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Plant and Cyanobacterial Hemoglobins Reduce Nitrite to Nitric Oxide under Anoxic Conditions

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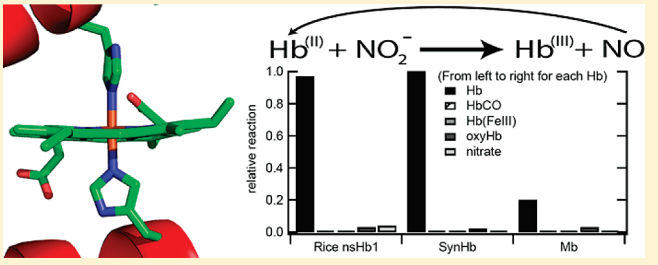
ABSTRACT: The ability of ferrous hemoglobins to reduce nitrite to form nitric oxide has been demonstrated for hemoglobins from animals, including myoglobin, blood cell hemoglobin, neuroglobin, and cytoglobin. In all cases, the rate constants for the bimolecular reactions with nitrite are relatively slow, with maximal values of ~5 M^{-1} s^{-1} at pH 7. Combined with the relatively low concentrations of nitrite found in animal blood plasma (normally no greater than 13 μM), these slow reaction rates are unlikely to contribute significantly to hemoglobin oxidation, nitrite reduction, or NO production. Plants and cyanobacteria, however, must contend with much higher (millimolar) nitrite concentrations necessitated by assimilatory nitrogen metabolism during hypoxic growth, such as the conditions commonly found during flooding or in waterlogged soil. Here we report rate constants for nitrite reduction by a ferrous plant hemoglobin (rice nonsymbiotic hemoglobin 1) and a ferrous cyanobacterial hemoglobin from *Synechocystis* that are more than 10 times faster than those observed for animal hemoglobins. These rate constants, along with the relatively high concentrations of nitrite present during hypoxia, suggest that plant and cyanobacterial hemoglobins could serve as anaerobic nitrite reductases in vivo.



Hemoglobins (Hbs) with unknown functions have been found in most organisms. A hypothetical role common to many of them is nitric oxide (NO) scavenging,[1] which is grounded in the established contribution of Hbs to nitric oxide dioxygenase ($HbO_2 + NO \rightarrow metHb + NO_3^-$) (NOD) activity in bacterial flavohemoglobins,[2] the effects of blood cell Hb on NO metabolism,[3] and the general reactivity of oxy, deoxy, and ferric hemoglobins with NO.[4] In plants, the NOD function for Hbs[5,6] is supported by increases in the level of Hb expression in response to nitrate, nitrite, and nitric oxide,[7] improvements in NO scavenging ability in plants overexpressing Hb,[8,9] decreases in the levels of NO-sensitive enzymes in plants with down-regulated Hb,[10] and the encouragement of cell growth by cyanobacterial Hb in the presence of high concentrations of reactive nitrogen species.[11]

The importance of NO scavenging is clear in bacteria and yeast, where the organisms must defend themselves against oxidative bursts from the immune systems of their hosts.[1,2] The role of Hb NOD activity in plants and animals could involve scavenging to mitigate cell damage resulting from elevated NO levels during hypoxia and could affect NO-mediated signaling. Alternatively, as proposed for myoglobin (Mb), red blood cell Hb, and neuroglobin in animals, Hbs could be responsible for production of NO via reduction of nitrite.[12,13] However, the relatively low rates of NO production by wild-type and native animal Hbs[14,15] and the modest (low micromolar) levels of nitrite typically found in mammalian tissues[16] diminish the likelihood that nitrite reductase activity is a major function of these proteins.

The metabolism of nitrogen is quite different in autotrophs such as plants and cyanobacteria, which must assimilate nitrogen through the reduction of nitrate under conditions ranging from normoxic to anoxic. In these organisms, nitrate and nitrite can accumulate to very high (millimolar) concentrations,[17] particularly when oxygen concentrations are low.[18] Such conditions are associated with plant Hb upregulation,[19,20] and thus, reactions of nitrate and nitrite with plant and cyanobacterial Hbs are potentially physiologically significant. Here we test the reactions of class 1 rice nonsymbiotic Hb (rice nsHb1)[21] and the Hb from the cyanobacterium *Synechocystis* (SynHb)[22−24] with nitrate and nitrite. These Hbs are not oxygen transporters and have hypothetical roles in nitrogen metabolism. Our results show that the deoxyferrous forms of each react rapidly with nitrite to form ferric Hb and ferrous-nitrosyl Hb in a fixed ratio, indicative of the production of nitric oxide from the reduction of nitrite by ferrous Hb.

MATERIALS AND METHODS

Protein expression and purification for rice nsHb1 and SynHb were conducted by previously described methods,[23,25] and horse heart myoglobin (Mb) was prepared by dissolving the lyophilized protein (Sigma) in 0.1 M sodium phosphate (pH 7.0), followed by centrifugation and desalting over a G-25 column equilibrated in the same buffer. Deoxyferrous protein samples were prepared

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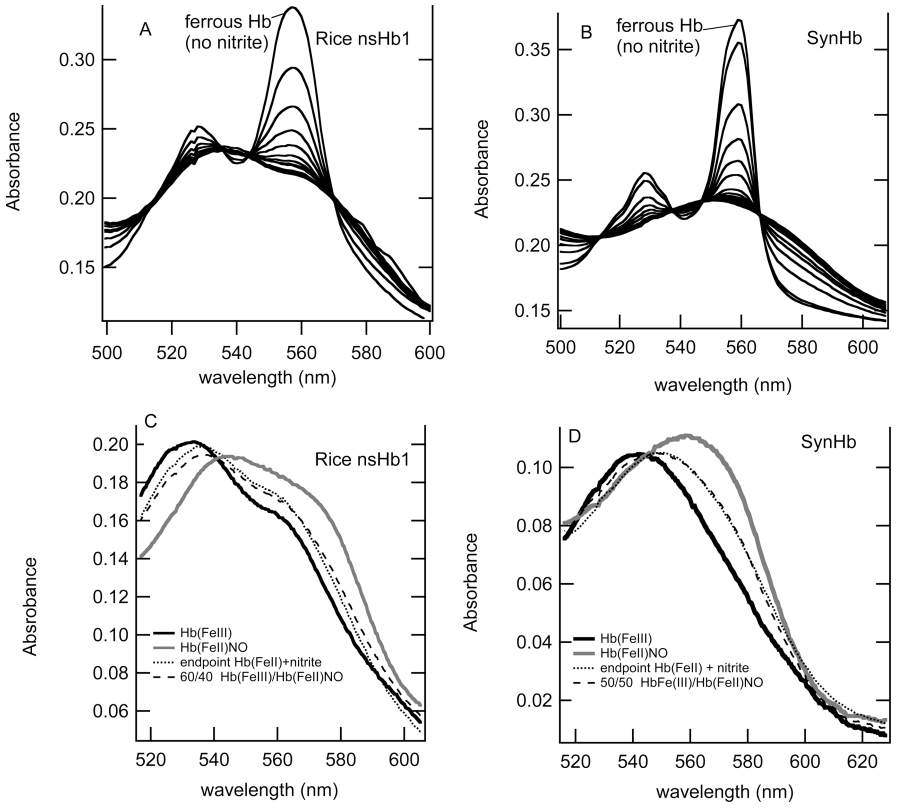

Figure 1. Reaction of rice nsHb1 and SynHb with nitrite. The spectral changes associated with Rice nsHb1 (A) and SynHb (B) oxidation are shown at 30 s intervals following the addition of 50 μM sodium nitrite. Panels C and D show ferric and ferrous nitrosyl (HbNO) reference spectra, the final experimental spectrum, and a sum of the reference spectra to determine the contribution of each species to the final experimental spectrum for rice nsHb1 and SynHb, respectively.

under anaerobic conditions by adding a liquid stock of 100 mM sodium dithionite (DT) in a slight excess of the protein concentration and then passing the sample over a medium G-25 desalting column (to remove excess sodium dithionite) that was poured and equilibrated in an anaerobic chamber (95% argon and 5% hydrogen, Coy Laboratories). Deoxyferrous samples were sealed in airtight cuvettes, confirmed using a USB 200 spectrophotometer (Ocean Optics), and diluted to a final concentration of 15 μM using deoxygenated 100 mM potassium phosphate buffer (pH 7).

Aliquots from an anaerobic 100 mM sodium nitrite solution were added to the cuvette using a gastight Hamilton syringe to achieve the experimental nitrite concentrations. Reaction progress was monitored by observing the decrease in the amount of deoxyferrous heme at 558 nM for rice nsHb1 and at 559 nM for SynHb. These time courses were collected using the "scanning kinetics" mode on a Cary 50 spectrophotometer. The reactions in Figure 3, including the reactivity of ferric Hbs with nitrite, those bound to different ligands, and the comparative reaction of deoxyferrous proteins with 100 μM nitrate, were conducted under the same conditions as the reactions with nitrite and monitored in the same way.

Single-exponential fits of the reaction progress were conducted using Igor Pro, and the k_{obs} values at each nitrite concentration were plotted versus nitrite concentration and fitted to a line to measure observed bimolecular rate constants. Analyses of the end point spectra in Figure 1 were performed by adding together the ferric and ferrous-nitrosyl components for each Hb in Igor Pro.

RESULTS

Reaction of Rice nsHb1 and SynHb with Nitrite. Sodium nitrite reacts with ferrous deoxy blood cell Hb to produce a combination of ferric and ferrous nitrosyl-Hb.[12,13,26] To determine if such a reaction occurs with rice nsHb1 and SynHb, the deoxy forms of each were reacted with nitrite under anaerobic conditions. Panels A and B of Figure 1 show the spectral changes associated with the reaction at 50 μM nitrite collected at 30 s intervals, demonstrating that each is oxidized relatively rapidly compared to other Hbs[12] at this nitrite concentration. Panels C and D of Figure 1 show the absorbance spectra for each Hb at the end of the reaction along with the spectra of the $Hb^{Fe(III)}$ and $Hb^{Fe(II)}NO$ components for each. The end point spectrum for each Hb could be fit to the sum of its respective $Hb^{Fe(III)}$ and $Hb^{Fe(II)}NO$ components to measure the contribution of each form to the products at equilibrium. In the case of rice nsHb1, the final ratio is 60% $Hb^{Fe(III)}$ and 40% $Hb^{Fe(II)}NO$, which is consistent with what is observed for murine neuroglobin and human Hb in this reac tion.[15,26] For SynHb, the final spectrum is composed of 50% $Hb^{Fe(III)}$ and 50% $Hb^{Fe(II)}NO$, corresponding exactly to the final product predicted and observed for other members of the globin family.[26,27]

Kinetics of the Reaction of Rice nsHb1 and SynHb with Nitrite. Measurements of the reaction of neuroglobin, Mb, and blood cell Hb have shown a range of observed rate constants for the wild-type proteins that are relatively slow (0.12−6 M^{-1} s^{-1} for neuroglobin and blood cell Hb and 2.9−5.6 M^{-1} s^{-1} for Mb).[12,15,27] To measure the rate constant for the reactions with rice nsHb1 and SynHb, we measured time courses for the nitrite-induced progression of each ferrous Hb to its equilibrium end

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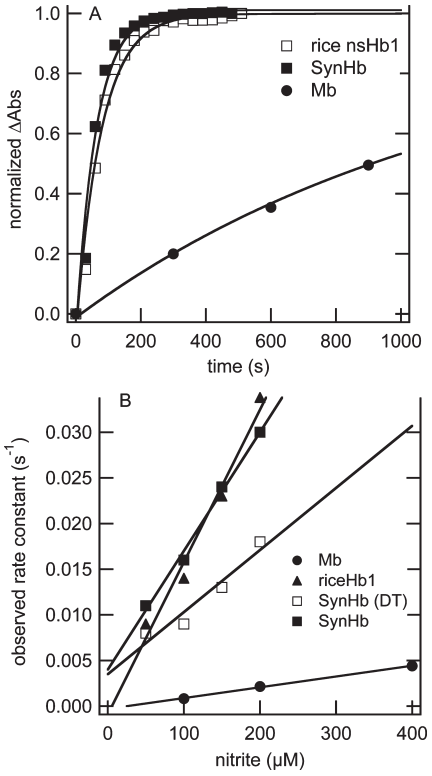

Figure 2. Kinetics of the reaction of rice nsHb1 and SynHb with nitrite. (A) Time courses for the reaction with each Hb and Mb at 100 μM nitrite. The absorbance change is normalized to that associated with the transition from each deoxyferrous Hb to its end point spectrum. (B) Observed reaction rates for each Hb as a function of nitrite concentration. Linear fits of these plots yield observed bimolecular rate constants of 166, 130, and 11 M^{-1} s^{-1}. Also included are the reaction rates for SynHb in the presence of sodium dithionite (DT), demonstrating that the slope is half that observed without DT.

point as a function of nitrite concentration (Figure 2). A comparison of the time courses for reactions of rice nsHb1, SynHb, and Mb (the fastest of the previously reported wild-type Hbs[12]) at 100 μM nitrite is shown in Figure 2A, demonstrating that the two autotroph Hbs are significantly faster than Mb. Figure 2B shows the dependence of the observed rate constant on nitrite concentration, yielding observed bimolecular rate constants for rice nsHb1 and SynHb of 166 and 130 M^{-1} s^{-1}, respectively, and a value for Mb of 11 s^{-1}.

Specificity of the Reaction of Rice nsHb1 and SynHb with Nitrite. Hbs will participate in many reactions because of the innate reactivity of the heme iron. To test the specificity of the reaction of nitrite with rice nsHb1 and SynHb, we have reacted nitrite with the ferric [$Hb^{Fe(III)}$], CO-bound [$Hb^{Fe(II)}CO$], and oxygenated [$Hb^{Fe(II)}O_2$] species of each along with the same derivatives of Mb. Additionally, the ferrous forms of each protein were reacted with nitrate. Figure 3 shows the progress of these reactions with 100 μM nitrite (or nitrate) after 300 s. Only the reaction of nitrite with the deoxyferrous form of each Hb occurs with any degree of completion on this time scale.

DISCUSSION

Doyle[26] and others[14,15] have previously investigated the mechanism of the reduction of nitrite by several animal deoxyferrous

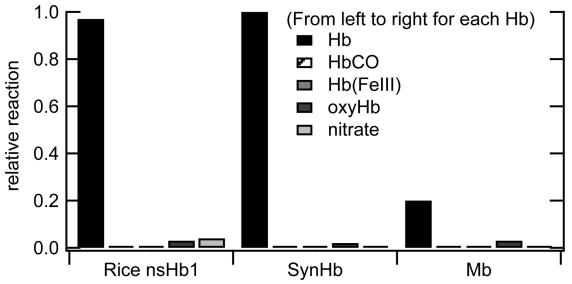

Figure 3. Reactivity of nitrite and nitrate with other forms of rice nsHb1, SynHb, and Mb. The deoxyferrous (Hb), CO (HbCO), ferric (metHb), and oxy (oxyHb) forms of each Hb were reacted with 100 μM nitrite, and the reaction progress was measured after 300 s. Each deoxyferrous Hb was also reacted with 100 μM nitrate over the same period of time. In each case, only the reaction of deoxyferrous Hb with nitrite shows any appreciable reaction.

Hbs. On the basis of the pH dependence of the reaction, it is thought that the protonated form of nitrite, nitrous acid (HNO_2), is the species that reacts directly with Hb, but the equilibrium between nitrite and nitrous acid is rapid and does not limit the relatively slow bimolecular reaction with Hbs. Thus, it is convenient to report the reaction with respect to nitrite concentration, which has become the custom in the literature. With this understanding, the reaction of nitrite with Hbs can be described by reactions I and II and eq 1.

$$Hb^{Fe(II)} + NO_2^- + H^+ \xrightarrow{k_{NO_2^-}} Hb^{Fe(III)} + NO + OH^- \quad (I)$$

$$Hb^{Fe(II)} + NO \xrightarrow{k_{NO}} Hb^{Fe(II)}NO \quad (II)$$

Reactions I and II are simplified by two assumptions. First, reaction I is rate-limiting and probably proceeds via the formation of $Hb^{Fe(III)}NO$, followed by NO release. As long as NO release is rapid relative to $k_{NO_2^-}$ (as is the case with ferric Mb and neuroglobin, where k_{-NO} = 12 and 6.2 s^{-1},[12] respectively), this will not prevent binding of NO to deoxyferrous Hb (reaction II). The second assumption is the irreversibility of reaction II, which is valid on these time scales because of the rapid (~100 μM^{-1} s^{-1}) bimolecular rate constant for binding of NO to ferrous Hbs,[28] and the extremely slow dissociation rate constant (~10^{-4} s^{-1}). The resulting high affinity of ferrous Hbs for NO is such that NO will always be bound at equilibrium under any reasonable experimental conditions.

The relatively slow rate of reaction I compared to that of reaction II means that two $Hb^{Fe(II)}$ molecules are consumed in the reaction with each NO_2^- (one in reaction I and the second upon binding to NO in reaction II). This leads to eq 1, which describes the expected velocity of the overall reaction as measured by monitoring the disappearance of deoxyferrous Hb.

$$\frac{-dHb^{Fe(II)}}{dt} = 2k_{NO_2^-}[Hb^{Fe(II)}][NO_2^-] \quad (1)$$

Equation 1 shows that "k_{obs}", as measured in Figure 2B by following the disappearance of ferrous Hb in the absence of sodium dithionite (DT), is actually equal to $2k_{NO_2^-}$. In the presence of DT [which rapidly reduces the $Hb^{Fe(III)}$ produced in reaction I], k_{obs} will equal $k_{NO_2^-}$ and should be half of the value measured in its absence, as observed by others for Mb and

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Hb.[29,30] As such, our value of 11 M^{-1} s^{-1} for Mb in the absence of DT is consistent with the value of 2.9 s^{-1} reported in its presence.[12]

To directly test this relationship, we conducted the reaction of nitrite with SynHb in the presence of 3 mM sodium dithionite (DT), and the result is included in Figure 2B. As expected, the slope in the presence of DT (68 M^{-1} s^{-1}) is half that observed in its absence (130 M^{-1} s^{-1}). Such consideration means that the actual rates of reduction of nitrite ($k_{NO_2^-}$) by rice nsHb and SynHb are 83 and 68 M^{-1} s^{-1}, respectively. These rates are the highest observed for a native or wild-type Hb by at least 1 order of magnitude.

The consequence of rapid, tight rebinding of NO to ferrous Hb is that, absent other factors, nitrite reduction by Hb is not an efficient means for generating free NO. In fact, to be useful for other purposes, NO produced in blood cells would also have to avoid binding to the previously existing high concentration of ferrous Hb, and reacting with oxyHb, which would result in its destruction. The latter two reactions occur on microsecond time scales at ~20 mM Hb in erythrocytes,[31] whereas reduction of nitrite by Hb occurs in minutes to hours at low nitrite concentrations. If fact, spectral analysis was chosen in our experiments as the primary means of observing the product because attempts to directly measure NO release using an NO electrode were unsuccessful. In efforts to measure NO production, others have adjusted experimental parameters specifically to minimize the extent of binding of NO to the remaining deoxyferrous Hb and even then observed relatively low levels of free NO gas.[27]

Consequences of Hb Hexacoordination for Nitrite Reduction. Rice nsHb1, SynHb, neuroglobin, and cytoglobin are all "hexacoordinate" hemoglobins, meaning that the distal histidine reversibly coordinates the heme iron.[32] The hemes of ferrous neuroglobin and SynHb bind tightly to the distal histidine (K_{H2} > 100, where K_{H2} is the association equilibrium constant for histidine coordination in the ferrous oxidation state), but those of rice nsHb and cytoglobin bind relatively weakly (K_{H2} < 100). Ferrous neuroglobin and cytoglobin react very slowly with nitrite,[15] and rice nsHb1 and SynHb react much more rapidly. Furthermore, pentacoordinate E7L neuroglobin (in which the distal histidine is replaced with leucine) is a very effective nitrite reductase,[12] but pentacoordinate Mb and blood cell Hb are not.

The only correlation between the strength of hexacoordination and reactions with nitrite is found in the plots of k_{obs} versus nitrite concentration for SynHb (Figure 2B) and murine neuroglobin.[15] Both of these plots have non-zero *y*-intercepts that are similar in magnitude (0.004 s^{-1}), and both Hbs have large values of K_{H2}. It is possible that this phenomenon reflects some aspect of the nitrite reaction mechanism with hexacoordinate Hbs, but there is no clear relationship between the overall nitrite reductase activity and the equilibria or kinetics of intramolecular heme coordination.

Physiological Significance of Hb Nitrite Reductase Activity in Plants and Cyanobacteria. A current hypothesis for plant and cyanobacterial Hbs is that they scavenge NO or peroxynitrite to detoxify these or other reactive oxygen species (ROS), which are particularly damaging under hypoxic conditions.[6,11,18] When bound to oxygen, these Hbs are certainly capable of reacting with NO to form nitrate,[4,5] and overexpression of plant nsHbs has been shown to reduce NO levels in vivo.[8,9] The results presented here suggest that under conditions of extreme hypoxia, plant and cyanobacterial Hbs could also function as nitrite reductases. Because these organisms often face such anoxic conditions,[11,18] during which nitrate and nitrite concentrations can rise to millimolar levels and the pH can drop several tenths of a unit,[33,34] it is likely that this activity is physiologically relevant, where it might help in preventing the generation of ROS from excess nitrite or in the continuation of nitrate assimilation. In fact, it is under just such conditions that cyanobacterial Hb knockouts show the largest amount of ROS buildup.[11]

There are a few questions that must be addressed in consideration of this hypothesis. (1) Why would plants want an alternative nitrite reductase active under anoxic conditions? (2) Could Hbs have a dual function as NO scavengers in the presence of oxygen and nitrite reductases during anoxia? (3) How are plant Hbs reduced in vivo, and how could they maintain catalytic nitrite reduction in the presence of potential inhibition by the NO they produce?

During hypoxia, nitrite can accumulate to toxic levels.[17,35,36] Plant Hbs could help to reduce nitrite levels under such conditions, using the reductive power accumulated as a result of anaerobic glycolysis. However, $Hb^{Fe(II)}NO$ formed in the reaction would inhibit catalytic nitrite reduction, and there would need to be either a mechanism in vivo for its removal prior to Hb binding, or $Hb^{Fe(II)}NO$ must react further to regenerate free Hb. Long-term plant survival requires continuation of protein synthesis even under anoxic conditions.[37,38] It is possible that $Hb^{Fe(II)}NO$ is an intermediate in the production of a further reduced nitrogen oxide, which could lead to the ammonia needed for protein synthesis in the absence of O_2.

It is also possible that Hbs are NO scavengers when oxygenated, nitrite reductases under anoxic conditions, or a combination of the two if partially saturated with oxygen. Igamberdiev et al.[6] have presented a strong argument for NOD activity during fermentative growth, during which Hb reduction is achieved by reduced monodehydroascorbate reductase.[39] Oxygen binding follows, and the oxyHb scavenges NO using the NOD reaction. Under anoxic conditions, the NOD reaction will not occur, and the resulting deoxyferrous Hb could function as a nitrite reductase. However, if the Hb were partially oxygenated (for rice nsHb1 this would be at ~2 nM O_2 and for SynHb at ~20 nM O_2[32]), it is possible that nitrite reduction could lead to NO that is subsequently scavenged by reacting with oxyHb.

Regardless of whether plant and cyanobacterial Hbs are NO scavengers or nitrite reductases, their rates of activity are certainly limited by the rates of reduction of each ferric Hb in vivo.[4,39] Even in the case of monodehydroascorbate reductase, the most likely candidate for Hb reduction in plant roots, the rate of reduction is slower than that observed here for the nitrite reductase reaction. Thus, it is important to characterize the reductase half-reactions carefully when evaluating the proposed activity of any Hb reactions involving changes in the heme iron oxidation state. It will also be necessary to compare nitrite reductase activities in the other classes of plant Hbs,[21] such as the leghemoglobins, which are not associated with surviving hypoxia. Results from such experiments will test the specificity of the reaction and will be an important consideration in evaluating the physiological significance of Hb nitrite reductase activity in these organisms.

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■ ABBREVIATIONS

Hb, hemoglobin; rice nsHb1, rice class 1 nonsymbiotic hemoglobin; SynHb, *Synechocystis* hemoglobin; Mb, myoglobin; NOD, nitric oxide dioxygenase; ROS, reactive oxygen species.

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