The hemerythrin-like diiron protein from Mycobacterium kansasii is a nitric oxide peroxidase

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The hemerythrin-like protein from Mycobacterium kansasii (Mka HLP) is a member of a distinct class of oxo-bridged diiron proteins that are found only in mycobacterial species that cause respiratory disorders in humans. Because it had been shown to exhibit weak catalase activity and a change in absorbance on exposure to nitric oxide (NO), the reactivity of Mka HLP toward NO was examined under a variety of conditions. Under anaerobic conditions, we found that NO was converted to nitrite (NO$_2^-$) via an intermediate, which absorbed light at 520 nm. Under aerobic conditions NO was converted to nitrate (NO$_3^-$). In each of these two cases, the maximum amount of nitrite or nitrate formed was at best stoichiometric with the concentration of Mka HLP. When incubated with NO and H$_2$O$_2$, we observed NO peroxidase activity yielding nitrite and water as reaction products. Steady-state kinetic analysis of NO consumption during this reaction yielded a $K_m$ for NO of 0.44 μM and a $k_{cat}/K_m$ of 2.3 × 10$^5$ M$^{-1}$s$^{-1}$. This high affinity for NO is consistent with a physiological role for Mka HLP in deterring nitrosative stress. This is the first example of a peroxidase that uses an oxo-bridged diiron center and a rare example of a peroxidase utilizing NO as an electron donor and cosubstrate. This activity provides a mechanism by which the infectious Mycobacterium may combat against bacteria, as well as to produce NO$_2^-$ to adapt to hypoxic conditions.

Certain mycobacteria possess oxo-bridged diiron proteins with active site features similar to those of hemerythin (1). These are a distinct class of hemerythrin-like proteins (HLPs) that are found in mycobacterial species that cause respiratory disorders in humans. Unlike true hemerythins (2), these HLPs do not function as oxygen carriers or oxygen-storage proteins. The first of these mycobacterial HLPs to be characterized was the Rv2633c protein from Mycobacterium tuberculosis (3). That protein was shown to exhibit catalase activity. Subsequently, the crystal structure was determined of the orthologous protein from Mycobacterium kansasii (4). This HLP from M. kansasii (Mka HLP) exhibited weak catalase activity and an additional reactivity toward nitric oxide (NO), as judged by an NO-dependent change in its absorbance spectrum (4). Understanding the precise activity of this protein is important, because the gene for the orthologous protein in M. tuberculosis is rapidly upregulated after phagocytosis of the bacteria by macrophages during infection (5, 6). As NO is generated in the macrophage to kill the infectious Mycobacterium, elucidation of the precise reactivity of the Mka HLP toward NO is of particular interest and could support future design of antimycobacterial drugs.

Hemerythins and HLPs share a common structural feature of a four α-helix bundle that contains the oxo-bridged diiron site. However, their overall structures vary, as do the identity of the ligands that coordinate the two irons. The structure of the Mka HLP (4) shows it to be a monomer comprised of a similar four helix bundle with an additional fifth helix (Fig. 1A). The oxo-bridged diiron site in the mycobacterial HLPs is coordinated by the side chains of six amino acids; four histidines, two glutamic acids, and a tyrosine (1, 4) (Fig. 1B). The use of a tyrosine ligand and the pattern of amino acid ligation are conserved among the mycobacterial HLPs. These features are not seen in hemerythins or HLPs from any other sources (1, 7). A bridging solvent oxygen completes the coordination. The coordination environment leaves one iron coordinatively saturated and the other with one open coordination site, which could accommodate NO or H$_2$O$_2$ binding.

The previously observed NO-dependent change in the absorbance spectrum of the Mka HLP (4) is noteworthy as NO is used as a defense against mycobacterial infections. A lethal cocktail of NO and superoxide (O$_2^{-}$) is generated by macrophages in response to the mycobacterial infections (8). Disproportionation of O$_2^{-}$ results in a large reservoir of H$_2$O$_2$, which can participate in Fenton chemistry that causes lethal DNA damage. In addition, NO reacts with O$_2^{-}$ to form peroxynitrite (ONOO$^-$), which subsequently decomposes to form radical species that also mediate lethal DNA damage. Some pathogenic bacteria express NO detoxification enzymes to survive this host response (8, 9). These enzymes most commonly exhibit NO reductase or NO dioxygenase activities (10, 11). Thus, it is likely that reaction of NO with the Mka HLP also protects the infectious Mycobacterium from this host...
defense mechanism. An NO oxidase could convert NO to nitrite (NO$_2^-$). An NO dioxygenase could convert NO to nitrate (NO$_3^-$). While rare, NO peroxidase activity could convert NO to nitrite (12, 13). Enzymes catalyzing these reactions typically use heme iron (10, 14). The NO reactivity of the Mka HLP is atypical for a nonheme diiron site and thus of mechanistic as well as physiological relevance.

This study examined the reactivity of the Mka HLP under a variety of reaction conditions to determine the exact nature of the reactivity toward NO and its possible physiological relevance. Under anaerobic conditions, some NO was converted to NO$_2^-$, and under aerobic conditions, some NO was converted to NO$_3^-$. However, in each case, the Mka HLP was unable to catalyze multiple turnovers. Significantly, the Mka HLP does function as an effective high-affinity NO peroxidase with catalytic formation of NO$_2^-$ and conversion of H$_2$O$_2$ to water. This is the first example of a peroxidase that uses an oxo-bridged diiron center, rather than a heme for catalysis, and is a rare example of a peroxidase that utilizes NO as an electron donor. Consistent with the role of NO as an electron donor for the peroxidase reaction, evidence is also presented that NO can react with one of the ferric irons and transfer an electron to generate Fe(II) via a process known as reductive nitrosylation (15). To our knowledge, this reactivity is unprecedented for a nonheme iron protein. In fact, binding of NO to the Fe(III) center of a nonheme iron protein, which is a prerequisite for reductive nitrosylation, has not been reported (16). The observed NO peroxidase activity of the Mka HLP, which is likely common to mycobacterial HLPs, would provide an activity that is an ideal defense mechanism for protection from NO and H$_2$O$_2$, which are produced in the macrophage during infection to combat the invading Mycobacterium (9). Furthermore, the NO$_2^-$ produced in the peroxidase reaction is a signaling molecule in mycobacteria that allows adaptation to the hypoxic conditions that are experienced by the Mycobacterium within the macrophage (17).

**Results**

**Oxidation state of the Mka HLP**

The absorbance spectrum of the as-isolated Mka HLP exhibits a 350-nm feature (Fig. 2A) that is characteristic of an oxo-bridged nonheme diferric site (18). Absorbance changes on addition of dithionite to reduce the protein were difficult to interpret as absorbance in this region remained, even after lengthy incubations with excess dithionite. This suggested that the Mka HLP could not be fully reduced to a diferrous site. Electron paramagnetic resonance (EPR) spectroscopy established the oxidation state. The as-isolated Mka HLP lacks any EPR signal. This result is consistent with an EPR silent species such as an antiferromagnetically coupled diferrous state or a diferrous center (Fig. 2B). These two possibilities are differentiated in dithionite-reduced samples of the Mka HLP. Addition of one equivalent of dithionite results in a sharp rising feature with g-values of 1.99, 1.79, and 1.60. That these values are less than 2.0 is consistent with the presence of a mixed-valence diiron center (Fe$^{II}$–Fe$^{III}$). It follows that the as-isolated Mka HLP is in the diferrous oxidation state. Surprisingly, even after treatment with additional reducing equivalents, only the 1-electron reduction of the diiron site to the mixed-valence state is observed. This suggests that the protein favors the mixed-valence oxidation state over the diferrous oxidation state. This finding distinguishes the Mka HLP from true hemerythrins that cycle between diferrous and diferrous states (2, 19).

**Anaerobic reaction of the Mka HLP with NO to form NO$_2^-$**

The Mka HLP was mixed with NO under anaerobic conditions. Analysis for reaction products in the mixture after this reaction indicated that the product was primarily NO$_2^-$ (Table 1). Intermediate species with distinct spectroscopic features were observed during this reaction. A species exhibiting a broad absorbance centered at 520 nm (Fig. 3) formed within the time of manual mixing of the Mka HLP with NO.
The 520-nm absorbance feature decayed slowly over several minutes. Analysis for reaction products in the reaction mixture at this early time point, after formation of the 520-nm intermediate, revealed that most of the NO\(_2^-\) had been formed, and that there was negligible additional formation during the decay of this intermediate. The total amount of NO\(_2^-\) that was formed was approximately equivalent to the concentration of the Mka HLP. Thus, at best, stoichiometric conversion of NO was observed and the protein was unable to catalyze multiple turnovers. In these experiments, no reductant was added to the reaction mixture to prereduce the Mka HLP, and therefore, the results indicate that NO reacts with the diferric protein. When the reaction was repeated with the Mka HLP that was first reduced with dithionite to the mixed-valence state, NO\(_2^-\) formation was significantly reduced (Table 1). This strongly suggests that the initial step in the anaerobic reaction is reductive nitrosylation to form an Fe(II)-NO intermediate, which reacts with water to yield NO\(_2^-\). While the latter reaction step has been observed for heme iron in proteins, including hemoglobin (15), this has not typically been seen in nonheme diiron proteins. For the Mka HLP, this allows the diiron site to oxidize NO.

**Aerobic reaction of Mka HLP with NO to form NO\(_3^-\)**

The primary change in the absorbance spectrum of the as-isolated Mka HLP on addition of NO under aerobic conditions is the formation of a weak absorbance feature in the 300 to 350 nm range that overlaps the shoulder of the 280 nm protein absorbance. There is also a broad absorption peak centered around 500 nm in the as-isolated protein, which decreases in intensity on reaction with NO. An aerobic titration of the Mka HLP with NO is shown in Figure 6.

Determination of the product of the reaction of the Mka HLP with NO under aerobic conditions required careful consideration. A significant experimental complication is that NO at high concentrations and under aerobic conditions reacts with O\(_2\) to form NO\(_2^-\); a process known as NO autoxidation (20). Under these conditions, it is difficult to differentiate between NO\(_2^-\) produced by the Mka HLP and that produced from nonenzymatic autoxidation. However, the rate of NO autoxidation is second order with respect to NO concentration (20), and therefore, at micromolar NO concentrations, NO has a half-life of several minutes. For this reason,

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**Table 1**

**Reaction products of the Mka HLP with NO under anaerobic conditions**

| Sample          | [Nitrite, NO\(_2^-\)], \(\mu\)M | [Nitrate, NO\(_3^-\)], \(\mu\)M |
|-----------------|---------------------------------|---------------------------------|
| NO only         | 0                               | 0                               |
| Mka HLP only    | 0                               | 0                               |
| Mka HLP + NO    | 74 ± 7                          | 8 ± 3                           |
| Dithionite-reduced | 18 ± 4                         | 6 ± 1                           |

Concentrations of nitrite and nitrate were determined by ion chromatography. The Mka HLP was present at 60 \(\mu\)M and NO at 450 \(\mu\)M in deoxygenated 50 mM MOPS, pH 7.5. Reactions were performed in triplicate.
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Figure 3. Changes in the absorbance spectrum of the Mka HLP after addition of NO gas under anaerobic conditions. The spectra were recorded before NO addition (red), after mixing with NO gas (green) and 1500 s after NO addition (blue). Samples contained 60 μM HLP. HLP, hemerythrin-like protein; Mka, Mycobacterium kansasi.

previous studies of enzymatic NO dioxygenase activity utilized low micromolar concentrations of NO to avoid the competing autoxidation (21, 22). Even so, it is not possible to completely eliminate nonenzymatic side reactions. To assure accurate interpretation of our results, controls and with all other reaction components except the Mka HLP were always performed to correct for background formation of NO2.

When NO was mixed with the Mka HLP in air saturated buffer, analysis of the reaction mixture indicated that NO3 was the primary product (Table 2). Some NO2 was also present, but the amount of NO2 that was formed was actually less than the background level in the absence of the protein, 290 μM versus 320 μM. An explanation for this is that in the presence of the Mka HLP, some of the NO was diverted from nonenzymatic NO autoxidation to be specifically converted to NO3 by the Mka HLP. The maximum amount of NO3 detected after completion of the reaction was at best stoichiometric with the concentration of Mka HLP. Thus, as observed for the anaerobic reaction, the Mka HLP cannot catalyze multiple turnovers of this aerobic reaction either.

The mechanism of aerobic conversion of NO to NO3 by the Mka HLP is unclear. In NO dioxygenase, which contains flavin and heme cofactors, the conversion of NO to NO3 is initiated by O2 binding to a heme Fe(II) to generate an Fe(II)-O2 intermediate that then reacts with NO to ultimately form NO3 via an O-bound ferric-peroxynitrite intermediate, Fe(III)-OONO. For this Mka HLP NO dioxygenase activity, we propose that the sequence of O2 and NO binding is reversed. For the Mka HLP, what can be said with certainty is that NO3 formation is produced in the presence of O2, whereas NO2 is the sole product of the anaerobic reaction. In each of these cases, the mechanisms of transformation of NO to NO3 and NO to NO2 by the oxo-bridged diiron center are highly unusual, if not novel.

NO peroxidase activity of the Mka HLP

Two observations suggested the possibility that the Mka HLP could have NO peroxidase activity. First, it was previously shown that Mka HLP possessed weak catalase activity (4). This indicated that H2O2 could bind to at least one of the iron in the diiron site. Second, the anaerobic reaction with NO, described in this study, indicated that NO can reduce one of the iron by reductive nitrosylation. This suggests the possibility that NO could serve as the electron donating substrate in a peroxidase reaction. To test for this activity, the Mka HLP was mixed with H2O2 and NO. The reaction was performed under anaerobic conditions to minimize NO autoxidation. These conditions also approximate physiological conditions for a Mycobacterium inside the macrophage or granuloma during infection. The O2 levels within the Mycobacterium can be extremely low in this hypoxic environment where high levels of NO and H2O2 are generated to attack the bacterium (17, 23, 24). Analysis of the reaction mixture after completion of the reaction yielded NO3 as the primary product, with product formation well in excess of the concentration of the Mka HLP (Table 3). Thus, in contrast to the other single-turnover activities described above for the anaerobic NO oxidase and aerobic NO dioxygenase activities, the NO peroxidase reaction is an actual multiturnover enzymatic activity of the Mka HLP.

This reaction was repeated under aerobic conditions. In these aerobic reactions, NO2 production by NO autoxidation was observed, as expected; nevertheless, it was still possible to observe Mka HLP-dependent, H2O2-dependent NO2 production (Table 3). While the amount is less than was observed anaerobically, it still represents multiple enzymatic turnovers and indicates that the Mka HLP-dependent peroxidase activity can compete with the spontaneous NO autoxidation reaction under aerobic conditions.

To further characterize this NO peroxidase activity, NO consumption by the Mka HLP under anaerobic conditions was monitored using an NO electrode (Fig. 7). A background rate
of NO consumption was observed prior to addition of the Mka HLP that is attributed to nonenzymatic reaction of NO with H₂O₂ or residual O₂ in the chamber. Addition of the Mka HLP resulted in an immediate increase in the NO consumption rate. This Mka HLP-dependent rate increased with increasing H₂O₂ concentration (Table 4). In addition, 1 μM HLP reproducibly consumed 5 to 6 μM NO within minutes under these conditions. This result provides further evidence that NO consumption is catalytic with multiple turnovers with respect to NO consumption.

The Mka HLP does exhibit some weak catalase activity to produce O₂ (4). Thus, it is possible that even under anaerobic conditions, nonenzymatic autoxidation of NO to form NO₂⁻ could result from the reaction of the catalase-produced O₂. Therefore, it was important to rule this out as a possible alternative explanation to actual peroxidase activity. Comparison of the NO consumption rate in Table 4 with that of the catalase activity of the Mka HLP at 10 mM H₂O₂ precludes this possibility. Addition of the Mka HLP increased the NO consumption rate under these conditions from 15 ± 1 nM NO/s to 36 ± 6 nM NO/s. The Mka HLP-dependent NO consumption rate is therefore 21 nM NO/s. By comparison, the steady-state catalase activity of 1 μM Mka HLP under these conditions was 4.1 ± 0.3 μM H₂O₂/s, which is equivalent to production of 2 μM O₂/s. Because the initial NO consumption rates were calculated within 5 s of HLP addition, only up to 10 μM O₂ could accumulate within this interval. The rate of NO autoxidation at 25 °C was calculated using Equation 1 (25). The fastest NO consumption rate at 10 μM O₂ and 5 μM NO was calculated as 2.3 nM NO/s or approximately tenfold slower than the observed rate of 21 nM NO/s in the presence of the Mka HLP. This analysis confirms the conclusion that the observed NO consumption and subsequent nitrite formation is the result of an enzymatic NO peroxidase activity and not an artifact related to the catalase activity of the Mka HLP.

\[-\frac{d[NO]}{dt} = 9 \times 10^6 \text{M}^{-2}\text{s}^{-1}[NO]^2[O_2]\]  

**Steady-state kinetic analysis of Mka HLP-dependent NO peroxidase activity**

Kinetic studies of the NO peroxidase reaction could monitor formation of either the nitrite product or consumption of the NO substrate. There is not a continuous assay available with which to monitor NO₂⁻ production; however, the rate of consumption of the NO substrate could be monitored using an NO electrode as in Figure 7. The reactions were initiated by addition of the Mka HLP to the mixture containing NO and H₂O₂. The order of addition of H₂O₂ and HLP to NO did not affect the rate of the reaction. Initial rates of NO consumption, corrected for background nonenzymatic loss of NO, were determined at different concentrations of NO to determine the Kₘ for NO and kₗ for the reaction (Fig. 8). The results indicate that the enzyme is saturated at low micromolar concentrations. Analysis of the data yielded values kₗ of 9.2 ± 0.3 × 10⁻² s⁻¹ (5.5 min⁻¹), Kₘ of 0.44 ± 0.08 μM, and a kₗ/Kₘ of 2.3 × 10⁷ M⁻¹s⁻¹.

The studies described in Table 4 confirmed that nitrite was the product of the reaction. While it would be desirable, it was not possible to also monitor NO₂⁻ production in parallel with NO consumption. As described under Experimental procedures, for each time point the reaction would need to

| Sample          | [Nitrite, NO₂⁻], μM | [Nitrate, NO₃⁻], μM |
|-----------------|---------------------|---------------------|
| NO only         | 320 ± 20            | 0                   |
| Mka HLP only    | 0                   | 0                   |
| Mka HLP + NO    | 290 ± 7             | 59 ± 13             |

Concentrations of nitrate and nitrite were determined by ion chromatography. The Mka HLP was present at 60 μM and NO at 450 μM in air saturated 50 mM MOPS, pH 7.5. Reactions were performed in triplicate.
be quenched and then analyzed by the multistep chemical reactions of the Griess assay. The limit of detection of nitrite by the Griess assay is approximately 2.5 μM nitrite. This is far greater than the K_m for NO of 0.44 μM. Furthermore, H_2O_2 interferes with the Griess reaction further decreasing its sensitivity. As such, it would not be possible to monitor the steady-state reaction by monitoring nitrite production in the relevant range of substrate concentration. One cannot simply remove H_2O_2 by addition of catalase as that would produce O_2, which would then react nonenzymatically with NO to produce nitrite. Similarly, preparations of samples for analysis by ion chromatography also require H_2O_2 removal, an anaerobic overnight incubation, and chromatography for each sample. This is why the nitrite product results presented in Tables 1–4, which were performed at higher concentrations of NO, were end point determinations after the completion of the reaction, which were corrected for nonenzymatic background reactions.

**Possible NO peroxidase mechanisms**

Two possible mechanisms for the NO peroxidase reaction that is catalyzed by the Mka HLP are proposed (Fig. 9). Each is consistent with the sum of the data and observations from this study. In each, the starting and end points of the reaction cycle are the Fe(III)-Fe(III) state. One mechanism utilizes only the diiron site for catalysis (Fig. 9A). The initial two steps are the same as proposed for the anaerobic reaction with NO (see Fig. 5). The reaction is initiated by reductive nitrosylation to yield NO_2^{-}. In contrast to the anaerobic mechanism in Figure 5, H_2O_2 then reacts with the mixed-valence species to generate a Compound II-like species plus water. This is followed by a second NO reacting with the Fe(III) of Compound II to form a second NO_2^{-} and regenerate the diferric site. The other possible mechanism is patterned after the mechanism for heme-dependent peroxidases (Fig. 9B). The first step in this mechanism is reaction with H_2O_2 to yield a Compound I-like intermediate. Compound I in heme iron sites is typically described as a ferryl Fe(IV)=O with a porphyrin cation radical. In this proposed mechanism for the Mka HLP with nonheme irons, the cation radical is centered on the Tyr that provides a ligand for one of the irons. This intermediate then undergoes reductive nitrosylation by NO, which leads to formation of a NO_2^{-} and oxidation of the ferrous iron by the Tyr radical. This yields a Compound II-like intermediate that reacts with a second NO to generate a second NO_2^{-}, as also occurred in the other proposed mechanism.

**Discussion**

The Mka HLP is a representative of a distinct class of HLPs found in infectious mycobacteria. That distinction was originally based on the sequence of the proteins. Hemerythrins function as oxygen carriers or storage proteins. The other HLPs have a variety of other functions. These include activity as sensors for oxygen (26) or iron (27), signal transduction (28) and repair of Fe-S centers (29). Characterization of the Mka HLP as an NO peroxidase further distinguishes it based on function. While each of these proteins possess an oxo-bridged diiron site, the identity and pattern of amino acid residues that provide ligands for the irons in the mycobacterial HLPs are distinct from the others. This is likely the basis for the unprecedented reactivity of the Mka HLP. The overall structure of the Mka HLP also differs from that of hemerythrin and other HLPs. It is a monomer with a core structure of five α-helices, rather than four α-helices. The majority of hemerythrins are multimers of six or eight four-helix subunits (2, 30). In the other HLPs, the four-helix
domain is fused to another protein domain that dictates its function. The Mka HLP is a monomer, which is not fused to another protein domain.

The characterization of the NO peroxidase activity of the Mka HLP is not only an unprecedented activity for a hemerythrin or HLP, but also unexpected as peroxidases typically use heme cofactors to catalyze their reactions. Furthermore, the Mka HLP is a high-affinity NO peroxidase with a submicromolar $K_m$ for NO. Similar micromolar or submicromolar $K_m$ values have been reported for other NO detoxifying enzymes, including NO dioxygenases (10, 21), and NO reductases (31–33). This high affinity for NO is consistent with a role of the Mka HLP to scavenge NO at low NO concentrations to prevent accumulation of this potential toxic compound. The $K_m$ value determined for NO in this study is in the range of concentration of NO needed for nitrosative stress (34). It is also consistent with the upregulation of this enzyme after phagocytosis by macrophages, which will expose the bacterium to NO. The high affinity could also allow this reaction to persist, even under aerobic conditions, as the Mka HLP-catalyzed peroxidase reaction was shown to be able to compete with the spontaneous NO decay under aerobic conditions.

In addition to the ability to catalyze an NO peroxidase reaction, the oxo-bridged diiron site of the Mka HLP exhibits chemical reactivity that is not typically seen in hemerythrins and other HLPs. The two irons are not readily reduced to a diferrous form, but instead to a stable mixed-valence species. Initial reaction with NO results in reductive nitrosylation, a process before only seen with heme iron. Furthermore, the mixed-valence state could not be readily reduced to the

![Figure 8. Steady-state kinetic analysis of NO consumption during the NO peroxidase reaction catalyzed by the Mka HLP.](image)

**Figure 8.** Steady-state kinetic analysis of NO consumption during the NO peroxidase reaction catalyzed by the Mka HLP. The concentration of NO was monitored using an NO electrode. NO was varied in the presence of 100 nM Mka HLP and 100 mM H$_2$O$_2$ in 50 mM potassium phosphate, pH 7.5, at 20°C, under anaerobic conditions. Points are the average of three replicates. The line is a fit of the data to Equation 2 with an $R^2$ of 0.98. HLP, hemerythrin-like protein; Mka, *Mycobacterium kansasii*; NO, nitric oxide.

![Figure 9. Proposed mechanisms for the NO peroxidase reaction that is catalyzed by the Mka HLP.](image)

**Figure 9.** Proposed mechanisms for the NO peroxidase reaction that is catalyzed by the Mka HLP. Mechanism A utilizes only the oxo-bridged diiron site. Mechanism B also utilizes the Tyr residue that provides a ligand for iron. The bridging O is not shown for simplicity. HLP, hemerythrin-like protein; Mka, *Mycobacterium kansasii*; NO, nitric oxide.
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differens state that is commonly seen as an intermediate form of hemerythrins and other HLPs. These unusual properties of the Mka HLP are likely related to the nature of the amino acid side chains that coordinate the two iron atoms and their geometry. This combination of amino acid residue ligands is unique to mycobacterial HLPs (1, 7). This information points to a novel mechanism for the catalysis of the peroxidase reaction by a diiron center. Taking into account the novel reactivity described above, two possible mechanisms for the reaction are proposed (Fig. 9) that are each consistent with the data presented herein.

The biological relevance of the Mka HLP goes beyond removal of NO. The NO peroxidase activity of the Mka HLP is well suited for a mycobacterial host. The two substrates for the reaction, H₂O₂ and NO, are each produced in macrophages to defend against an infectious Mycobacterium (35, 36). This occurs in a relatively hypoxic environment similar to the anaerobic conditions under which the Mka HLP was studied and shown to catalyze the peroxidase reaction. The NO peroxidase activity converts H₂O₂ to water and NO to nitrite, thus neutralizing both of the molecules used as the host defense mechanism. Furthermore, production of nitrite can also be advantageous, as nitrite is a signaling molecule in mycobacteria that slows the growth of the organism under hypoxia conditions (17, 23, 24). Thus, the action of the Mka HLP not only protects the host bacterium from oxidative and nitrosative damage, but also allows the host to adapt to the hostile hypoxic environment by virtue of the nitrite produced by the peroxidase reaction.

These studies provide a framework for future structure-function studies to determine the precise contributions of each of the amino acid ligands, as well as other residues in the protein that are unique to mycobacterial HLPs, to the unusual reactivity described herein. The results also provide a rationale for physiologic studies of M. kansasii, as well as other infectious mycobacteria. For example, it is known that the gene for this protein is upregulated in M. tuberculosis after phagocytosis of the bacterium by macrophages. This raises the question of what might happen if the gene were knocked out or inactivated. Once the consequences of this are understood, it could further implicate these mycobacterial HLPs as potential targets for drugs to combat tuberculosis and other mycobacterial infections.

Experimental procedures

Protein expression and purification

The methods for expression and purification of the Mka HLP were as described previously (4). One difference is that the previous study used the gene that had been cloned from M. kansasii. In the current study, the protein was expressed using a commercially synthesized gene (Genewiz) that was codon-optimized for expression in Escherichia coli and to which an N-terminal His tag was added. This was cloned into a pET15b vector and transformed into E. coli Rosetta2(DE3) cells.

Preparation of solutions

Dithionite (Na₂S₂O₄) solutions were prepared by dissolving Na₂S₂O₄ powder with deoxygenated water or buffer in an anaerobic glovebox. Dithionite solutions were quantified by UV-visible absorption spectroscopy using a known extinction coefficient (ε₂₃₅ = 8 mM⁻¹ cm⁻¹).

Solutions of NO were prepared by two alternative methods. Saturated solutions of NO were prepared by bubbling NO gas into solution after passage through a NaOH solution to remove the impurities. Alternatively, the NO precursor PROLI NONOate (Cayman Chemical) was used. It was prepared by dissolving PROLI NONOate into 0.01 N NaOH solution and quantitated from the absorbance at 252 nm (ε = 8400 M⁻¹ cm⁻¹). Each molecule of the NONOate released two NO molecules after addition to the buffer.

Sample preparation

Aerobic samples were generated using air-saturated 50 mM MOPS or 50 mM phosphate at pH 7.5. Anaerobic samples were generated in a Genesis glovebox (Vacuum Atmospheres Company) in an anaerobic N₂ atmosphere. Buffers containing 50 mM MOPS or 50 mM phosphate at pH 7.5 were deoxygenated by three vacuum and N₂ purge cycles on a Schlenk line. Reactions were initiated by addition of NO gas in the form of buffered NO. Buffered NO in septum-sealed headspace vials was transferred through the septum and to the samples by use of a 100-μL Hamilton syringe. Samples containing NO gas were prepared in a 2-mL septum sealed Starna cuvette. Reactions with NO gas were initiated by replacing the headspace of the cuvette with purified NO gas and inverting the cuvette to introduce NO into solution. The solution NO concentration in samples prepared in this manner was 1200 μM NO. UV-vis absorption spectra were collected on an Ocean Optics USB 2000+ spectrometer in the glovebox to prevent contamination with O₂.

Determination of nitrite and nitrate concentrations formed by reaction with NO.

Determination of nitrite concentrations during assays was achieved using the Griess assay (37). For determination of the time course for nitrite formation, samples were collected by quenching 100 μl aliquots of each reaction sample with 50 μl of deoxygenated Griess reagent R1 (1% sulfanilamide in 5% H₃PO₄) and mixing by pipetting. Development of the Griess assay was then immediately initiated thereafter by addition of 50 μl of deoxygenated Griess reagent R2 (0.1% napthylethylenediamine dihydrochloride in water). The concentration was determined by using an extinction coefficient, ε₅₄₂ = 50 mM⁻¹ cm⁻¹ as well as from a standard curve that was generated from reactions with known concentrations of nitrite.

Quantitation of nitrite in samples containing H₂O₂ during the peroxidase reaction required additional preparation steps because H₂O₂ concentrations greater than 1 mM interfere with the Griess assay. After incubation of these samples in the
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glovebox, the samples were purged with N₂ gas to remove excess NO. Afterward, excess H₂O₂ was removed by addition of 50 µg of catalase and incubated at room temperature for 5 min. The H₂O₂ concentration was determined spectrophotometrically by monitoring the absorbance at 240 nm using the extinction coefficient of ε₂₄₀ = 43.6 M⁻¹ cm⁻¹. The resulting samples were then analyzed by Griess assay as described above.

Formation of nitrite and nitrate was also quantitated by ion chromatography. Samples for ion chromatography were prepared and incubated overnight in an anaerobic glovebox. This incubation ensured complete removal of solution NO from the samples. After overnight incubation, the samples were removed from the glovebox and the protein that was present was removed using three MWCO Amicon microcentrifugal filters (Millipore). The filtrate was then analyzed by ion chromatography for nitrite and nitrate using a Dionex Integrity High-Pressure Ion Chromatography (ThermoScientific) equipped with a 4 mm anionic exchange column (IonPac AS20), suppressor (Dionex ADRS 600 Suppressor) and a conductivity detector, operated at constant voltage (4.0 V). The sample loop was 20 µl and was first degassed in an internal oven at 30 °C and then carried through the column by 35 mM NaOH (ultrapure, carbonate free, Acros Organics). The elution times under the conditions studied were 4 min and 4.9 min for nitrates and nitrates, respectively.

Electron paramagnetic spectroscopy

X-band (9.51 GHz) EPR spectra were acquired using a Freiberg Instrument Miniscope MS5000 spectrometer equipped with an Advanced Research Systems LTR helium flow cryostat. Standard collection parameters were 1.0 mT modulation amplitude and a 100 s sweep time. Temperature and microwave powers during collection are noted in the figure captions.

NO electrode studies

Experiments that monitored NO consumption were performed in a multiport measurement chamber with a magnetic stir bar (World Precision Instruments). The reactions were performed in 1.5 ml of 50 mM phosphate buffer, pH 7.5. The buffer was degassed prior to the experiment by bubbling N₂ gas through a 22 G needle threaded through the cap of the reaction chamber and into the buffer for 5 to 10 min. The needle was removed prior to addition of NO to the chamber and the cap lowered to the liquid level to eliminate the headspace in the reaction vessel. The fastest rotation rate on the magnetic stir plate resulted in the smallest background NO consumption rate. Under this configuration, the NO electrode was calibrated using quantified PROLI-NONOate solutions. Final HLP and H₂O₂ concentrations in each NO electrode experiment are listed in the figure captions.

Steady-state kinetic studies

An NO electrode was used to monitor [NO] as described above during the time course of the NO peroxidase reaction catalyzed by the Mka HLP. For steady-state kinetic analysis of the reactions, the initial linear rate of the decrease in [NO] was determined and plotted. Data were fit to Equation 2 where v is the initial rate, S is NO, and E is the Mka HLP.

\[ \frac{v}{[E]} = k_{\text{cat}} [S] / (K_m + [S]) \]

Data availability

All data are contained within the manuscript.

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Abbreviations—The abbreviations used are: EPR, electron paramagnetic resonance; HLP, hemerythrin-like protein; Mka, Mycobacterium kansasi; NO, nitric oxide.

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