/L144F as compared to WT *Tt* H-NOX. The proximal histidine residue was found to have rotated around the iron–imidazole bond into a more eclipsed conformation (Figure 2B). An eclipsed histidine conformation has previously been shown to result in decreased O_2-binding energy and contribute to lower O_2 affinity by decreasing the strength of the iron–histidine bond.[12] The mixture of five- and six-coordinate Fe^{II}–NO observed spectrally for I75F/L144F was likely also the result of the weaker iron(II)–histidine bond. Furthermore, although within the coordinate error of most WT structures,[4,13] a slight increase in the iron–histidine bond distance was observed that may contribute to the decreased O_2 affinity. Calculations previously carried out on an imidazole-ligated model system found that an increase in the iron–imidazole bond distance by as little as around 0.1 Å, which is difficult to resolve crystallographically, resulted in an approximately 3 kcal mol^{-1} decrease in binding energy.[12] The combined changes to the distal and proximal pockets provide an explanation for the observed 126-fold decrease in O_2 affinity for *Tt* I75F/L144F with respect to WT *Tt* H-NOX. In a previous study, these factors were found to be important for controlling the energetics of O_2 binding in Mb and leghemoglobin;[12] they thus appear to be key determinants of O_2 affinity in widely varying protein architectures.

The effects of distal-pocket bulk on the reactivity of ferrous oxy *Tt* H-NOX were further investigated using NO as a probe. The reaction of the Fe^{II}–O_2 protein with NO (sometimes called NO dioxygenation; Scheme 1) appears to be an inherent property of the globins and may provide insight as to how the reactivity of the Fe–O_2 bond in *Tt* H-NOX is altered by the mutations. WT *Tt* H-NOX was found to have an NO dioxygenation rate of 0.051 μM^{-1}s^{-1} (Table 1), which is the lowest rate that has been reported.[14,15] The I75F, L144F, and I75F/L144F mutants were found to have NO dioxygenation

Table 1: Kinetic parameters for *Tt* H-NOX and sperm-whale myoglobin.

Protein	K_d [nM]	k_{off} [s^{-1}]	k_{on} [μM^{-1}s^{-1}]	k_{ox} ($\times 10^{-5}$) [s^{-1}]	$k'_{NO,ox}$ [μM^{-1}s^{-1}]
Tt WT	88.2 ± 0.67	1.20 ± 0.02	13.6 ± 1.0	stable	0.051 ± 0.002
Tt I75F	497 ± 16	11.19 ± 0.12	22.5 ± 0.7	stable	0.19 ± 0.01
Tt L144F	2360 ± 50	16.06 ± 0.21	6.8 ± 0.1	1.67 ± 0.03	0.64 ± 0.02
Tt I75F/L144F	11150 ± 330	45.7 ± 0.9	4.1 ± 0.1	17.5 ± 1.7	2.0 ± 0.1
Mb	910[9]	15[9]	17[9]	1.53[9]	34[9]
Mb V68F	2100[9]	2.5[10]	1.2[10]	1.94[9]	9.4[9]
Mb L29F	67[10]	1.4[10]	21[10]	0.014[10]	8.1[9]
Mb L29F/V68F	13[9]	0.17[9]	13[9]		2.9[9]

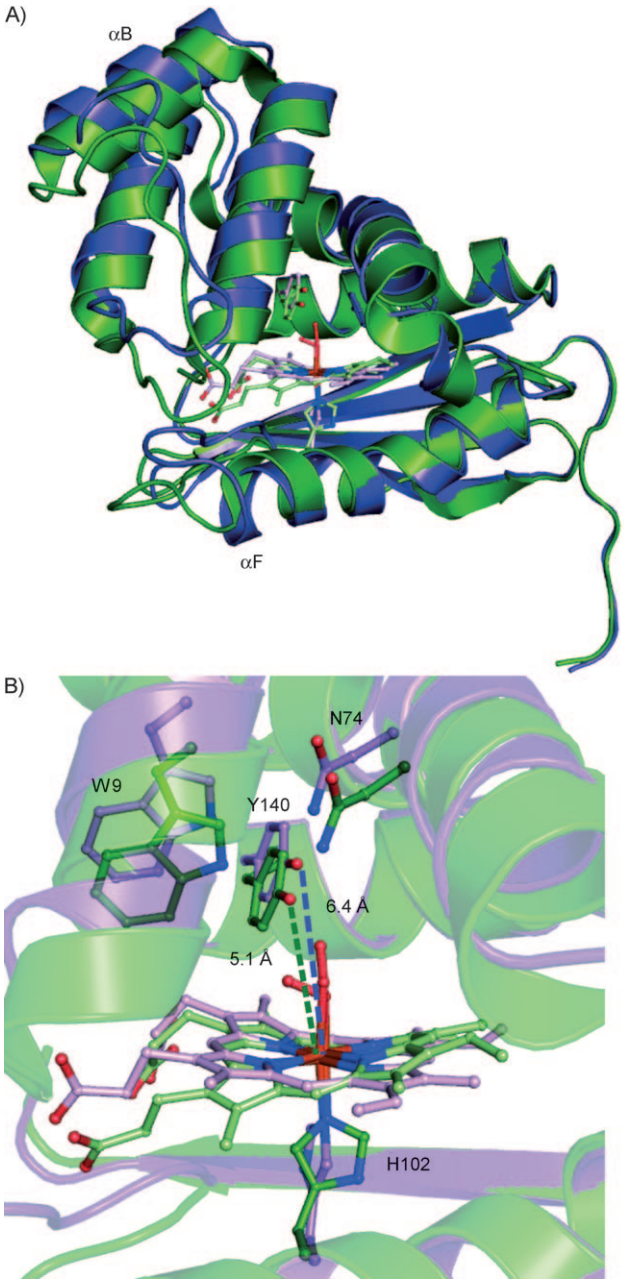


Figure 2. A) Overlay of the structures of *Tt* WT (green) and I75F/L144F (purple). The structures were aligned along the C-terminal β sheets, since this alignment resulted in the approximate superposition of the heme groups. A calculated distance-difference matrix (see Figure S2 in the Supporting Information) showed that there is very little difference in the C-terminal β sheets between the two structures. B) Structures of the heme environments of WT and I75F/L144F highlighting changes in interatom distances and heme planarity and tilt.

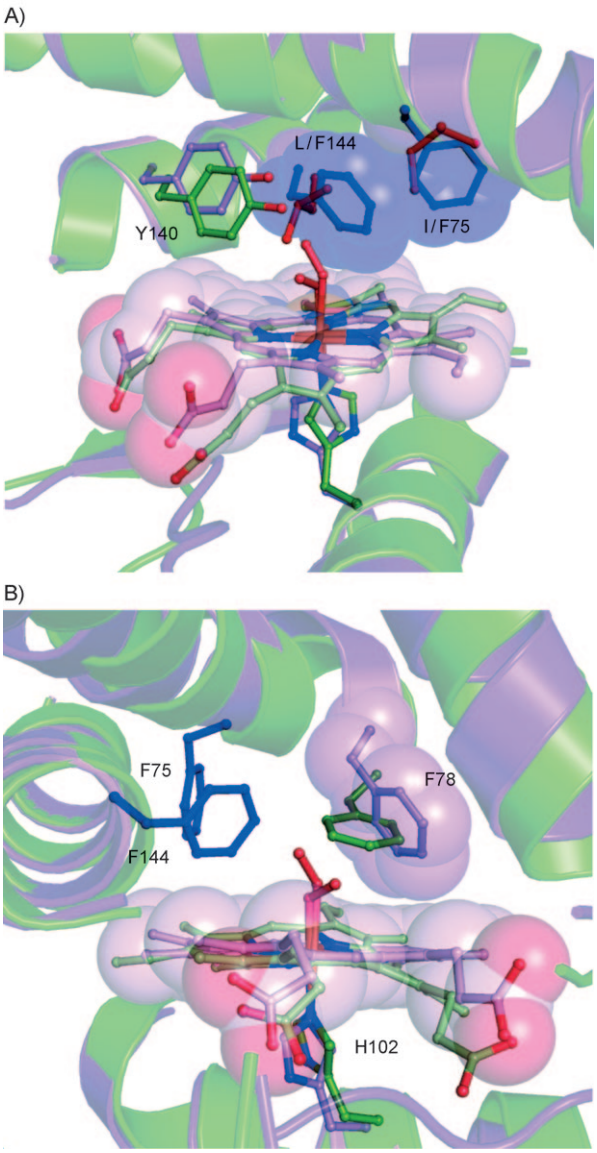


Figure 3. Heme environments of the I75F/L144F mutant (purple) and WT *Tt* H-NOX (green). A) Arrangement of F75 and F144 (blue) in the distal pocket. B) Change in the positioning of the adjacent residue F78 upon introduction of the phenylalanine mutations.

rates of 0.19, 0.64, and 2.0 $\mu M^{-1} s^{-1}$, respectively. All of these rates are among the lowest reported.[14,15]

It is interesting that the dioxygenation rates for the *Tt* H-NOX proteins described above are orders of magnitude lower than those of the globins, even though the two protein families utilize protoporphyrin IX heme groups for O_2 binding and have similar O_2 affinities. Previous studies have suggested that the ferric-superoxide-like character of the Fe^{II}–O_2 complexes and the half-life of O_2 at the iron center, as the Fe^{II}–O_2 protein is a required reactant, are important for controlling NO dioxygenation rates.[8,16]

The O–O stretch can be measured by resonance Raman spectroscopy to determine the bond order of bound O_2. *Tt* H-NOX has been found to have an O–O stretch of 1131 ν^{-1} (O=O: 1556 ν^{-1}),[17] which is very similar to that observed for the globins (1122–1155 ν^{-1}) and indicates a metal–superoxide species.[18] Therefore, differences in the superoxide character of bound O_2 are probably not fully responsible for the dramatic differences in the NO dioxygenation rates of the globins and *Tt* H-NOX. Furthermore, a long residence time of O_2 on the heme group clearly does not dictate NO reactivity in this family of mutants, as both the lowest NO dioxygena-

tion rate and the lowest k_{off} value were observed for WT *Tt* H-NOX.

Within this family of *Tt* H-NOX mutants, a possible reason for the difference in NO dioxygenation rates is access to the heme-bound O_2. The I75F/L144F structure has a small cavity near the bound O_2 molecule that could be occupied by NO, whereas no cavities were found in the WT structure. Studies with Mb have shown that the blocking of cavities in the distal pocket can decrease NO reactivity; the reverse may occur upon the introduction of distal-pocket bulk in *Tt* H-NOX. Furthermore, a previous study found that hydrogen bonding of the bound O_2 molecule by a tyrosine residue decreases the NO reactivity of a protein,[15] probably by changing the electronics of the heme iron center. WT *Tt* H-NOX, with a hydrogen-bonding tyrosine residue, was found to have a reduction potential of 167 mV versus the standard hydrogen electrode (SHE),[19] as compared to a reduction potential of 59 mV versus SHE for Mb,[20] which has a hydrogen-bonding histidine residue. The more difficult one-electron oxidation of WT *Tt* H-NOX likely makes formation of the ferric product of NO dioxygenation more unfavorable. Investigations into the heme electronics of the I75F, L144F, and I75F/L144F *Tt* H-NOX distal-pocket mutants are currently underway.

The results reported herein show that distal-pocket bulk significantly decreases the O_2 affinity of *Tt* H-NOX domains. However, in contrast to results with Mb, the addition of distal-pocket bulk leads to a more elongated structure and changes in the Y140–Fe bond distance and histidine-residue orientation. The dramatic decrease in O_2 affinity in *Tt* I75F/L144F suggests that these structural features may provide a general method of controlling O_2 affinity.

Received: August 27, 2009
Revised: October 28, 2009
Published online: December 16, 2009

Keywords: bioinorganic chemistry · heme proteins · iron · ligand affinity · structural biology

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