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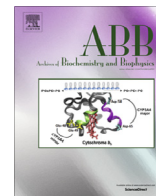
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# Mechanistic studies on the flavin-dependent $N^6$ -lysine monooxygenase MbsG reveal an unusual control for catalysis

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## ABSTRACT

The mechanism of *Mycobacterium smegmatis* G (MbsG), a flavin-dependent L-lysine monooxygenase, was investigated under steady-state and rapid reaction conditions using primary and solvent kinetic isotope effects, substrate analogs, pH and solvent viscosity effects as mechanistic probes. The results suggest that L-lysine binds before NAD(P)H, which leads to a decrease in the rate constant for flavin reduction. L-lysine binding has no effect on the rate of flavin oxidation, which occurs in a one-step process without the observation of a C4a-hydroperoxyflavin intermediate. Similar effects were determined with several substrate analogs. Flavin oxidation is pH independent while the  $k_{cat}/K_m$  and  $k_{red}/K_D$  pH profiles for NAD(P)H exhibit single  $pK_a$  values of  $\sim 6.0$ , with increasing activity as the pH decreases. At lower pH, the enzyme becomes more uncoupled, producing more hydrogen peroxide and superoxide. Hydride transfer is partially rate-limiting at neutral pH and becomes more rate-limiting at low pH. An inverse solvent viscosity effect on  $k_{cat}/K_m$  for NAD(P)H was observed at neutral pH whereas a normal solvent viscosity effect was observed at lower pH. Together, the results indicate a unique mechanism where a rate-limiting and pH-sensitive conformational change occurs in the reductive half-reaction, which affects the efficiency of lysine hydroxylation.

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## Introduction

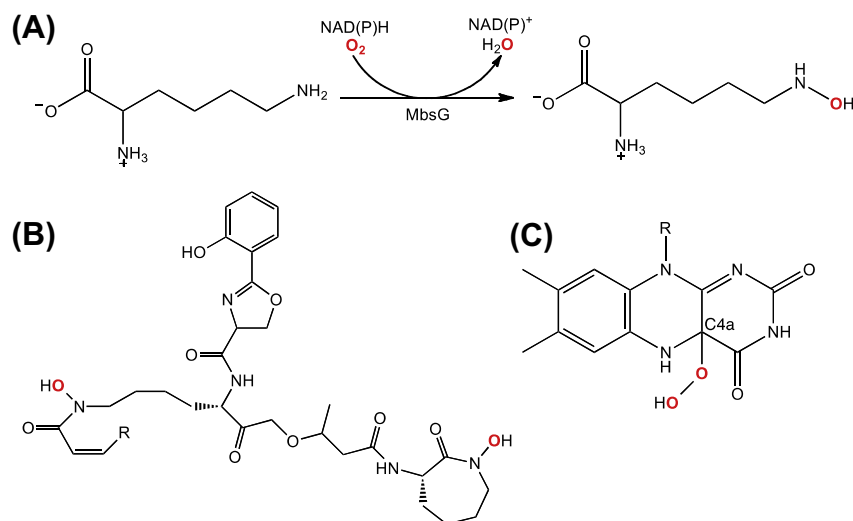
Iron is an essential nutrient required by most living organisms [1]. However, ferric iron is very insoluble, forming iron-hydroxide complexes [2]. This, along with iron sequestration by iron-binding proteins in human serum, presents a barrier that invading microbes must overcome in order to establish infection [3,4]. Many microbes have developed an elegant solution to this problem through the synthesis and secretion of low molecular-weight compounds called siderophores. Siderophores are structurally diverse and contain a number of functional groups, including catechols, phenols, hydroxamates, and carboxylates, which coordinate to chelate ferric iron [5,6]. Siderophores have been shown to be essential for virulence in a number of human pathogens including *Aspergillus fumigatus*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* [7–10]. Because of their important role in pathogenesis and the absence of human homologs, enzymes involved in the biosynthesis of siderophores are promising drug targets.

One group of enzymes critical for hydroxamate-containing siderophore biosynthesis is the microbial *N*-hydroxylating monooxygenases (NMOs).<sup>1</sup> These are flavin adenine dinucleotide (FAD) dependent enzymes that selectively catalyze the hydroxylation of the soft nucleophilic terminal amine groups of L-ornithine, L-lysine, or several aliphatic diamines [11–16]. NMOs are typically highly coupled, selective for NADPH, and stabilize long-lived C4a-hydroperoxyflavin intermediates [17–20]. *Mycobacterium smegmatis* G (MbsG) is an L-lysine monooxygenase involved in the biosynthesis of the siderophore mycobactin (Scheme 1). This enzyme is a homolog of the L-lysine monooxygenase from *M. tuberculosis* MbtG ( $\sim 75\%$  identity), which is essential for virulence [9,10]. Previous biochemical characterization has shown that MbsG does not share the same characteristics as other NMOs [21]. MbsG is non-specific for reduced pyridine dinucleotide, as it can utilize both NADH and NADPH with similar catalytic efficiency. While this enzyme hydroxylates L-lysine,

<sup>1</sup> Abbreviations used: NMO, *N*-hydroxylating monooxygenase; KIE, kinetic isotope effect; pL, pD or pH; MbsG, *Mycobacterium smegmatis* G; Sida, siderophore A; PvdA, pyoverdine A; PHBH, *para*-hydroxybenzoate hydroxylase; FAD, flavin adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FMO, flavin-containing monooxygenase; PAMO, phenylacetone monooxygenase.

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**Scheme 1.** (A) NAD(P)H- and oxygen-dependent reaction catalyzed by MbsG. (B) Structure of the siderophore mycobactin. (C) Structure of the C4a-hydroperoxyflavin. This intermediate is the hydroxylating species that is commonly stabilized by Class B flavin-monooxygenases.

it is highly uncoupled, producing unusually high levels of superoxide and hydrogen peroxide. To gain insight into the mechanism of action of this unique flavin monooxygenase, we investigated the reaction with both NAD(P)H and molecular oxygen in the stopped-flow spectrophotometer. Furthermore, the mechanism of MbsG was probed using primary and solvent kinetic isotope effects, substrate analogs, pH, and viscosity effects. Together, the results suggest the presence of a rate-limiting conformational change that is sensitive to pH and solvent viscosity. This conformational change, which is coupled to flavin reduction, is modulated by L-lysine binding. We propose that this represents an unusual mechanism of regulation among flavin monooxygenases.

## Materials and methods

### Materials

L-Lysine, L-ornithine, 6-amino-1-hexanol, L-arginine, putrescine, cadaverine, NADH, NADPH, NAD<sup>+</sup>, NADP<sup>+</sup>, glycerol, buffers, and salts were purchased from Fisher Scientific (Pittsburgh, PA). WST-1 was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD). Glucose oxidase was purchased from MP Biomedical (Solon, OH). MbsG was expressed and purified as described previously [21].

### Steady-state activity assays

The steady-state reaction catalyzed by MbsG was monitored either by measuring the concentration of oxygen consumed in the reactions or by measuring the concentration of L-N<sup>6</sup>-hydroxyl-lysine produced. In addition, hydrogen peroxide and superoxide, produced as a result of the uncoupling of the reaction, were also quantified as previously described [21]. Oxygen consumption was monitored using a Hansatech Oxygraph (Norfolk, UK) and hydroxylated lysine was quantified by a variation of the Csaky iodine oxidation assay following procedures previously reported [21,22]. These assays were performed in 100 mM sodium phosphate, pH 7.5, at 25 °C.

### Monitoring flavin reduction

Flavin reduction by NAD(P)H was performed under anaerobic conditions on an Applied Photophysics SX20 stopped-flow spectrophotometer (Leatherhead, UK) housed in a Coy glove box (Grass

Lake, MI). Reactions were performed at 25 °C in 100 mM sodium phosphate, pH 7.5. Buffer was made anaerobic by 5 cycles of vacuum and argon flushing in 20 min intervals with constant stirring. Oxygen was removed from the stopped-flow by flushing with 1 mL of anaerobic 100 mM sodium acetate, pH 5.0, containing 100 mM glucose and 100 µg/mL glucose oxidase Type-X. This solution remained in the stopped-flow overnight to completely remove any residual oxygen. MbsG was made anaerobic by degassing with five, 20 min cycles of vacuum and argon flushing. Oxidized MbsG (15 µM after mixing) was mixed with various concentrations of NAD(P)H (0.25–7.5 mM after mixing) and monitored with a photodiode array spectrophotometer until the enzyme was fully reduced. This experiment was repeated in the presence of L-lysine at a final concentration of 3 mM after mixing.

### Monitoring flavin oxidation

Flavin oxidation of MbsG was studied by first reducing the enzyme with near stoichiometric concentrations of NADH by mixing 30 µM of MbsG with 1.5-fold (45 µM) NADH. This was allowed to reduce in the glove box for 45 min under anaerobic conditions. Double mixing on the stopped-flow spectrophotometer was unable to be utilized for this experiment due to the slow reduction of MbsG with NADH at stoichiometric concentrations. Oxidation reactions were performed at 25 °C in 100 mM sodium phosphate, pH 7.5. Oxygen saturated buffer (1.2 mM) was obtained by bubbling 100% oxygen gas into a closed vial for 30 min at 25 °C [23]. Various concentrations of oxygen were obtained by mixing 100% oxygen saturated buffer with anaerobic buffer. Reduced MbsG (15 µM after mixing) was mixed with various concentrations of oxygenated buffer (100–600 µM after mixing). The reaction was monitored with a photodiode array spectrophotometer until the enzyme was fully oxidized. This experiment was repeated with L-lysine present at a final concentration of 3 mM after mixing.

### Synthesis of deuterated coenzymes

4-pro-R-4<sup>2</sup>H-NADPH was synthesized by the method of Jeong and 4-pro-R-4<sup>2</sup>H-NADH was synthesized by the method of Sucharitakul [24,25]. 4-pro-S-4<sup>2</sup>H-NADH and 4-pro-S-4<sup>2</sup>H-NADPH were synthesized by the method of Viola et al. [26], with minor modifications as previously described [27]. NADH and NADPH were synthesized by the same methods as controls.

### pL profile and solvent kinetic isotope effects

The effect of pH on the activity of MbsG was determined under steady-state conditions following oxygen consumption, lysine hydroxylation, and hydrogen peroxide and superoxide formation. MbsG was used at a concentration of 2  $\mu$ M for all steady-state assays. The effects on enzymatic activity were monitored between pH values 5.4 and 9.0 as MbsG was found to be stable in this pH range. For experiments at pH 5.4–7.4, a citrate/phosphate buffering system was used and prepared by mixing different ratios of 100 mM citric acid and 200 mM sodium phosphate [28]. At pH 7.5–8.0, 100 mM sodium phosphate was used, and between pH values 8.5 and 9.0, 100 mM sodium pyrophosphate was used. Tests were performed to ensure that the activity of MbsG did not change between the different buffers. For pH studies using the stopped-flow spectrophotometer, MbsG was stored in 10 mM sodium phosphate, pH 7.5, and mixed in the stopped-flow spectrophotometer with the appropriate buffer to rapidly equilibrate to the desired pH value. To measure the solvent kinetic isotope effect in the pL-independent region, the pD profile was also determined in the same buffer solutions made in 99% D<sub>2</sub>O (all pD values were calculated by adding 0.4 to the value on the pH meter, which is the variation from the change in the equilibrium on a hydrogen-selective glass electrode) [29]. All solutions were checked for their proper pL values with a Fisher Scientific Accumet AB15+ Basic pH meter.

### Effects of ligand binding

The effect of different ligands on activity was monitored by following the rate of oxygen consumption. 10 mM each of L-lysine, L-ornithine, L-arginine, 6-amino-1-hexanol, putrescine, or cadaverine were present with 5 mM NADH in 100 mM sodium phosphate pH 7.5. The reaction was initiated by addition of MbsG at a final concentration of 2  $\mu$ M. Formation of hydroxylated products were also tested for all of these ligands using the Csaky assay in the presence of 5 mM NADH and 10 mM ligand with 2  $\mu$ M MbsG incubated for 10 min. To determine if the presence of these ligands influenced the rate constant for flavin reduction, oxidized MbsG (15  $\mu$ M after mixing) was mixed in the stopped-flow spectrophotometer with various concentrations of NADH (1–5 mM after mixing) and either no ligand, or 10 mM (after mixing) of the tested ligands. The characteristic decrease at 452 nm for flavin reduction was recorded in the stopped-flow spectrophotometer. Effects of pre-incubating L-lysine with MbsG before mixing with NADH were also performed by incubating 10 mM L-lysine with oxidized MbsG for ~5 min before determining the reduction rates at various concentrations of NADH containing 10 mM L-lysine present, to keep the concentration of L-lysine constant after mixing. The effect of L-lysine and analog binding on the rate of flavin oxidation was also investigated in the stopped-flow spectrophotometer. In these experiments, reduced MbsG (15  $\mu$ M after mixing) was mixed with oxygenated buffer (300  $\mu$ M after mixing) with either no ligand or 10 mM of the tested ligands. When L-lysine was pre-equilibrated with MbsG, 10 mM L-lysine was incubated with 30  $\mu$ M reduced MbsG for ~5 min (concentrations before mixing) and the MbsG:L-lysine solution was mixed with oxygen and L-lysine, present at 300  $\mu$ M and 10 mM after mixing, respectively.

### Solvent viscosity effects

The effects of solvent viscosity on the rate of oxygen consumption were determined in either 100 mM sodium phosphate, pH 7.5, or in citrate/phosphate, pH 5.6, at 25 °C using glycerol as the viscosogen. The values for the relative glycerol viscosities ( $\eta_{\text{rel}}$ ) were obtained from Weast [30].

### Data analysis

All data were fit using KaleidaGraph (Synergy Software, Reading, PA). The rates of reduction at different concentrations of NAD(P)H were determined by fitting the decrease in absorbance at 452 nm to Eq. (1) which describes a single exponential decay equation. The resulting  $k_{\text{obs}}$  values were plotted as a function of NAD(P)H concentration. These data were fit using Eq. (2) in order to determine  $k_{\text{red}}$  and  $K_{\text{D}}$  values.

$$v = c + ae^{-(k \times t)} \quad (1)$$

$$k_{\text{obs}} = \frac{k_{\text{red}} \times [\text{S}]}{K_{\text{D}} + [\text{S}]} \quad (2)$$

For flavin oxidation studies, the increase in absorbance at 452 nm was fit to Eq. (3), which describes a single exponential rise equation. The resulting  $k_{\text{obs}}$  values were plotted as a function of oxygen concentration. These data were fit to a linear equation to determine the bimolecular rate constant of flavin oxidation ( $k_{\text{ox}}$ ).

$$v = c + a(1 - e^{-(k \times t)}) \quad (3)$$

The  $k_{\text{cat}}/K_{\text{m}}$  and  $k_{\text{red}}/K_{\text{D}}$  pL profiles were fit to Eq. (4), which describes a curve with a slope of 1 at low pL (pH or pD) and an independent region at high pL values.

$$\log(k) = \log[k(\text{lim}) \times (1 + 10^{\text{pK}_a - \text{pL}})] \quad (4)$$

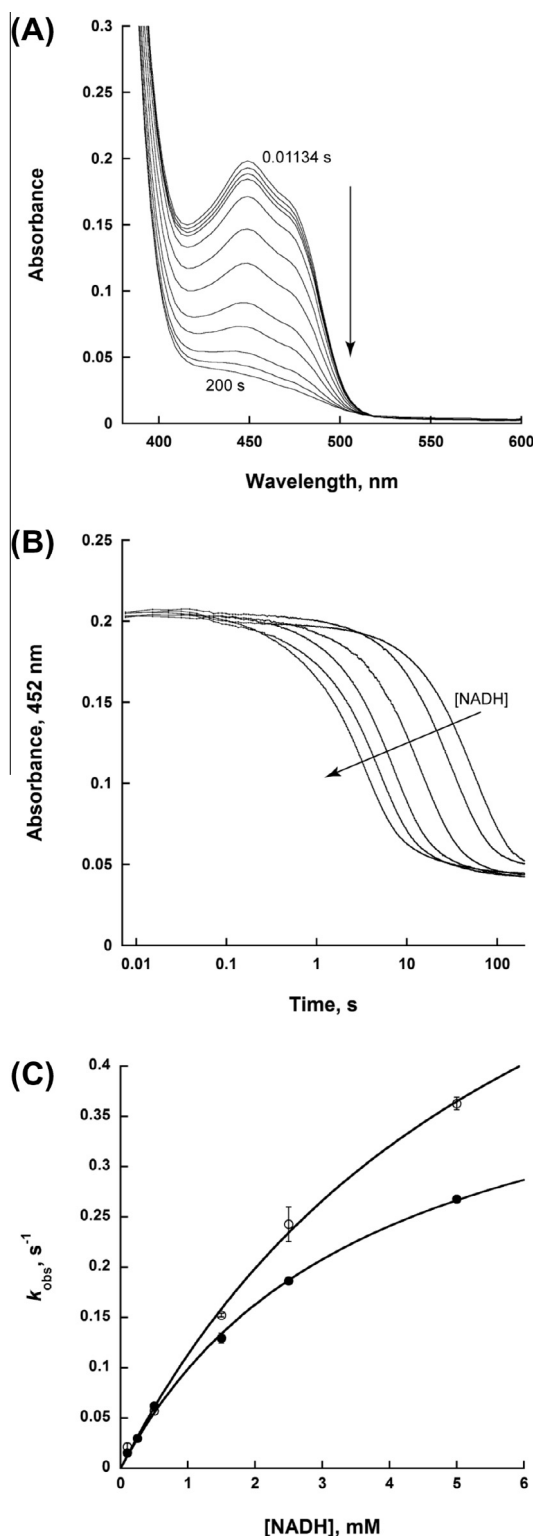
## Results

### Flavin reduction

The rate constants for flavin reduction ( $k_{\text{red}}$ ) were determined by mixing oxidized MbsG with varying amounts of NAD(P)H in both the presence and absence of 3 mM L-lysine in the stopped-flow spectrophotometer under anaerobic conditions at pH 7.5. The decrease in absorbance at 452 nm best fit to Eq. (1), which describes a single exponential decay (Fig. 1A and B). Fig. 1C shows the dependence of flavin reduction as a function of increasing concentrations of NADH, in both the absence and presence of 3 mM L-lysine (the data for NADPH is shown in Fig. S1). The results are summarized in Table 1. The  $k_{\text{red}}$  value for NADH is slightly higher than for NADPH in the absence of L-lysine. In the presence of L-lysine, the  $k_{\text{red}}$  value decreases to ~0.4 s<sup>-1</sup> for both coenzymes. The  $K_{\text{D}}$  value does not change for NADPH in the presence of L-lysine; however, it decreases 2-fold for NADH. The decrease in the  $k_{\text{red}}$  value in the presence of L-lysine is similar to the decrease in steady-state  $k_{\text{cat}}$  values determined by measuring rates of oxygen consumption (Table 1) [21].

### Flavin oxidation

The rates of flavin oxidation were determined by mixing varying concentrations of oxygen with reduced MbsG in both the presence and absence of L-lysine. The reaction of reduced MbsG with oxygen occurs in a one-step process, with no detectable intermediates (Fig. 2A and B). In Fig. 2B, the increase in absorbance at 452 nm best fit to Eq. (3), which describes a single exponential rise. The observed rates of flavin oxidation were plotted as a function of molecular oxygen concentration (Fig. 2C). The bimolecular rate constant of flavin oxidation is  $3.64 \times 10^3 \pm 0.06 \text{ M}^{-1} \text{ s}^{-1}$  in the absence of L-lysine and  $3.1 \times 10^3 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$  in presence of 3 mM L-lysine.



**Fig. 1.** Reaction of oxidized MbsG with NADH. (A) Time resolved flavin reduction spectra with 500  $\mu$ M NADH and 3 mM L-lysine collected over 200 s. (B) Traces of flavin reduction at 452 nm with 0.1–5 mM NADH and 3 mM L-lysine. The absorbance changes at 452 nm were fit to Eq. (1). (C)  $k_{\text{obs}}$  values of flavin reduction as a function of NADH in the absence (○) or presence (●) of 3 mM L-lysine. The data was fit to Eq. (2).

#### Primary kinetic isotope effects

The steady-state primary kinetic isotope effect (KIE) was measured to determine if hydride transfer is rate-limiting using both

**Table 1**

Pre-steady state and steady-state kinetic parameters for MbsG.

	NADH		NADPH	
	No L-lysine	3 mM L-lysine	No L-lysine	3 mM L-lysine
$^a k_{\text{red}} \text{ (s}^{-1}\text{)}$	$0.82 \pm 0.08$	$0.46 \pm 0.02$	$0.54 \pm 0.07$	$0.44 \pm 0.04$
$^a K_{\text{D}} \text{ (mM)}$	$6.3 \pm 0.9$	$3.7 \pm 0.4$	$6.0 \pm 1.4$	$6.2 \pm 1.1$
$^a k_{\text{red}}/K_{\text{D}} \text{ (M}^{-1} \text{s}^{-1}\text{)}$	$131 \pm 23$	$125 \pm 6$	$90 \pm 10$	$71 \pm 6$
$^b k_{\text{cat}} \text{ (s}^{-1}\text{)}$	$0.90 \pm 0.02$	$0.43 \pm 0.01$	$0.73 \pm 0.07$	$0.55 \pm 0.02$
$^b K_{\text{m}} \text{ (mM)}$	$7.0 \pm 0.2$	$2.4 \pm 0.1$	$12 \pm 1$	$12 \pm 1$
$^b k_{\text{cat}}/K_{\text{m}} \text{ (M}^{-1} \text{s}^{-1}\text{)}$	$128 \pm 2$	$183 \pm 5$	$67 \pm 5$	$47 \pm 2$

Conditions: Reactions were performed in 100 mM sodium phosphate at pH 7.5 and 25 °C.

<sup>a</sup> Kinetic parameters of flavin reduction.

<sup>b</sup> Steady-state kinetic parameters determined by monitoring oxygen consumption [21].

4-*pro-R*-4<sup>2</sup>H-NADH and 4-*pro-R*-4<sup>2</sup>H-NADPH (Fig. S2) at saturating concentrations of L-lysine (3 mM).  $^D k_{\text{cat}}$  values of  $1.6 \pm 0.1$  for 4-*pro-R*-4<sup>2</sup>H-NADH and  $1.3 \pm 0.2$  for 4-*pro-R*-4<sup>2</sup>H-NADPH were determined at pH 7.5.  $^D(k_{\text{cat}}/K_{\text{m}})$  values at pH 7.5 of  $1.4 \pm 0.1$  and  $1.7 \pm 0.1$  were also obtained for 4-*pro-R*-4<sup>2</sup>H-NADH and 4-*pro-R*-4<sup>2</sup>H-NADPH, respectively. At pH 5.6, the  $K_{\text{m}}$  value for NADH is very high and saturation was not achieved at the highest concentration tested (7.5 mM). Thus, the  $k_{\text{cat}}$  value could not be accurately determined, which prevented analysis of the KIE on this kinetic constant (see Fig. S2C). However, the  $k_{\text{cat}}/K_{\text{m}}$  could be accurately measured yielding a higher  $^D(k_{\text{cat}}/K_{\text{m}})$  value for 4-*pro-R*-4<sup>2</sup>H-NADH at pH 5.6 of  $2.0 \pm 0.1$ . Assays with 4-*pro-S*-4<sup>2</sup>H-NADH and 4-*pro-S*-4<sup>2</sup>H-NADPH resulted in KIE values not different from unity at pH 7.5 (data not shown).

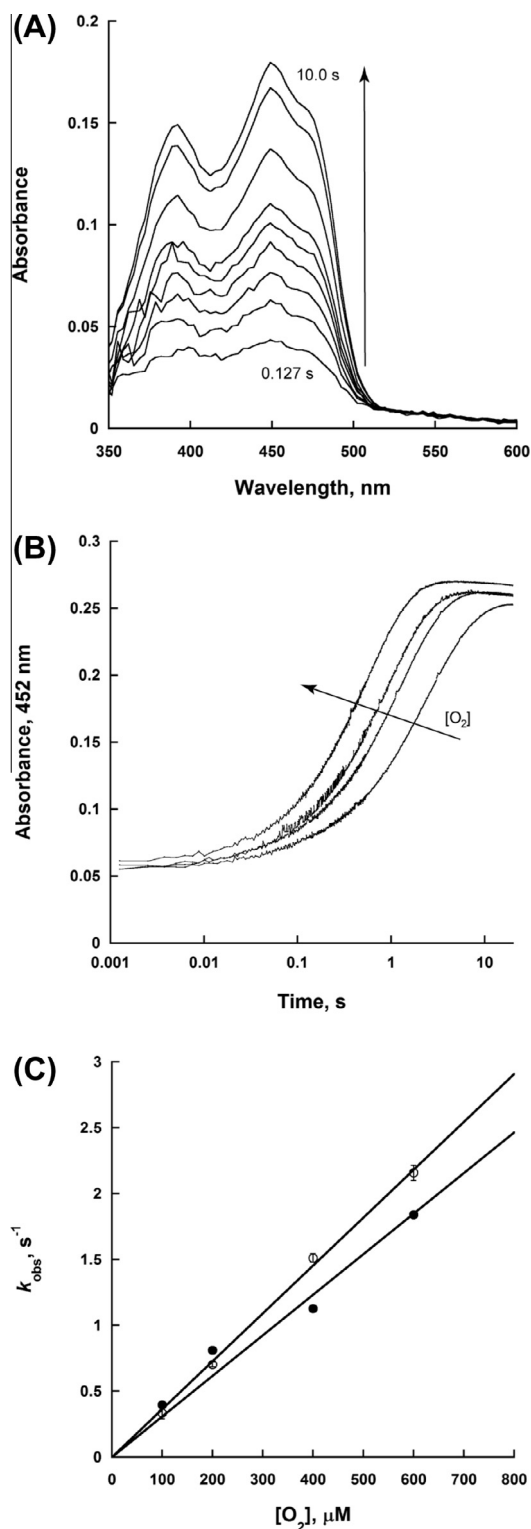
#### pH profile

The effect of pH on the activity of MbG was determined by following the rate of oxygen consumption. At lower pH values, the rate of oxygen consumption is linear as a function of NADH concentration (data not shown). Coenzyme saturation was not achieved even at 10 mM coenzyme. Thus, the  $k_{\text{cat}}$  values could not be accurately determined (data not shown).  $k_{\text{cat}}/K_{\text{m}}$  for NAD(P)H increases at low pH and is relatively unchanged at pH values greater than 7.5. A  $\text{p}K_{\text{a}}$  value of  $5.9 \pm 0.1$  was calculated from the  $k_{\text{cat}}/K_{\text{m}}$  pH profile (Fig. 3A closed circles). The pH dependence of flavin reduction with NADH was also determined. Here, a single ionizable group with a  $\text{p}K_{\text{a}}$  value of  $6.1 \pm 0.1$  was determined for the  $k_{\text{red}}/K_{\text{D}}$  pH profile. This  $\text{p}K_{\text{a}}$  value is very similar to the  $\text{p}K_{\text{a}}$  value determined in the oxygen consumption assay under steady-state conditions (Fig. 4A). Similar pH experiments with NADPH yielded consistent results (data not shown). The bimolecular rate constant for flavin oxidation did not significantly change as a function of pH, indicating that flavin oxidation is independent of pH (Fig. 4B). Similarly, formation of hydroxylated lysine showed very little change as a function of pH (Fig. S3A). In contrast, the rates of superoxide and hydrogen peroxide formation increased with decreasing pH (Fig. S3B).

#### Effect of ligand binding

We previously showed that the presence of L-lysine and several substrate analogs slow the rate of oxygen consumption of MbsG [21]. To determine what step of the reaction is affected, both flavin reduction and oxidation were monitored in the presence of 10 mM each of L-ornithine, 6-amino-1-hexanol, L-arginine, L-lysine, putrescine, and cadaverine. The rate constants obtained in the presence of the second substrate (or analog) were compared to the values





**Fig. 2.** Reaction of reduced MbsG with O<sub>2</sub>. (A) Time resolved flavin oxidation spectra with 300 μM O<sub>2</sub> over 10 s. (B) Traces of flavin oxidation at 452 nm with 100–600 μM O<sub>2</sub> and 3 mM L-lysine. The absorbance changes at 452 nm were fit to Eq. (3). (C)  $k_{\text{obs}}$  values of flavin oxidation as a function of molecular oxygen in the absence (○) or presence (●) of 3 mM L-lysine.

obtained in the absence of a ligand (with only NADH) (Fig. 5). Oxygen consumption and amine hydroxylation with these ligands were tested to determine the extent to which the rate of oxygen consumption was decreased and to measure whether hydroxylation occurred. The results indicate that all ligands tested

decreased the rate of oxygen consumption, while only L-lysine is hydroxylated by MbsG (Fig. 5A and B). Addition of L-lysine results in a decrease of ~50% in the rate of oxygen consumption. Similarly, a decrease of ~40% is observed in the  $k_{\text{red}}/K_{\text{D}}$  value. Thus, L-lysine binding is controlling the reaction of oxidized MbsG and NADH. With the exception of L-ornithine, all other ligands decreased the oxygen consumption rate by >30% and  $k_{\text{red}}/K_{\text{D}}$  by >15%, with putrescine and cadaverine showing the highest decrease (down to ~40% in the rate of oxygen consumption and >50% in  $k_{\text{red}}/K_{\text{D}}$ ). Incubation of L-lysine with MbsG before reacting with NADH had an equal effect on reduction, suggesting that L-lysine binding occurs more rapidly than NADH binding. All tested ligands did not have a significant effect on the rate constant for flavin oxidation (Fig. 5D). The data indicates that L-lysine/ligand binding modulates the reactivity of MbsG with NAD(P)H leading to a decrease in the rate of hydride transfer.

#### Solvent kinetic isotope effects

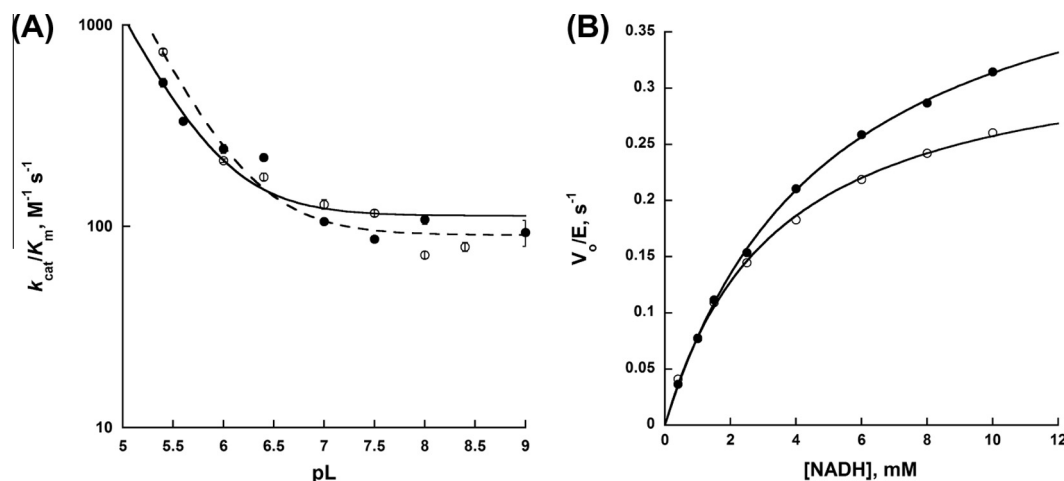
The solvent kinetic isotope effect was measured in order to determine if protons are exchanged with solvent in a rate-limiting transition state. The pL profile indicated that the kinetic parameters were independent at pL values equal to or greater than 7.5 (Fig. 3A). The  $k_{\text{cat}}/K_{\text{m}}$  pD profile for NAD(P)H shows a  $pK_{\text{a}}$  value of  $6.25 \pm 0.07$  (Fig. 3A open circles). The solvent kinetic isotope effect determined at pL 7.5, resulted in a  $^{2}\text{D}_0 k_{\text{cat}}$  value of  $1.36 \pm 0.03$  and a  $^{2}\text{D}_0(k_{\text{cat}}/K_{\text{m}})$  value of  $0.93 \pm 0.03$  (Fig. 3B). The low  $^{2}\text{D}_0 k_{\text{cat}}$  value possibly indicates one or more solvent exchangeable protons in flight during a partially rate-limiting transition state, possibly flavin oxidation. A possible explanation for the slight inverse solvent kinetic isotope effect on  $k_{\text{cat}}/K_{\text{m}}$  is the presence of an isomerization step that is enhanced due to the higher viscosity of D<sub>2</sub>O.

#### Solvent viscosity effects

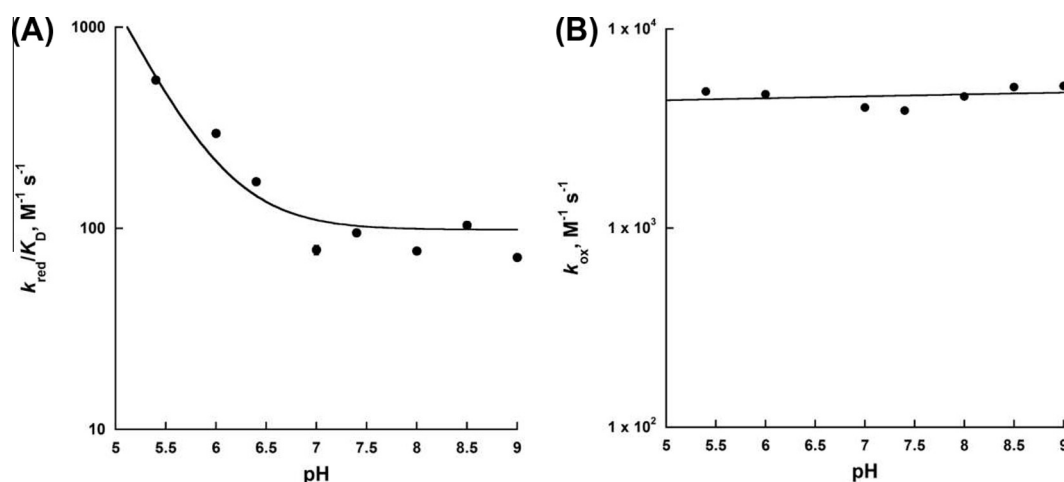
Results from the solvent kinetic isotope effect experiments provided clues to the presence of an isomerization step in MbsG. To test this possibility, the effect of solvent viscosity was determined as a function of NADH in the presence of 3 mM L-lysine at both pH 7.5 and pH 5.6. In a plot of normalized  $k_{\text{cat}}$  versus relative glycerol viscosity, a slope of 0.10 was calculated at pH 7.5 (Fig. 6A). This indicates that product release is partially rate-limiting in the reaction at pH 7.5. Calculation of  $k_{\text{cat}}$  values at pH 7.5 required less extrapolation due to the relatively low Michaelis constant (~2 mM) in the presence of glycerol at this pH (data not shown). A plot of normalized  $k_{\text{cat}}$  could not be calculated at pH 5.6 due to the inability to saturate with NADH at lower pH, thus, only  $k_{\text{cat}}/K_{\text{m}}$  was analyzed. In a plot of normalized  $k_{\text{cat}}/K_{\text{m}}$  versus relative viscosity, a slope of –0.13 was calculated at pH 7.5 (Fig. 6B closed circles). A plot of normalized  $k_{\text{cat}}/K_{\text{m}}$  versus relative viscosity at pH 5.6 resulted in a slope of 0.10 instead of a negative slope (Fig. 6B open circles). An inverse effect on solvent viscosity has also been reported in other flavoenzymes, where it has been interpreted as the participation of a viscosity sensitive isomerization or conformational change in the reaction [31,32]. The data is consistent with a conformational change that is favored in solutions of higher viscosity at a pH of 7.5. At pH 5.6, this conformational change is no longer sensitive to solvent viscosity and the reaction decreases as a function of increasing glycerol concentration suggesting that NADH binding is more rate-limiting under these conditions.

#### Discussion

The two most comprehensively studied NMOs to date are the L-ornithine monooxygenases, SidA and PvdA. These enzymes are



**Fig. 3.** (A) Effects of pH (● solid line) on the  $k_{cat}/K_m$  values for NADH following oxygen consumption. The same experiment was performed in D<sub>2</sub>O (○ dashed line). Experiments at each pH value were performed as a function of NADH with 3 mM L-lysine present. (B) Steady-state oxygen consumption of MbsG in H<sub>2</sub>O (●) and D<sub>2</sub>O (○) as a function of NADH in the presence of 3 mM L-lysine at pH 7.5.



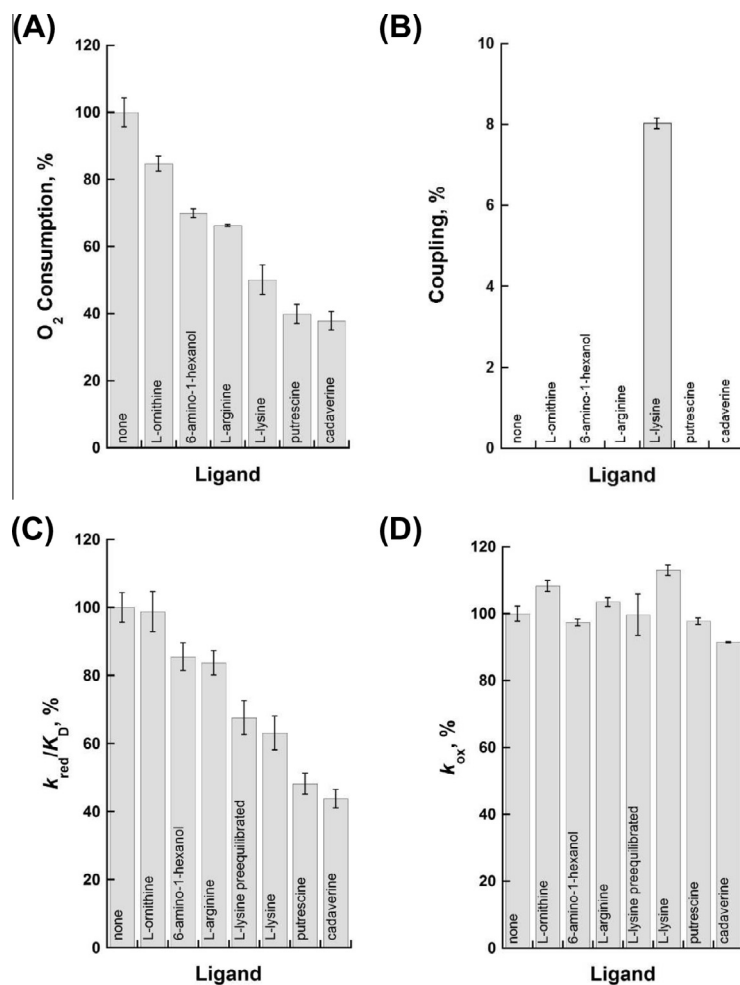
**Fig. 4.** pH dependence of flavin reduction and oxidation. (A) pH profile of  $k_{red}/K_D$  for NADH in the presence of 3 mM L-lysine and (B) pH profile of flavin oxidation in the presence of 3 mM L-lysine.

capable of stabilizing long-lived C4a-hydroperoxyflavin intermediates, which is a common characteristic of members of the Class B flavin monooxygenases (Scheme 1C) [17,18,33–35]. Stabilization of this intermediate is required to ensure coupling of oxygen activation to formation of hydroxylated product, rather than the release of hydrogen peroxide or other reactive oxygen species [20,36,37]. MbsG is an NMO that does not function like related Class B flavin monooxygenases. This L-lysine monooxygenase is highly uncoupled, producing high levels of reactive oxygen species during turnover (Table 2) [21].

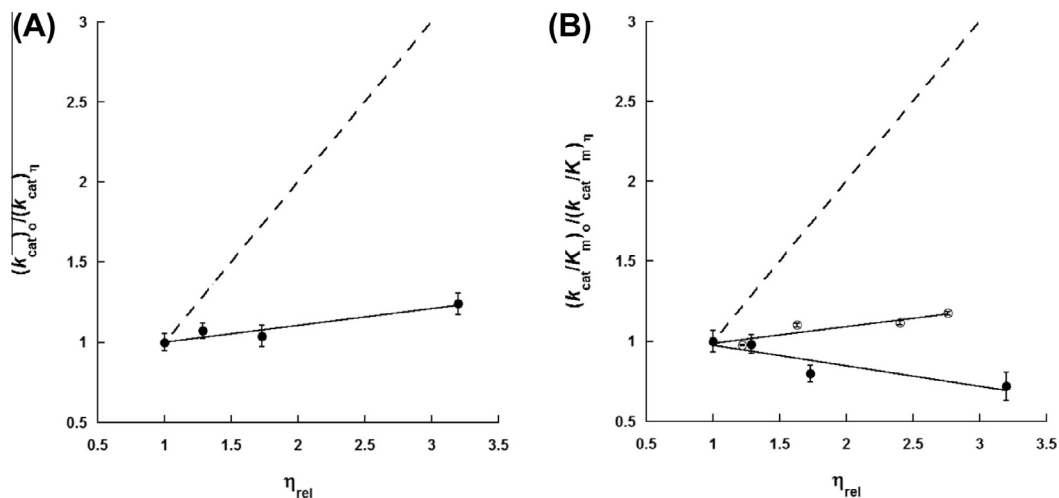
Various mechanisms of regulation for flavin monooxygenases have been determined. For *p*-hydroxybenzoate hydroxylase (PHBH), a member of the Class A flavin monooxygenases, the activity is regulated in the reductive half reaction. In this enzyme, deprotonation of the hydroxyl group of *p*-hydroxybenzoate triggers movement of the flavin isoalloxazine ring from an *in* position to an *out* position, enhancing the reaction with NADPH by  $\sim 10^5$ -fold [38,39]. The reduced flavin swings back into the active site where it is protected from solvent and reacts with molecular oxygen forming the C4a-hydroperoxyflavin, which is the hydroxylating species. Thus, the activity of PHBH is regulated by binding of substrate, which *enhances* reduction of the flavin. Class B flavin monooxygenases utilize a different mechanism of regulation. In

this group of enzymes, the catalytic cycle has been well characterized in bacterial and mammalian flavin monooxygenase (FMOs), phenylacetone monooxygenase (PAMO), and in the ornithine monooxygenase, SidA. In these enzymes, the reaction of oxidized flavin with NADPH is independent of binding of the hydroxylatable substrate yielding reduced enzyme in complex with NADP<sup>+</sup>. This complex reacts with molecular oxygen to form a C4a-(hydro)peroxyflavin, which is stabilized in some cases for more than 1 h [18,33,37,40–42]. The presence of NADP<sup>+</sup> is absolutely necessary for stabilization of the C4a-(hydro)peroxyflavin. Only upon binding of the hydroxylatable substrate is rapid turnover observed (i.e., ornithine increases turnover of SidA by  $\sim 80$ -fold) [43]. This mechanism has been referred to as the “cocked-gun mechanism” where the stable C4a-(hydro)peroxyflavin is ready to react with an appropriate substrate [41].

As mentioned, MbsG does not function as other flavin-dependent monooxygenases. In addition to being highly uncoupled, the presence of L-lysine and other ligands results in a decrease in the rate of oxygen consumption. Here, we show that the  $k_{red}$  value decreases in the presence of L-lysine and non-hydroxylatable analogs (Figs. 1 and 5C). Pre-incubation of MbsG with L-lysine before reaction with NADH or reaction of MbsG with NADH/L-lysine results in a similar decrease in the  $k_{red}$  value. This is consistent



**Fig. 5.** Effect of ligand binding to MbsG. All experiments were normalized to the values obtained in the absence of ligand with the exception of the hydroxylation experiment where all values were normalized to the value obtained with L-lysine. (A) Normalized effect on oxygen consumption. (B) Coupling of MbsG (oxygen consumption to L-lysine hydroxylation). (C) Normalized effect on flavin reduction. (D) Normalized effect on flavin oxidation. 5 mM NADH and 10 mM ligand were used in the oxygen consumption and hydroxylation experiments. NADH was varied between 1 and 5 mM while ligands were used at 10 mM in the flavin reduction experiment. 300  $\mu$ M oxygen and 10 mM ligand were used in the flavin oxidation experiment.



**Fig. 6.** Effect of solvent viscosity on MbsG. Effect of solvent viscosity on (A)  $k_{cat}$  at pH 7.5 and (B) on  $k_{cat}/K_m$  at pH 7.5 (●) and pH 5.6 (○). Steady-state values were normalized to the values calculated for 0% glycerol. The  $k_{cat}/K_m$  values are those calculated for NADH at various concentrations of glycerol in the presence of 3 mM L-lysine. The dashed line with a slope of one describes a case in which the reaction is completely diffusion-controlled.



**Table 2**

Activity of MbsG following L-lysine hydroxylation, superoxide, and hydrogen peroxide formation.

	NADH		NADPH	
	No L-lysine	3 mM L-lysine	No L-lysine	3 mM L-lysine
$k_{\text{hydroxylation}} (\text{s}^{-1})$	–	$0.075 \pm 0.002$	–	$0.070 \pm 0.002$
$k_{\text{superoxide}} (\text{s}^{-1})$	$0.50 \pm 0.04$	$0.024 \pm 0.001$	$0.16 \pm 0.04$	$0.028 \pm 0.002$
$k_{\text{hydrogen peroxide}} (\text{s}^{-1})$	$0.30 \pm 0.15$	$0.07 \pm 0.02$	$0.14 \pm 0.05$	$0.014 \pm 0.001$

Conditions: Reactions were performed in 100 mM sodium phosphate at pH 7.5 and 25 °C.

with rapid equilibrium binding of L-lysine, most likely before NAD(P)H binding. The down regulation on the rate of flavin reduction by substrate binding has not been reported for any other flavin monooxygenase.

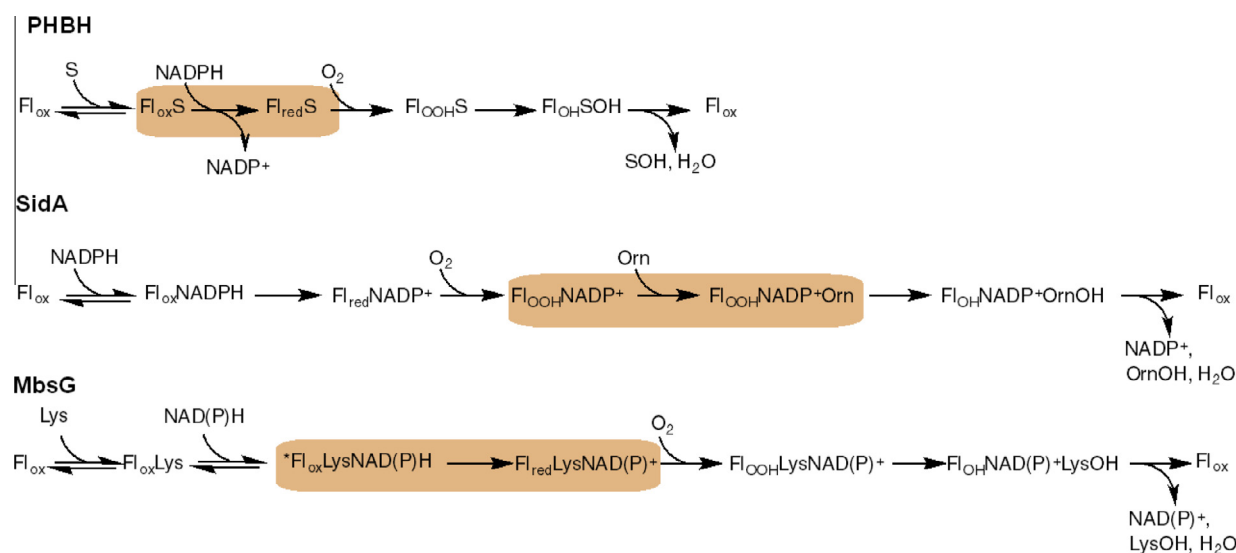
Stopped-flow experiments on the oxidation step show that the reaction of reduced MbsG with oxygen occurs in a one-step process without the observation of a C4a-hydroperoxyflavin intermediate (Fig. 2). This is in stark contrast to SidA where the half-life of the C4a-hydroperoxyflavin is ~30 min [18,20,44]. The lack of stabilization of this intermediate is consistent with the high level of uncoupling in MbsG (Table 2). Furthermore, in the related NMOs SidA and PvdA, as well as other Class B monooxygenases, addition of substrate enhances flavin oxidation [17,19]. Our results clearly indicate that MbsG does not utilize the same mechanism of stabilization, as the oxidation step is not affected by the addition of L-lysine or non-hydroxylatable analogs (Figs. 2 and 5D).

Probing the hydride transfer step by measuring the primary deuterium KIE showed that this step is partially rate-limiting at pH 7.5 since the effect on the  $D(k_{\text{cat}}/K_m)$  value is only  $1.4 \pm 0.1$ . The magnitude of the isotope effect is significantly different between MbsG and other Class B flavin-dependent monooxygenases where hydride transfer is the rate-determining step with KIE values of 3–6; however, the *pro*-(R)-stereoselectivity is conserved [18,35,36].

To gain further insight into the catalytic cycle of MbsG, pH studies were conducted. The results showed that as the pH decreases, the  $k_{\text{cat}}/K_m$  values increase. Fitting the data to Eq. (4) results in a  $pK_a$  value of ~6 for a group(s) that need(s) to be protonated for

activity (Fig. 3A). Similar results were obtained when the  $k_{\text{red}}/K_D$  pH profile was determined, indicating that the pH sensitive step occurs during or before flavin reduction. pH studies on the oxidative half-reaction indicate that reaction with molecular oxygen is independent of pH (Fig. 4B). In other flavin monooxygenase systems, the rate of oxidation increases as a group with a  $pK_a$  value >9 becomes deprotonated [44,45]. This was proposed to be due to deprotonation of the N5 atom of the flavin, or due to a residue that is involved in hydrogen peroxide elimination from the C4a-hydroperoxyflavin. Thus, the degree of coupling decreases at higher pH in these enzymes as the C4a-hydroperoxyflavin becomes less stable. The mechanism by which MbsG achieves optimal coupling appears to be very different from other studied systems. While the rate of lysine hydroxylation is largely unchanged by pH, the rates of formation of reactive oxygen species increases ~10-fold at low pH when compared to more basic pH values (Fig. S3).

The viscosity and solvent kinetic isotope effect results on  $k_{\text{cat}}/K_m$  are consistent with the presence of a conformational change in the catalytic cycle of MbsG. A solution viscosity sensitive conformational change has been reported in other flavoenzyme systems; however, MbsG is the first monooxygenase [31,32]. The fact that hydride transfer or flavin oxidation are not significantly rate-limiting suggests that perhaps the conformational change is the slow step in the reaction. Furthermore, based on the pH results, we hypothesized that the conformational change might be pH sensitive, becoming faster at lower pH values. To test this hypothesis, the viscosity effects were performed at pH 5.6. In contrast to the observed increase in the  $k_{\text{cat}}/K_m$  values at pH 7.5, the  $k_{\text{cat}}/K_m$  values at pH 5.6 decrease as a function of increasing concentrations of glycerol. We interpret these data to indicate a change in the rate-limiting step in the reaction, with a conformational change being rate-limiting at pH 7.5 and becoming less rate-limiting as pH decreases. To further test if a pH sensitive conformational change controls the reaction of MbsG, we measured the KIE at pH 5.6. A  $D(k_{\text{cat}}/K_m)$  value of  $2.0 \pm 0.1$  was determined at pH 5.6, which is significantly higher than the value of  $1.4 \pm 0.1$  measured at pH 7.5. These results further support our hypothesis that a conformational change limits the reaction of MbsG at pH > 7.5, while at lower pH values this step becomes less rate-limiting.



**Scheme 2.** Kinetic mechanisms of flavin monooxygenases. The regulatory steps modulated by substrate binding are highlighted in apricot colored boxes. In PHBH, substrate binds before reaction with NADPH, which results in an increase in the rate of flavin reduction. SidA stabilizes a long-lived C4a-hydroperoxyflavin intermediate, which is ready to hydroxylate L-ornithine upon binding. Here, L-ornithine enhances the oxidation of the flavin (similar mechanisms apply to FMO, PAMO, and PvdA). In MbsG, L-lysine binds before NAD(P)H. L-lysine binding induces a conformational change that decreases the flavin reduction step and the production of reactive oxygen species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In summary, the mechanism of regulation of MbsG is unusual among flavin-dependent monooxygenases (Scheme 2). In MbsG,  $\epsilon$ -lysine binding decreases the rate of flavin reduction while having no effect on flavin oxidation. The pH studies show that isomerization of an MbsG-lysine-NADPH complex is pH sensitive, where at high pH (>7.5) the enzyme has a lower rate of flavin reduction, but is more coupled. At acidic pH, MbsG reacts faster with NAD(P)H; however, the increased rate mainly channels oxygen consumption towards formation of superoxide and hydrogen peroxide. Thus, it appears that slowing the conformational change prior to reduction increases the efficiency of lysine hydroxylation. Recent structural investigations on related Class B flavin monooxygenases have provided clues about the mechanism of stabilization of the C4a-hydroperoxyflavin. It has been shown that after flavin reduction, NADP<sup>+</sup> moves into a conformation that is essential for stabilization of the C4a-hydroperoxyflavin [33,41,46]. This ensures efficient hydroxylation of the substrate and prevents the futile utilization of NADPH. In MbsG,  $\epsilon$ -lysine binding might induce conformational changes that primes the active site for proper interaction with NAD(P)H, such that reduction is slightly decreased, but activation of molecular oxygen does not yield excess reactive oxygen species.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.abb.2014.04.006>.

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