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Substrate Binding Modulates the Activity of *Mycobacterium smegmatis* G, a Flavin-Dependent Monooxygenase Involved in the Biosynthesis of Hydroxamate-Containing Siderophores

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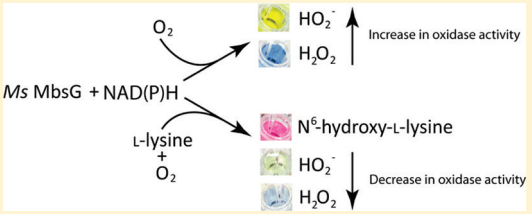
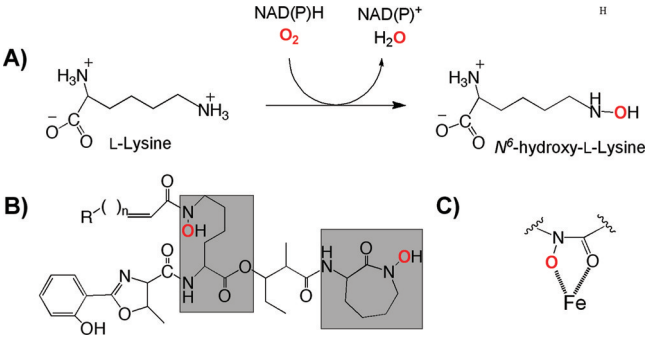
ABSTRACT: *Mycobacterium smegmatis* G (MbsG) is a flavin-dependent monooxygenase that catalyzes the NAD(P)H- and oxygen-dependent hydroxylation of the terminal amino group on the side chain of L-lysine in the biosynthetic pathway of the siderophore mycobactin. Mycobactins are essential for mycobacterium growth under iron-limiting conditions encountered during infection in mammals. Thus, enzymes involved in the biosynthesis of mycobactin represent potential drug targets. MbsG was expressed in *Escherichia coli* and purified using metal affinity and ionic exchange chromatographies. Recombinant MbsG represents the first member of this class of enzymes isolated in the active form, with a tightly bound FAD cofactor. The k_{cat} value for formation of hydroxylated L-lysine under steady-state conditions was 5.0 min^{-1}, and K_m values of 0.21 mM for L-lysine, 1.1 mM for NADH, and 2.4 mM for NADPH were calculated. The enzyme functioned as an oxidase when the activity of MbsG was measured by monitoring oxygen consumption in the absence of L-lysine, oxidizing NADH and NADPH with k_{cat} values of 59 and 49 min^{-1}, respectively. Under these conditions, MbsG produced both hydrogen peroxide and superoxide. In contrast, when L-lysine was present, the reaction became more coupled, producing hydroxylated L-lysine and decreasing the oxidase activity. These results suggest that substrate binding modulates the function of MbsG from an oxidase to a monooxygenase.



Iron is an essential nutrient and is typically present at concentrations too low to support proliferation of microbial pathogens in mammalian hosts.[1] The low concentration of iron is partly due to the fact that it is present as insoluble ferric hydroxides and is sequestered by mammalian iron-binding proteins such as ferritin and lactoferrin, which further reduce the availability of iron.[1,2] To overcome this iron deficiency, many invasive pathogens such as *Mycobacterium tuberculosis* and *Aspergillus fumigatus* obtain iron via siderophores. These compounds are low-molecular mass metal chelators that are involved in iron uptake.[3]

Siderophores vary among species and are diverse in many respects, particularly in the presence of catechol, carboxylate, or hydroxamate functional groups at the iron-binding site.[4] *Mycobacteria* spp., including *M. tuberculosis*, synthesize and secrete the hydroxamate-containing siderophore mycobactin. Biosynthesis of hydroxamate-containing siderophores involves the action of novel flavin-containing N-hydroxylating monooxygenases (NMO). The genes encoding NMOs in *M. tuberculosis* and *Mycobacterium smegmatis* have been identified as MbtG and MbsG, respectively. These enzymes catalyze the NAD(P)H- and oxygen-dependent hydroxylation of the side chain amino group of L-lysine that is incorporated into the siderophore mycobactin (Scheme 1). Mycobactins have been shown to be essential for growth under iron-limiting conditions.[5,6] The importance of this enzyme in mycobactin biosynthesis and the lack of homologous enzymes in humans suggest that MbtG is an attractive drug target for the treatment of tuberculosis.

Scheme 1. (A) Reaction Catalyzed by MbsG, (B) Structure of Mycobactin,[a] and (C) Role of the Hydroxamate Residues in Metal Binding



[a]Hydroxylated L-lysines are shown in gray boxes.

Here, we present the recombinant expression and characterization of the N^6-lysine hydroxylase from *M. smegmatis*, a homologue of MbtG that is 75% identical. This enzyme was isolated in the active form with a bound FAD cofactor and represents the first member of the bacterial NMO that acts on

Received: June 17, 2011
Revised: August 2, 2011
Published: August 26, 2011

L-lysine to be isolated in its functional form. Kinetic characterization shows that in the absence of L-lysine, MbsG functions as an oxidase and the addition of the amino acid substrate converts the activity to a monooxygenase. This represents a novel mechanism of regulation for flavin-dependent monooxygenases.

MATERIALS AND METHODS

Materials. *Pme*I and *Sgf*I restriction endonucleases were from Promega Corp. (Madison, WI). *Escherichia coli* TOP10 and BL21-T1^R chemically competent cells were from Invitrogen. Plasmids pVP55A and pVP56K were obtained from the Center for Eukaryotic Structural Genomics (University of Wisconsin, Madison, WI). DNA sequencing was performed at the Virginia Bioinformatics Institute DNA sequencing facility. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Gold Biotechnology (St. Louis, MO), and protein purification was performed on an ÄKTA Prime FPLC system (GE Healthcare). L-Lysine, substrates, NADPH, NADH, buffers, and salts were purchased from Thermo Fisher Scientific and used without further purification.

Cloning. The *M. smegmatis mbsG* gene was amplified directly from genomic DNA using oligos 5′-ggttgcgatcgccatgagcgagacgaccccacggctg-3′ (*Sgf*I site underlined) and 5′-gggggtttaaactcgaacggattggtgctcatcgg-3′ (*Pme*I site underlined). The polymerase chain reaction product was digested with *Sgf*I and *Pme*I endonucleases for 20 min at 37 °C. The digestion reaction was terminated by incubation at 65 °C for 30 min. The digested *mbsG* gene was ligated into the pVP55A or pVP56K plasmid, each previously digested with *Sgf*I and *Pme*I enzymes. Plasmid pVP55A carries an ampicillin resistant gene, while pVP56K carries a kanamycin resistant gene. Ligation reaction mixtures were transformed in *E. coli* TOP10 cells and plated on LB-agar plates with the appropriate antibiotic. Positive clones were identified by DNA sequencing.

Protein Expression and Purification. Initial expression tests with the pVP55A plasmid, which produced an MbsG N-terminally fused to a His_8 tag, yielded insoluble protein. Therefore, protein was expressed using plasmid pVP56K. This plasmid expressed MbsG as a fusion with maltose binding protein (MBP). An overnight culture of *E. coli* BL21-T1^R cells containing the *pVP56K:mbsG* plasmid was used to inoculate six 1.5 L flasks of LB broth containing kanamycin (50 μg/mL). Cultures were grown at 37 °C with agitation, set at 250 rpm, to an optical density at 600 nm (OD_{600}) of ∼0.6. Protein expression was initiated by addition of 300 μM IPTG. Cells were harvested after induction for 4 h at 20 °C by centrifugation and stored at −80 °C. For protein isolation, all of the steps were performed at 4 °C. A 30 g cell pellet was resuspended in 90 mL of buffer A [25 mM HEPES, 300 mM NaCl, 25 mM imidazole, and 5% glycerol (pH 7.5)], and the cell suspension was incubated with constant stirring for 30 min in the presence of 1 mM phenylmethanesulfonyl fluoride and DNase I, RNase, and lysozyme (25 μg/mL each). Cells were then lysed by sonication, and the cellular debris and insoluble material were precipitated by centrifugation at 35000*g* for 45 min. The resulting supernatant was loaded onto two-in-tandem 5 mL HisTrap columns (GE Healthcare) previously equilibrated with buffer A. The columns were washed with buffer A, and MbsG was eluted with a 150 mL imidazole gradient (from 25 to 300 mM) at a flow rate of 5 mL/min. Fractions were analyzed by SDS−PAGE, and those containing His_8-MBP-MbsG were pooled and dialyzed in 2 L of buffer A overnight. MbsG was liberated from the His_8-MBP tag by the addition of tobacco etch virus (TEV) protease (approximately 0.5 mg of TEV/mg of MbsG based on flavin content) during dialysis. After overnight cleavage, the protein solution was centrifuged at 35000*g* for 45 min to remove traces of precipitate, and the sample was loaded onto two-in-tandem 5 mL HisTrap columns equilibrated with buffer A. In this step, His_8-MBP and His_8-TEV remained bound to the column but MbsG did not. The yellow flow-through containing MbsG was pooled and dialyzed for 2 h in 2 L of buffer B [25 mM HEPES and 5% glycerol (pH 7.5)]. The sample was then loaded onto two-in-tandem 5 mL DEAE columns (GE Healthcare) equilibrated with buffer B. MbsG was eluted with a 200 mL NaCl gradient (from 10 to 400 mM) at a flow rate of 3 mL/min. Fractions containing MbsG were pooled and concentrated to a volume of 2 mL. Aliquots were frozen in liquid nitrogen prior to being stored at −80 °C.

Determination of the Bound Flavin Extinction Coefficient. The spectrum of purified MbsG (40 μM) in 100 mM sodium phosphate (pH 7.5) was recorded. The flavin was extracted by addition of 10% SDS and incubated at 95 °C for 1 min, and the spectrum of the liberated FAD was recorded. An extinction coefficient at 450 nm of 12800 M^{-1} cm^{-1} was calculated for the FAD bound to MbsG from the extinction coefficient at 450 nm for free FAD, 11300 M^{-1} cm^{-1}.[7]

Identification of the Bound FAD Cofactor by Mass Spectrometry. MbsG was incubated at 95 °C for 5 min. Denatured protein was precipitated by centrifugation at 9300*g* for 5 min. The supernatant was removed, and a 1 μL aliquot was spotted onto a MALDI target plate and allowed to air-dry. Then, 1 μL of matrix solution (4 mg/mL α-cyano-4-hydroxycinnamic acid in a 50:50 water/acetonitrile mixture supplemented with 0.1% trifluoroacetic acid) was added to the spot and allowed to air-dry. The sample was analyzed using an AB Sciex 4800 MALDI TOF/TOF instrument operating in reflector negative ion mode following tuning and calibration utilizing commercial FAD. Both MS and MS/MS spectra were compared to that of commercial FAD to validate the identification of the bound FAD cofactor.

Iodine Oxidation Assay. The amount of hydroxylated product formed by MbsG was assayed by a variation of the Csaky iodine oxidation reaction.[8] The standard assay buffer contained 104 μL of 100 mM sodium phosphate (pH 7.5) and concentrations of L-lysine and NAD(P)H ranging from 0 to 20 mM and from 0 to 15 mM, respectively. In assays that were performed with varying amounts of L-lysine, NADH and NADPH concentrations were kept constant at 3 and 5 mM, respectively. In assays that were performed with varying amounts of NADH and NADPH, L-lysine concentrations were kept constant at 3 mM. The reaction was initiated by addition of MbsG (2.0 μM), and the reaction was allowed to proceed for 10 min at 25 °C with shaking at 750 rpm. The reaction was quenched by the addition of 52.5 μL of 2 N perchloric acid. Aliquots (47.5 μL each) of the terminated reaction mixture were transferred into a 96-well plate for color development. The pH values of the reaction mixtures were neutralized by the addition of 47.5 μL of a 10% (w/v) sodium acetate solution, followed by the addition of 47.5 μL of 1% (w/v) sulfanilic acid in 25% (v/v) acetic acid. Then, 19 μL of 0.5% (w/v) iodine in glacial acetic acid was added, and the reaction mixture was incubated with orbital shaking at 25 °C for 15 min. Excess iodine was removed by the addition of 19 μL of 0.1 N sodium thiosulfate, and the color was then developed

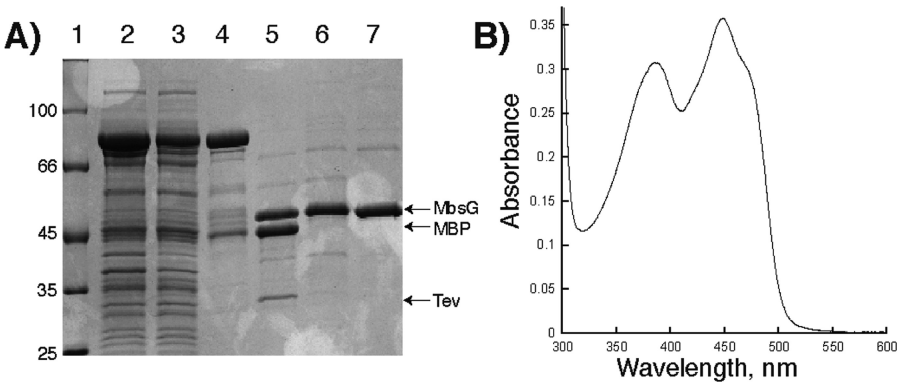


Figure 1. Summary of the purification of MbsG. (A) SDS–PAGE gel (10%) summarizing the purification of MbsG: (1) molecular weight markers, (2) complete lysate, (3) lysate supernatant, (4) IMAC, (5) sample after treatment with TEV protease, (6) second IMAC, and (7) sample from DEAE chromatography. (B) UV–visible spectrum of purified MbsG.

Table 1. Summary of the Purification of MbsG[a]

sample	total mass (mg)	total volume (mL)	activity (μmol min^{-1})	specific activity (μmol min^{-1} mg^{-1})	recovery (%)	*x*-fold change
complete lysate	3800	110	13200	3.5	100	1
supernatant	2500	108	16800	6.7	127.3	1.9
Ni^{2+}	100	75	7230	72.3	54.8	20.5
DEAE	32	35	3940	123.1	29.9	35.8

[a]Rate values were calculated using the iodine oxidation assay at 25 °C with 100 mM sodium phosphate (pH 7.5), 3 mM NADH, and 3 mM L-lysine. Protein quantities were calculated using the Bradford dye binding assay (Bio-Rad) using bovine serum albumin as a standard.

via addition of 19 μL of 0.6% (w/v) α-naphthylamine in 30% (v/v) acetic acid. The absorbance at 562 nm was measured after incubation for 45 min on a SpectraMax M5e plate reader (Molecular Devices). A standard curve of hydroxylamine hydrochloride was used to calculate the amount of hydroxylated product produced.

Oxygen Consumption Assay. The amount of molecular oxygen consumed by MbsG was monitored using a Hansatech Oxygraph (Norfolk, U.K.). The standard assay contained 1 mL of 100 mM sodium phosphate (pH 7.5), with concentrations of NAD(P)H ranging from 0 to 20 mM and an L-lysine concentration of either 0 or 3 mM. The reaction was initiated by addition of MbsG (2.0 μM), and the reaction was allowed to proceed for 1 min at 25 °C with constant stirring.

Hydrogen Peroxide Formation Assay. The amount of hydrogen peroxide formed from MbsG was measured using the Pierce Hydrogen Peroxide Detection Kit. The standard assay buffer contained 150 μL of 100 mM sodium phosphate (pH 7.5) and concentrations of L-lysine and NAD(P)H both ranging from 0 to 10 mM. In assays that were performed with varying amounts of L-lysine, NADH and NADPH concentrations were kept constant at 3 and 5 mM, respectively. In assays that were performed with varying amounts of NADH and NADPH, L-lysine concentrations were kept constant at either 0 or 3 mM. The reaction was initiated by MbsG (2.0 μM) and the mixture incubated at 25 °C with shaking at 750 rpm for 10 min. The assay was terminated by the addition of 52.5 μL of 2 N perchloric acid. Aliquots of 20 μL of the assay mixture were then added to 200 μL of working reagent [100 mM sorbitol, 125 μM xylenol orange, 250 μM ammonium ferrous(II) sulfate in water, and 25 mM H_2SO_4] in a 96-well plate. The mixture was incubated for 20 min at 25 °C with orbital shaking, at which time the absorbance at 595 nm was measured. A standard curve of hydrogen peroxide was used to calculate the amount of hydrogen peroxide produced by MbsG.

Superoxide Formation Assay. The amount of superoxide formed by MbsG was measured by coupling the assay mixture with WST-1 (*Dojindo* Molecular Technologies, Inc.). WST-1 was added directly to the reaction mixture in a 96-well plate at a concentration of 200 μM. The standard assay buffer contained 200 μL of 100 mM sodium phosphate (pH 7.5) and concentrations of L-lysine and NAD(P)H both ranging from 0 to 10 mM. In assays that were performed with varying amounts of L-lysine, NADH and NADPH concentrations were kept constant at 3 and 5 mM, respectively. In assays that were performed with varying amounts of NADH and NADPH, L-lysine concentrations were kept constant at either 0 or 3 mM. The reaction (200 μL) was initiated by MbsG (2.0 μM) and the mixture incubated at 25 °C with orbital shaking at 750 rpm. After 10 min, the absorbance of the assay mixture was measured at 438 nm. The extinction coefficient of the formed WST-1 formazan (ϵ_{438} = 37000 M^{-1} cm^{-1}) was used to calculate the amount of superoxide produced by MbsG.

Data Analysis. Kinetic data were analyzed using KaleidaGraph (Synergy Software, Reading, PA). Initial rate data were fit to the Michaelis–Menten equation to obtain k_{cat} and K_m values. The substrate inhibition constant for reduced nicotinamide dinucleotide and L-lysine (K_{is}) was determined by fitting the data to eq 1.

$$v = \frac{k_{cat}S}{K_m + S + S^2/K_{is}} \quad (1)$$

RESULTS

Expression and Purification of MbsG. Expression of MbsG in pVP55A, which expressed the protein as a fusion to a His_8 tag, resulted in large amounts of recombinant protein that was completely insoluble (data not shown). To increase protein solubility, MbsG was expressed as a fusion to His_8-MBP in plasmid pVP56K (Figure 1A). This fusion protein remained in

dx.doi.org/10.1021/bi200933h | *Biochemistry* 2011, 50, 8489–8496

the supernatant after centrifugation. The fusion protein contained a His_8 tag at the N-terminus of MBP, which permitted the use of an IMAC column. The partially purified fusion protein was treated with His_8-TEV protease, which cleaves at the recognition sequence engineered between the C-terminus of MBP and the N-terminus of MbsG. To isolate MbsG from His_8-MBP and the His_8-TEV protease, the sample was loaded back onto the IMAC column and the flow-through was collected. Because MbsG does not have affinity for the column, the His_8-MBP and His_8-TEV remained bound while MbsG eluted in the mobile phase. This protein was further purified using a DEAE anion exchange chromatographic step (Figure 1A). Upon purification of MbsG, a 36-fold increase in the specific activity was obtained. In general, a yield of 32 mg of homogeneous protein was obtained from 33 g of cell paste (Table 1). The purified MbsG was found to have a specific activity of 124.5 μmol min^{-1} mg^{-1}. The optical spectrum in Figure 1B shows the presence of an FAD cofactor bound to the purified enzyme. Absorbance maxima can be seen at 380 and 450 nm with a "shoulder" occurring at 472 nm. The percent of incorporation of flavin into MbsG was calculated to be 45% via comparison of the concentration of purified MbsG based on the extinction coefficient of the FAD cofactor at 450 nm (12800 M^{-1} cm^{-1}), and the total protein concentration which was determined using the Bradford dye binding assay. High-resolution MALDI-TOF MS analysis in negative ion mode of the isolated flavin cofactor indicated a mass of m/z 784.14, consistent with the mass of a deprotonated flavin adenine dinucleotide (data not shown). It is worth noting that MbsG is the first reported flavin-dependent N^6-lysine hydroxylase to be isolated in its active form with bound flavin.[9−11]

Kinetic Parameters Measuring N^6-hydroxy-L-lysine. The steady-state kinetic parameters of MbsG with respect to product formation are listed in Table 2. MbsG was shown to utilize NADH and NADPH promiscuously as dinucleotides. k_{cat} values of 4.5 ± 0.1 and 4.2 ± 0.1 min^{-1} were determined with NADH and NADPH, respectively. K_m values of 0.260 ± 0.023, 1.1 ± 0.2, and 2.4 ± 0.4 mM were calculated for L-lysine, NADH, and NADPH, respectively (Table 2 and Figure 2). These results suggest similarities in the first-order kinetic steps between these two reduced dinucleotides.

Table 2. Steady-State Kinetic Parameters of MbsG Measuring Product Formation[a]

parameter	NADH	NADPH
k_{cat} (min^{-1})	4.5 ± 0.1	4.2 ± 0.1
$K_{m,L\text{-}Lys}$ (mM)	0.260 ± 0.023	0.209 ± 0.020
$K_{I,L\text{-}Lys}$ (mM)	32 ± 4	42 ± 6
$k_{cat}/K_{m,L\text{-}Lys}$ (min^{-1} mM^{-1})	26 ± 5	27 ± 5
$K_{m,dinucleotide}$ (mM)	1.1 ± 0.2	2.4 ± 0.4
$K_{I,dinucleotide}$ (mM)	9 ± 2	11 ± 2
$k_{cat}/K_{m,dinucleotide}$ (min^{-1} mM^{-1})	13 ± 4	4 ± 1

[a]Conditions: 100 mM sodium phosphate at pH 7.5 and 25 °C.

The difference in the K_m values for reduced dinucleotides resulted in a >3-fold higher catalytic efficiency (k_{cat}/K_m) with NADH compared to that with NADPH. A decrease in activity was observed at high concentrations of L-lysine and reduced concentrations of the nucleotide, suggesting substrate inhibition. The K_I value for L-lysine was determined to be 32 ± 4 or 9 ± 2 mM for NADH and 11 ± 2 mM for NADPH. Intracellular concentrations of 410 μM for L-lysine, 83 μM for NADH, and 120 μM for NADPH have been reported in *E. coli*.[12] If we assume that similar values are present in *M. smegmatis*, then the inhibitory effects of both coenzyme and L-lysine are not physiologically relevant.

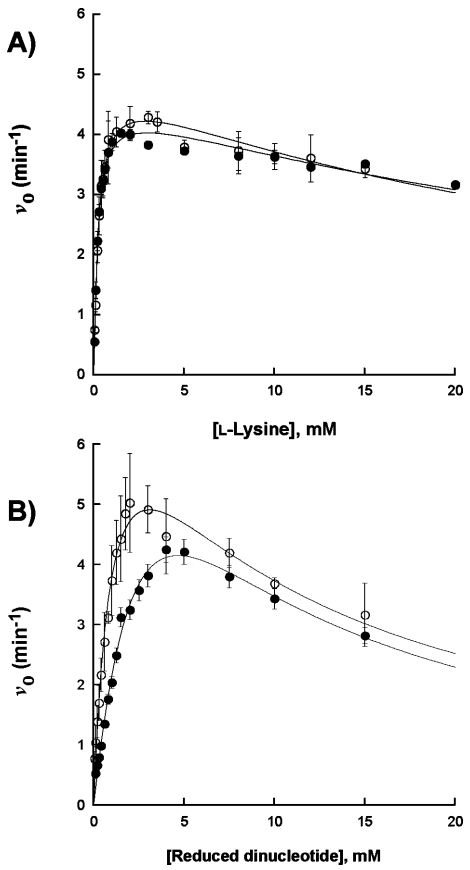


Figure 2. Steady-state kinetics of MbsG measuring the formation of N^6-hydroxy-L-lysine. (A) Formation of N^6-hydroxy-L-lysine as a function of L-lysine in the presence of 3 mM NADH (○) or 5 mM NADPH (●). (B) Formation of N^6-hydroxy-L-lysine as a function of NADH (○) or NADPH (●) in the presence of 3 mM L-lysine.

Kinetic Parameters Measuring Oxygen Consumption. Since the hydroxylation of L-lysine requires the utilization of a molecule of oxygen, MbsG activity can be monitored by measuring the changes in the concentration of oxygen over time. Steady-state kinetic traces of the rate of oxygen consumption are shown in Figure 3, and the parameters are summarized in Table 3. With L-lysine at saturating (3 mM) concentrations, a k_{cat} value of 26 ± 0.4 min^{-1} was obtained when the NADH concentration was varied. Under the same conditions, when NADPH was the variable substrate, a k_{cat} value of 33 ± 2 min^{-1} was obtained. The k_{cat}/K_m value for NADH is 4-fold higher than that for NADPH. This results from a much higher K_m value for NADPH. The relative promiscuity of either reduced dinucleotide with MbsG is a unique feature as in general, flavin-dependent monooxygenases are specific to or prefer NADPH.[13]

In the absence of L-lysine, MbsG displays a higher k_{cat} value, reacting with reduced dinucleotide and molecular oxygen. Under these conditions, MbsG functions as a flavin oxidase. Upon addition of 3 mM L-lysine, a lower k_{cat} value is measured. This shows that in the absence of substrate, MbsG reacts with reduced dinucleotide and is highly uncoupled, but in the

dx.doi.org/10.1021/bi200933h | *Biochemistry* 2011, 50, 8489−8496

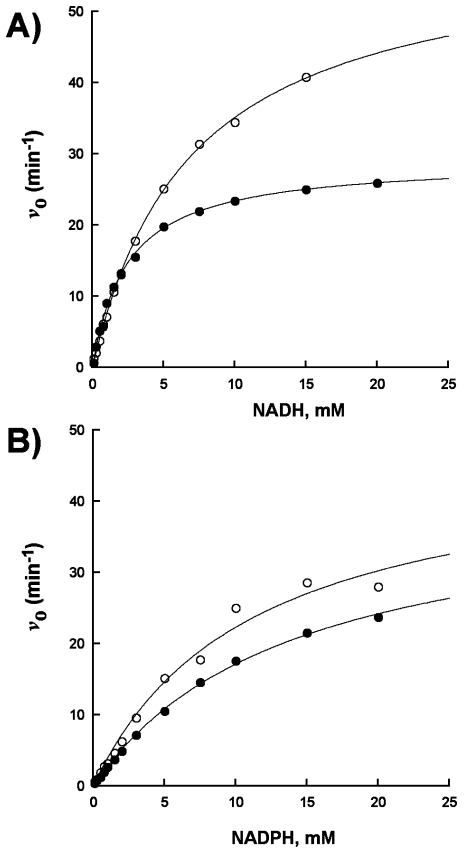


Figure 3. Steady-state kinetics of MbsG measuring the consumption of oxygen. (A) Oxygen consumption as a function of NADH in the absence (○) or presence (●) of 3 mM L-lysine. (B) Oxygen consumption as a function of NADPH in the absence (○) or presence (●) of 3 mM L-lysine.

presence of substrate, the reaction becomes more coupled and is driven toward product formation (N^6-hydroxy-L-lysine).

Regulation by Ligands. As previously mentioned, the oxidase activity of MbsG is regulated by the addition of L-lysine. Analogues of L-lysine were tested to determine if they also regulated the oxidase activity of MbsG. L-Lysine was found to regulate the activity most significantly, but 6-amino-1-hexanol was found to have similar effects (Figure 4B). L-Arginine and L-ornithine were determined to affect the oxidase activity of MbsG as well, but to a lesser extent. One possible explanation for the observed decrease in oxidase activity caused by these ligands is that they interact with the active site of MbsG and help stabilize the C4a-hydroperoxyflavin intermediate; however, hydroxylation was detected with only L-lysine (data not shown).

Hydrogen Peroxide Formation. The concentrations of hydrogen peroxide produced over a 10 min time interval with varying concentrations of NAD(P)H or substrate are shown in Figure 5. These graphs indicate that the enzymatic reaction of MbsG is significantly uncoupled as it produces large amounts of hydrogen peroxide. In Figure 5A, it can be seen that as the concentration of L-lysine is increased, the amount of hydrogen peroxide produced decreases, suggesting that the reaction becomes more coupled. This is consistent with the oxygen consumption assay results where, in the absence of L-lysine, MbsG rapidly consumes oxygen, but in the presence of L-lysine, the oxidase activity is significantly decreased. In panels B and C of Figure 5, it can be seen that as the concentration of either reduced dinucleotide is increased, greater amounts of hydrogen peroxide are produced. The amount of hydrogen peroxide produced in the presence of NADPH is ~3-fold smaller than the amount of hydrogen peroxide produced in the presence of NADH. Thus, with NADH, the formation of the hydroperoxyflavin intermediate is much less stable. This is consistent with the higher oxidase activity of MbsG with NADH compared to that with NADPH in the absence of L-lysine (Table 3).

Superoxide Formation. The differences in the k_{cat} values obtained by directly measuring the formation of hydroxylated lysine (~5 min^{-1}) and the values measured by monitoring oxygen concentration (~30 min^{-1}) show extensive uncoupling in this enzyme. Because a possible product of the reaction between reduced flavin and molecular oxygen is superoxide, we tested to see if this oxygen reactive species was also being produced by MbsG.[13,14] Superoxide production was measured at saturating concentrations of either NADH (3 mM) or NADPH (5 mM). In the absence of L-lysine, ~100 μM superoxide was detected. Varying the concentration of L-lysine reduced the amount of superoxide produced 2-fold (Figure 6). The level of formation of superoxide increased as the concentration of reduced dinucleotides was increased, while the presence of saturating amounts of L-lysine (3 mM) reduced the levels of superoxide produced. Similarly, as observed with the production of hydrogen peroxide, more superoxide was produced with NADH than with NADPH in the absence of L-lysine (Figure 6). However, using L-lysine and NADH concentrations found in *E. coli* (400 μM L-lysine and 100 μM NADH), we determined that for every 20 ± 3 nmol of NADH oxidized, 15 ± 1 nmol of superoxide, 6.8 ± 1 nmol of hydrogen peroxide, and 1.8 ± 1 nmol of N^6-hydroxy-L-lysine were measured (data not shown).[12] When 12 ± 1 nmol of NADPH was consumed, 9.0 ± 0.2 nmol of superoxide, 3 ± 0.5 nmol of hydrogen peroxide, and 0.9 ± 0.3 nmol of N^6-hydroxy-L-lysine were formed. These data give ~12 and ~7% couplings under "physiological conditions" with NADH and NADPH, respectively.

DISCUSSION

Iron is an essential element for most living organisms. The availability of iron in mammals is too low for pathogenic bacteria, such as *Mycobacteria* spp., to establish an infection. To overcome this barrier, pathogens have developed strategies for acquiring iron for their metabolic processes through the biosynthesis of low-molecular mass (<1500 Da) metabolites with high iron affinity, termed siderophores. These molecules can scavenge for iron in hosts and support virulence.

MbsG is an N^6-lysine hydroxylase that operates through the use of a bound FAD cofactor. This enzyme is responsible for the formation of the hydroxamate functional group found on siderophores that gives these molecules their high affinity for iron. Studies of flavin-dependent N-hydroxylating enzymes have been limited, in part, because of the fact that recombinant enzymes were isolated in the inactive form without the flavin cofactor.[10,11] We show the expression and isolation of the recombinant form of this enzyme with a tightly bound FAD cofactor.

High levels of stable and active enzyme were isolated, and detailed characterization was performed. MbsG, though, was found to be expressed in smaller amounts than a related enzyme, N^5-ornithine hydroxylase from *A. fumigatus* (SidA). The yield of soluble and active MbsG was ~3.5 mg/L of

Table 3. Steady-State Kinetic Parameters of MbsG Measured by Oxygen Consumption[a]

	NADH		NADPH	
	without L-lysine	with 3 mM L-lysine	without L-lysine	with 3 mM L-lysine
k_{cat} (min^{-1})	54 ± 1	26 ± 0.4	44 ± 4	33 ± 1
$K_{m,dinucleotide}$ (mM)	7 ± 0.2	2.4 ± 0.1	12 ± 1	12 ± 1
k_{cat}/K_m (min^{-1} mM^{-1})	7.7 ± 0.1	11 ± 0.3	4 ± 0.3	2.8 ± 0.1

[a]Conditions: 100 mM sodium phosphate at pH 7.5 and 25 °C.

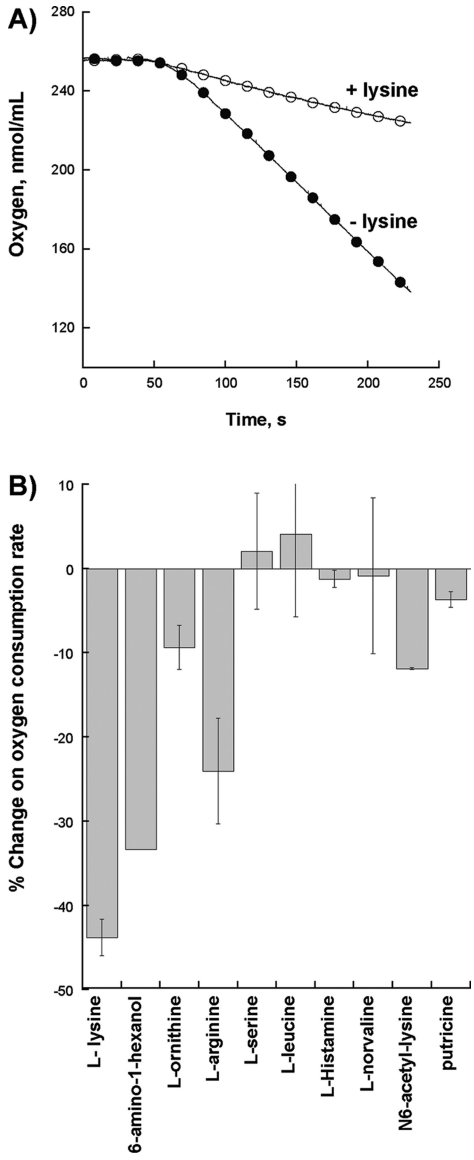


Figure 4. Effects of substrate or ligands on MbsG. (A) Oxygen consumption assay traces in the presence (●) or absence (○) of L-lysine. (B) Regulation of MbsG oxidase activity by various ligands.

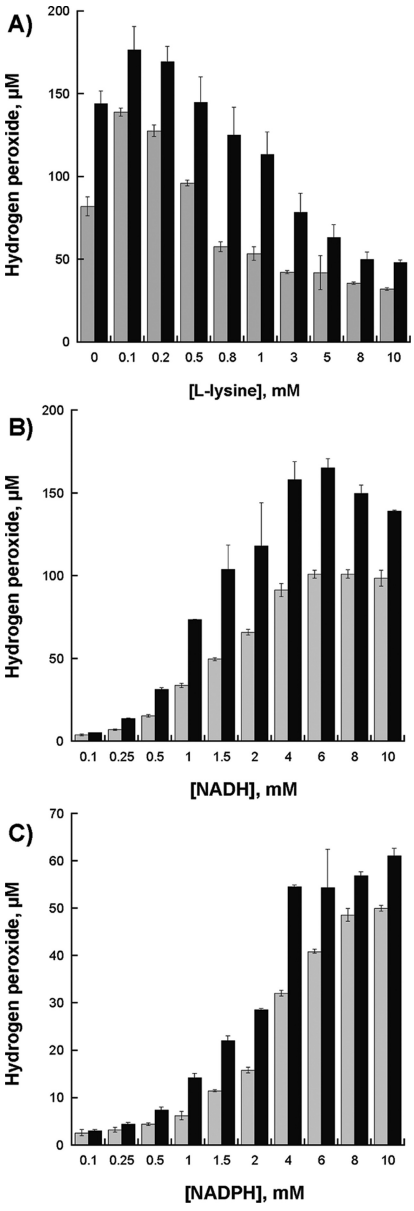


Figure 5. Hydrogen peroxide formation of MbsG. (A) Hydrogen peroxide formation as a function of L-lysine in the presence of 3 mM NADH (black bars) or 5 mM NADPH (gray bars). (B) Hydrogen peroxide formation as a function of NADH in the presence (gray bars) or absence of 3 mM L-lysine (black bars). (C) Same as panel B but with NADPH.

culture, while the yield of SidA was 15 mg/L of culture.[15] MbsG was shown to be specific to L-lysine, as no other amino acids or L-lysine analogues were hydroxylated. The enzyme can effectively use NADPH or NADH as the reduced dinucleotide, and in the absence of L-lysine, this monooxygenase has chemistry similar to that of an oxidase, producing hydrogen peroxide and superoxide. This oxidase activity can be compared to another FAD-containing enzyme, xanthine oxidase, which produces large amounts of superoxide and hydrogen peroxide.[16]

Interestingly, the activity of MbsG for product formation was determined to be slightly lower (6-fold) than that of SidA. MbsG also exhibits a small preference for NADH as the

dx.doi.org/10.1021/bi200933h | Biochemistry 2011, 50, 8489–8496

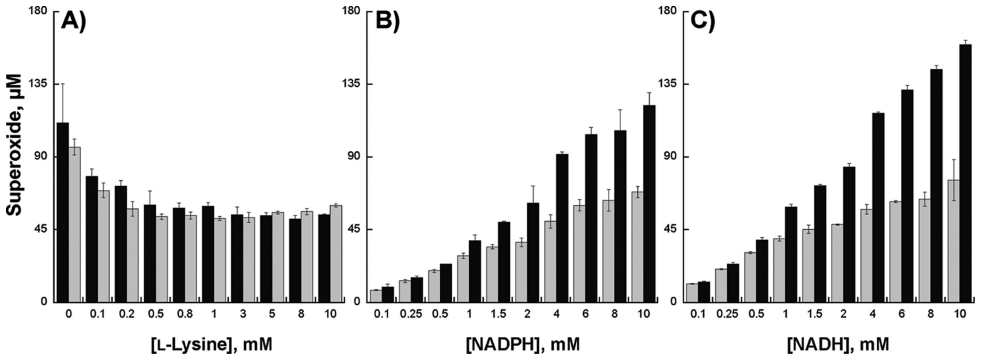
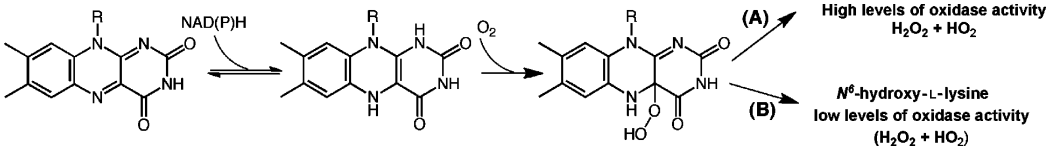


Figure 6. Quantification of superoxide produced by MbsG. The conditions are the same as those described in the legend of Figure 5.

Scheme 2. Substrate Regulation Mechanism of MbsG[a]



[a](A) High levels of oxidase activity observed in the absence of L-lysine. (B) In the presence of L-lysine, the oxidase activity of MbsG decreases and the monooxygenase activity increases.

dinucleotide, while SidA prefers NADPH. These results suggest differences in the rate-limiting step and a unique reduced dinucleotide preference among flavin monooxygenases. It is possible that because *Mycobacteria* spp. are slow-growing organisms the low activity of this NMO suffices for bacterial siderophore biosynthesis.

The byproducts of the MbsG reaction, superoxide and hydrogen peroxide, would be expected to be deleterious to the bacteria *in vivo*. However, it is well established that *Mycobacteria* spp. contain a number of enzymes to reduce the amount of oxygen reactive species, including the Fe, Mn superoxide dismutase, SodA, the Cu, Zn superoxide dismutase, SodC, and catalase-peroxidase, KatG.[19,20] The function of these enzymes is expected to minimize any cellular damage produced by the uncoupling reaction of MbsG.

The catalytic cycle of flavin monooxygenases includes the reaction with NAD(P)H to reduce the flavin cofactor. Subsequent reaction with molecular oxygen leads to the formation of a C4a-hydroperoxyflavin, which is required for hydroxylation.[13,14] Stabilization of this intermediate is key to ensuring coupling of the reaction. The mechanism of stabilization of oxygenated flavin intermediates in SidA is shown to involve interaction of $NADP^+$. In the absence of L-ornithine, $NADP^+$ stabilizes the flavin intermediate preventing the formation of hydrogen peroxide. Rapid turnover is observed only when L-ornithine is present.[15] This is markedly different in MbsG where rapid oxidase turnover occurs in the absence of L-lysine, but the extent of the reaction is decreased when L-lysine is introduced into the system. SidA is capable of reacting with NADPH in the absence of L-ornithine, which reduces the oxidized flavin cofactor.[17] This shows that L-ornithine is needed for oxygen reactivity in SidA but is not necessary in the reductive half-reaction. This is distinct from MbsG for which L-lysine does not control oxygen reactivity. Similar mechanisms have also been shown for other flavin monooxygenases such as the flavin monooxygenase from *Methylophaga* sp. strain SK1, and the phenylacetone monooxygenase from *Thermobifida fusca*.[21,22]

MbsG is shown to operate quite differently from another FAD-containing hydroxylase, *p*-hydroxybenzoate hydroxylase (PHBH). PHBH forms a ternary complex with its substrate, *p*-hydroxybenzoate, and NADPH.[18] This complex then proceeds to react with molecular oxygen and form the product 3,4-dihydroxybenzoate. Biding of *p*-hydroxybenzoate enhances the rate of flavin reduction in PHBH by 1.4×10^5-fold. This is in contrast to MbsG, for which the rate of oxidation of NAD(P)H is increased in the absence of its corresponding substrate, L-lysine.

The mechanism of stabilization of the C4a-hydroperoxyflavin in MbsG does not depend on only interactions with $NADP^+$ or NAD^+, because high levels of oxidase activity are observed when the enzyme reacts with reduced dinucleotides alone. Our results suggest that the C4a-hydroperoxyflavin in MbsG is regulated by the binding of the substrate, L-lysine. This binding modulates the activity of the enzyme converting it from an oxidase to a monooxygenase (Scheme 2). This mechanism represents a novel mechanism of regulation of activity among flavin monooxygenases.

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Funding

This work was supported, in part, by a grant from the National Science Foundation (MCB-1021384), the Allan T. Gwathmey Chemistry award from the Virginia Academy of Sciences, and the Ralph Powe Junior Faculty Enhancement award from the Oak Ridge Associated Universities.

ACKNOWLEDGMENTS

We thank Drs. Rich Helm and Keith Ray for the MS analysis. We also thank Wyatt Chocklett for help in the purification of *Ms* MbsG.

■ ABBREVIATIONS

MbsG, *M. smegmatis* G enzyme (N^6-lysine hydroxylase); MbtG, *M. tuberculosis* G enzyme (N^6-lysine hydroxylase); DEAE, diethylaminoethyl; WST-1, water-soluble tetrazolium-1; IMAC, immobilized metal affinity chromatography; MBP, maltose binding protein; FAD, flavin adenine dinucleotide; TEV, tobacco etch virus; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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