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Hydroxylamine Reduction to Ammonium by Plant and Cyanobacterial Hemoglobins

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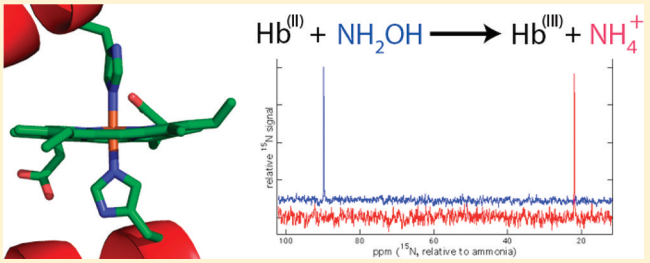
ABSTRACT: Plants often face hypoxic stress as a result of flooding and waterlogged soils. During these periods, they must continue ATP production and nitrogen metabolism if they are to survive. The normal pathway of reductive nitrogen assimilation in non-legumes, nitrate, and nitrite reductase can be inhibited during low oxygen conditions that are associated with the buildup of toxic metabolites such as nitrite and nitric oxide, so the plant must also have a means of detoxifying these molecules. Compared to animal hemoglobins, plant and cyanobacterial hemoglobins are adept at reducing nitrite to nitric oxide under anaerobic conditions. Here we test their abilities to reduce hydroxylamine, a proposed intermediate of nitrite reductase, under anaerobic conditions. We find that class 1 rice nonsymbiotic hemoglobin (rice nsHb1) and the hemoglobin from the cyanobacterium *Synechocystis* (SynHb) catalyze the reduction of hydroxylamine to ammonium at rates 100−2500 times faster than animal hemoglobins including myoglobin, neuroglobin, cytoglobin, and blood cell hemoglobin. These results support the hypothesis that plant and cyanobacterial hemoglobins contribute to anaerobic nitrogen metabolism in support of anaerobic respiration and survival during hypoxia.



Plants routinely face hypoxic and anoxic conditions, and survival varies greatly across different species, plant ages, and lengths of oxygen deprivation.[1,2] In order to survive these challenges, plants must generate ATP and cope with toxicity associated with increasing levels of nitrite (NO_2^-) and nitric oxide (NO).[3−5] The mechanisms by which NO_2^- and NO accumulation occur are not entirely understood but may result from the activities of nitrate reductase (NR)[6,7] and nitrite reductase (NiR)[8] as well as the reduction of nitrite by mitochondria and a membrane-bound nitrite/nitric oxide reductase (NiNOR).[9,10] Thus, in plants, hypoxia results in the production of nitrite and NO, which are harmful metabolites reflecting the failure to completely reduce nitrate to ammonium.

Hypoxia, nitrate, nitrite, and NO are known to up-regulate a specific class of plant hemoglobin (class 1 nonsymbiotic hemoglobins, or nsHbs[11]), which increases survival in the absence of oxygen.[5,12,13] The role of nsHbs in survival during hypoxia has previously been attributed to the scavenging and detoxification of NO and the concomitant oxidation of NADH to keep glycolysis functioning under these conditions.[5] The proposed mechanism of NO scavenging is the nitric oxide dioxygenation (NOD) reaction, which is common to hemoglobins (Hbs) in general.[14,15]

$$Hb^{2+}O_2 + NO \xrightarrow{NOD} Hb^{3+} + NO_3^- \quad (1)$$

Oxygen is required for the NOD reaction as well as a source of electrons to reduce the resulting ferric Hb. The source of reduction in plants has been attributed to NADH and the enzyme monodehydroascorbate reductase.[16]

It is counterintuitive, however, to waste the reductive power of NADH on the oxidation of NO, if it could instead be coupled to production of ammonium and/or generation of ATP. Furthermore, when oxygen levels are below ∼2 nM (the K_D for oxygen binding to class 1 nsHbs[11]), the cosubstrate of the NOD reaction is absent and the reaction cannot proceed. These ideas, along with the observation that nitrate helps to ameliorate the consequences of hypoxia,[17] led us to test the possibility that deoxygenated nsHb might instead reduce nitrite in support of cell protection during hypoxia. Those results show that plant and cyanobacterial Hbs reduce nitrite to NO very rapidly compared to animal Hbs.[18] However, converting nitrite to NO in isolation makes little sense metabolically as the NO produced binds to the remaining ferrous Hb, preventing continued nitrite reduction. Thus, for nitrite reduction to be a plausible hypothesis for the function of nsHb, they must influence additional chemistry associated with the overall goal of ammonium production.

We propose that during extreme hypoxia nsHbs catalyze the sequential reduction of nitrite to ammonium in a series of steps similar to that observed in siroheme containing nitrite/sulfite reductases.[19,20] The expected intermediates in this scheme are the one-electron reduction of nitrite to NO, the three-electron reduction of NO to NH_2OH (hydroxylamine, or HA), and the two-electron reduction of HA to ammonium, NH_4^+. It has long been known that deoxyferrous blood cell Hb reacts with HA to form N_2, N_2O, and ammonium and that the percentage of

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ammonium formed can be increased dramatically in the presence of an excess of reductant.[21,22] However, the reaction between HA and deoxyferrous blood cell Hb is very slow, having a bimolecular rate constant of 0.01 mM^{-1} s^{-1},[22] making blood cell Hb an unlikely candidate to function in HA reduction.

In the current work, we examine the ability of a deoxyferrous class 1 nsHb, rice nsHb1, to reduce HA. We compare this activity to that of three animal Hbs (myoglobin, neuroglobin, and cytoglobin) as well as a Hb from another photosynthetic autotroph, the cyanobacterium *Synechocystis* (SynHb). Our results show that deoxyferrous rice nsHb1 and SynHb convert HA specifically to ammonium at rates 100−2500 times faster than the animal Hbs. These results complement those of nitrite reduction by rice nsHb1 and SynHb[18] and support the hypothesis that they serve as an alternative means of reducing nitrogen metabolites, recycling NADH produced during anaerobic glycolysis, and continuing ammonium production during hypoxia.

MATERIALS AND METHODS

Protein and Reagent Preparation. Hbs were expressed, purified, and converted to the deoxyferrous forms as described previously.[18,23,24] These include rice nsHb1, SynHb, human neuroglobin, human cytoglobin, and horse heart myoglobin. Anaerobic buffers were prepared by boiling for 5 min followed by argon purging until cooled to room temperature, followed by equilibration in the anaerobic chamber overnight (95% argon and 5% hydrogen, Coy Laboratories). All dry chemical reagents were weighed outside of the anaerobic chamber, deoxygenated, and dissolved inside the chamber in anaerobic buffer. ^{15}N-Hydroxylamine, ^{15}N-ammonia, and ^{15}N-urea were purchased from Cambridge Isotope Laboratories.

Equilibrium Reactions. Anaerobic titrations of deoxyferrous Hbs with HA were carried out by adding a stock solution of 1 mM HA to a stirred cuvette containing deoxyferrous Hb in the anaerobic chamber and monitoring the equilibrated visible absorbance spectra with an Ocean Optics USB 2000 spectrophotometer. All reactions were carried out in 0.1 M phosphate buffer, pH 7.0, and room temperature. The progress of oxidation associated with these isosbestic transitions can be followed as the normalized decrease in absorbance at 557 nm.

NMR Experiments. NMR experiments were carried out on a Bruker DRX500 NMR spectrometer at the Iowa State University Biomolecular NMR Facility using a Nalorac 5 mm broadband probe configured for ^{15}N detection. 1D ^{15}N spectra were collected at 25 °C with inverse-gated ^{1}H decoupling. ^{15}N chemical shifts are referenced to urea as an indirect reference to liquid NH_3. Data were collected and processed using Bruker TopSpin 1.3 software, and Bruker data files were exported to Igor Pro for further analysis. Samples were prepared in the anaerobic chamber and sealed before being removed. NMR spectra were acquired from several minutes to several hours after sample preparation with little difference observed in the resulting spectra. To measure the yield of conversion of HA to ammonium (Figure 2C), 10 mM ^{15}N-urea was added just prior to measurement of NMR spectra, as an internal standard for peak integration. Thus, the values on the *y*-axis of Figure 2C are the peak area at 21 ppm divided by peak area at 77 ppm (for ^{15}N-urea). Each HA reduction reaction in Figure 2C contained the [HA] indicated on the *x*-axis, 2 μM Hb (either rice nsHb1 or SynHb), and 200 mM sodium dithionite (DT). As demonstrated in Figure 2A, DT alone has no effect on the ^{15}N NMR spectrum of ^{15}N-HA on these times scales.

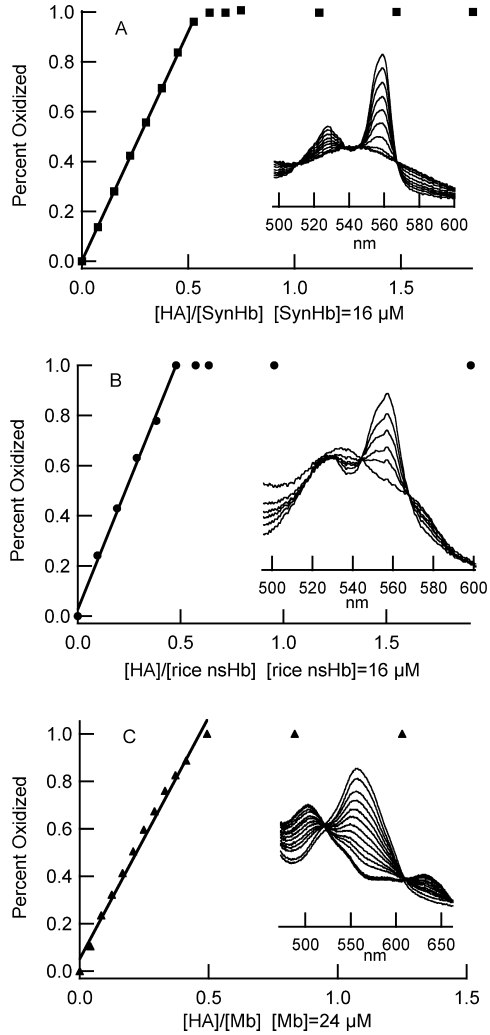

Figure 1. Reaction of deoxyferrous Hbs with HA: (A) rice nsHb1 (16 μM); (B) SynHb (16 μM); (C) Mb (24 μM). All titrations exhibit sharp deviations from linearity at [HA]/[Hb] = 0.5, at which point the titration reaches 100% completion. This shows that the reactions proceed by a mechanism in which two Hbs are oxidized by one HA. In each case the spectral transition is from deoxyferrous Hb^{2+} to ferric Hb^{3+}.

Kinetics Measurements. Kinetic traces for the reaction of HA with Hbs were measured using a Bio-Logic stopped flow SFM-400 reactor, a MOS-250 scanning spectrophotometer, and Biokine software. All reactions were carried out in 0.1 M phosphate buffer, pH 7.0, and room temperature. The reactor and all associated solutions were housed in the anaerobic chamber. Reactions were carried out under pseudo-first-order conditions by mixing deoxyferrous Hbs with equal volumes of HA to achieve final concentrations of 50, 100, 150, 200, and 500 μM HA. Reaction time courses were monitored at 557 nm and fitted to an exponential curve in Igor Pro to determine rate constants at each concentration (k_{obs}). These observed rate constants were plotted versus [HA] and fitted to a line to determine observed bimolecular rate constants (Figure 3B). The effect of moderate pH change on the reaction was measured under the same conditions, but with the phosphate buffer titrated to either pH 6.5 or 7.5.

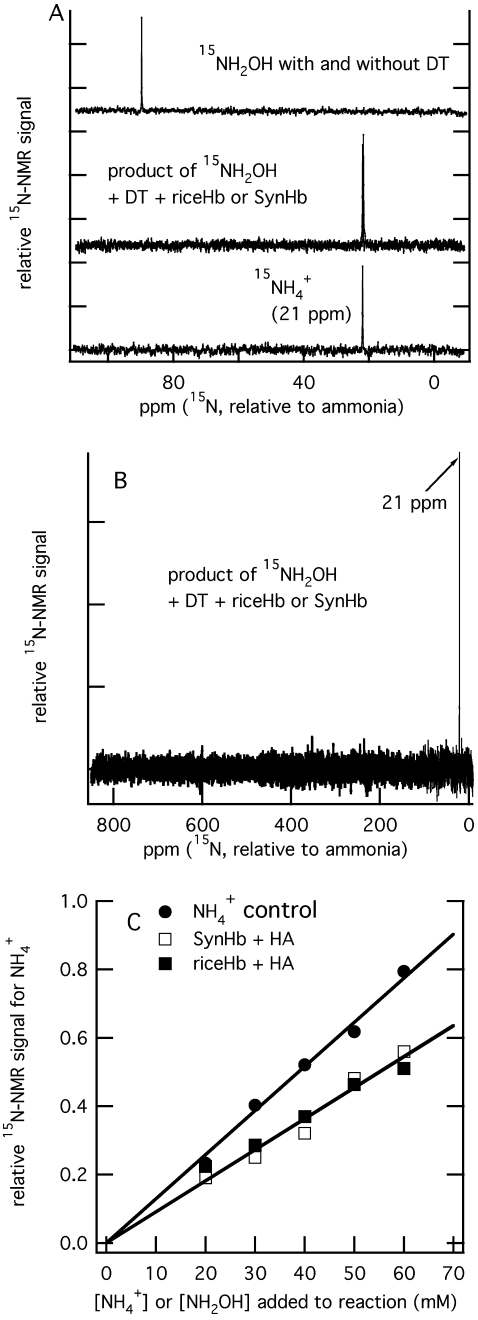

Figure 2. NMR detection and identification of the hydroxylamine reduction product. (A) ^{15}N NMR spectra of $^{15}NH_2OH$ in the presence and absence of sodium dithionite (DT) (upper panel), the product of the reaction of 50 mM $^{15}NH_2OH$ with ~2 μM nsHb1 or SynHb in the presence of 100 mM DT (middle panel), and $^{15}NH_4^+$ as a control (lower panel). (B) An expanded ^{15}N NMR spectrum of the reaction of $^{15}NH_2OH$ with rice nsHb1 (or SynHb) demonstrating that $^{15}NH_4^+$ is the only detectable soluble product. (C) A standard curve of $^{15}NH_4^+$ peak area compared to the $^{15}NH_4^+$ peaks generated by the reaction of $^{15}NH_2OH$ with ~2 μM rice nsHb1 (closed squares) and SynHb (open squares) in the presence of 100 mM DT. These results demonstrate that the reaction is both catalytic and specific, with at least 80% of starting $^{15}NH_2OH$ converted to $^{15}NH_4^+$.

RESULTS

Reaction of Hydroxylamine with Rice nsHb, SynHb, and Mb. Previous studies have demonstrated that deoxyferrous blood cell Hb reacts slowly with HA to catalyze a two-electron reduction to ammonium, along with the production of some N_2 and a small amount of N_2O.[21,22] To determine if a reaction also occurs with plant and cyanobacterial Hbs, the deoxyferrous forms of rice nsHb1, SynHb, and Mb were combined with HA under anaerobic conditions. Figure 1 shows absorbance spectra associated with these titrations. In each case the Hb (with concentrations in the 15−25 μM range) reacts stoichiometrically with HA, indicating that the K_D for binding must be no greater than ~1 μM. Furthermore, in each case the reaction is complete at a molar ratio of 0.5 HA/Hb, indicating a two-electron reduction of HA. These results are consistent with those observed for blood cell Hb,[22] leading to the hypothesis that the major product of the reaction is ammonium.

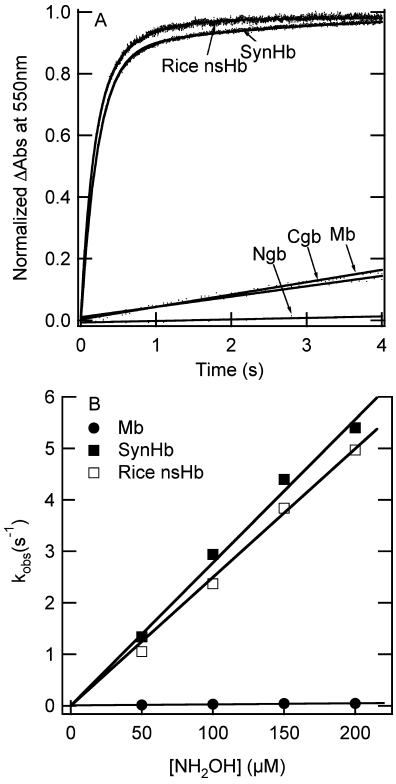

Figure 3. Relative rates of hydroxylamine reduction by different Hbs. (A) Time courses for the reaction of deoxyferrous rice nsHb1, SynHb, Mb, Cgb, and Ngb with 150 μM HA, as measured by the oxidation of the Hb (as in Figure 1). (B) Dependence of the reaction rate constant (k_{obs}) on [HA] for rice nsHb1, SynHb, and Mb, demonstrating that reaction rates for rice nsHb1 and SynHb are 2 orders of magnitude faster than Mb, having pseudo-first-order rates of 25 and 28 mM^{-1} s^{-1}, respectively, compared to 0.25 mM^{-1} s^{-1}.

Ammonium Is the Product of Hydroxylamine Reduction. The progress of the reactions associated with Figure 1 was measured by monitoring heme absorbance, which is convenient at micromolar concentrations. One product of the reaction is ferric Hb; the other, hypothesized to be ammonium, is presumably also produced and present at micromolar concentrations. Ammonium is difficult to detect directly at these low levels, so the product concentration was increased to concentrations necessary for detection via ^{15}N NMR spectroscopy with an acquisition time of 15 min (i.e., 20 mM ^{15}N). Because ammonium does not appear to bind the resulting ferric Hb (as indicated by the ferric end-point absorbance spectra in Figure 1), it was hypothesized that increasing product concentration might simply be a matter of adding more HA

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and reductant and letting the Hb catalyze the formation of higher product concentrations.

To test this possibility, 50 mM ^{15}N-HA was mixed with 2 μM rice nsHb1 or 2 μM SynHb in the presence of 100 mM sodium dithionite (DT, serving as the reductant). As a control to ensure that DT alone does not reduce HA, the ^{15}N NMR spectrum of ^{15}N-HA was measured in the absence and presence of 100 mM DT, and the chemical shift was found to be the same (90 ppm) (Figure 2A, upper panel). However, when the mixture included 2 μM rice nsHb1 or SynHb, all of the ^{15}N is converted to a product with a chemical shift of 21 ppm, identical to that of ammonium (Figure 3A, lower panel). Thus, in the presence of a reductant, rice nsHb1 and SynHb catalyze the complete conversion of HA to ammonium.

The ^{15}N chemical shift is determined by the shielding of the nitrogen nucleus and is generally directly proportional to its oxidation number. To verify the specificity of product formation, we also acquired ^{15}N NMR spectra over a large chemical shift range, including the locations of peaks for nitrogen compounds with more positive oxidation numbers than HA and ammonium (Figure 2B). No such peaks were observed, suggesting that ammonium is the principal soluble product of HA reduction by these Hbs. To test for the presence of gaseous products, GCMS was used to examine the headspace above the reaction. A small amount of N_2 could be detected but accounted for less than 1% of the total product (data not shown).

Quantification of Ammonium Production from Hydroxylamine. A standard curve for the ^{15}N NMR peak area was used to measure the yield of ammonium produced from HA by rice nsHb1 and SynHb (Figure 2C). The results for rice nsHb1 and SynHb were identical, with each Hb converting HA to ammonium with a yield of ~80%. In all samples, NMR detected no other soluble products, and only trace amounts of N_2 were detectable by GCMS.

The reverse reaction and the reaction of HA with ferric Hbs were also measured. Addition of ammonium to ferric Hb did not affect the Hb absorption spectrum and did not affect the ^{15}N NMR signal of ^{15}N-ammonium. HA was also equally unreactive with ferric Hb as measured spectrally or as the effect of ferric Hb on the ^{15}N-HA NMR spectrum. Thus, no reverse reaction, or reaction with ferric Hb, is detectable.

Kinetics of Ammonium Production from Hydroxylamine. Time courses for the reactions of HA with deoxyferrous rice nsHb1, SynHb, myoglobin, neuroglobin, and cytoglobin were measured in a stopped flow reactor housed in an anaerobic chamber. Figure 3A shows a comparison of time courses for each Hb at 150 μM [HA], monitoring the oxidation of the deoxyferrous Hb. The reactions with rice nsHb1 and SynHb are nearly complete in 2 s, while those of Mb, Ngb, and Cgb are no more than 10% complete over this period of time. Observed bimolecular rate constants for reactions with rice nsHb1, SynHb, and Mb were measured from the slopes of the plots of concentration dependence of the reaction (Figure 3B). Rice nsHb1 and SynHb have observed bimolecular rate constants for HA reduction that are 2 orders of magnitude faster than Mb, with values of 25 and 28 mM^{-1} s^{-1}, respectively, compared to 0.25 mM^{-1} s^{-1}.

Over the range of [HA] shown in Figure 3B, k_{obs} increases linearly with [HA]. However, at higher [HA] (>500 μM) some deviation from linearity is observed (data not shown) that is consistent with previous reports of the reaction by blood cell Hb.[21,22] In addition to the reaction with the deoxyferrous forms of each Hb in Figure 3, oxy, CO, and ferric forms of each were reacted with 150 μM HA, but no spectral changes were detected for any of these derivatives. Bazylinski and coworkers[22] reported a sharp increase in the rate of the reduction of HA by blood cell Hb as pH was raised from 6.5 to 7.5. As pH is lower in hypoxic root tissue,[25] we measured HA reduction by rice nsHb1 at pH 6.5 and 7.5 but observed no significant differences from the rate at pH 7.0 (data not shown).

DISCUSSION

The results presented in this study demonstrate that deoxyferrous rice nsHb1 and SynHb are capable of reducing HA to ammonium at rates much faster than other Hbs. In comparison to the fastest animal Hb tested, myoglobin (0.25 mM^{-1} s^{-1}), the observed bimolecular rate constants for rice nsHb1 (25 mM^{-1} s^{-1}) and SynHb (28 mM^{-1} s^{-1}) are at least 100 times faster. We have recently reported that deoxyferrous rice nsHb1 and SynHb are also particularly effective at reducing nitrite to NO.[18] Thus, rice nsHb1 and SynHb are adept at catalyzing two of the three reactions involved in the sequential conversion of nitrite to ammonium.

Other enzymes that will reduce nitrite to ammonium are the siroheme nitrite reductases (NiR) in plants and cyanobacteria[19,20] and the bacterial cytochrome *c* nitrite reductases (ccNiR).[26] NiR participates in the second step of assimilatory nitrate utilization, reducing nitrite to ammonium principally for amino acid production. NiR uses a [4Fe:4S] cluster and a siroheme prosthetic group to carry out the six-electron reduction presumably via sequential one-electron reductions and the formation of enzyme-bound NO and HA intermediates, though these molecules are not normally released from the enzyme.[27,28] However, when reductant is limited, these enzyme-bound intermediates may be released.[29] NiR also reacts directly with HA,[30] albeit with lower activity.[20,29]

ccNiR is responsible for dissimilatory nitrite ammonification in support of anaerobic respiration in many Gram-negative bacteria. It exists in the periplasmic space as a homodimer of 70 kDa subunits, each containing five heme groups. Four of the hemes are bis-histidine hexacoordinated, and the other is pentacoordinate with a lysine side chain binding one axial position of the heme iron.[31] Like NiR, ccNiR does not normally release NO or HA but will reduce exogenous HA with lower activity than nitrite.[32,33]

Hbs, NO Scavenging, and Nitrogen Metabolism. Over the past few decades, newly discovered Hbs have been identified in many organisms in all of the kingdoms of life. Most of these were identified from genomic sequencing, with little information indicating physiological function. Because of our familiarity with oxygen and NO reactions with Hbs, such reactions are natural starting hypotheses for their biological roles. However, most Hbs will bind oxygen and NO and are adept at the NOD reaction,[15] so observation of these reactions *in vitro* with nsHbs[34,35] does not distinctly support or refute hypothesized NOD or oxygen transport functions for these Hbs.

There are several reports of experiments testing the NOD hypothesis in nsHbs. Dordas et al.[36] measured ATP/ADP ratios in alfalfa root cell cultures overexpressing a nsHb. In these experiments, the authors presume that NOD activity is a reflection of NADH consumption in support of continued glycolysis[5] and thus results in increased ATP/ADP ratios. The ATP/ADP ratios of the overexpression lines increased 2.5-fold in comparison to the under-expressing lines and 1.3-fold

compared to wild-type cells when exposed to NO. Subsequently,[37] it was shown that NO concentrations reach only 250 nmol/(g fresh weight) in hypoxic nsHb-overexpressing maize cell cultures, compared to 500 in the underexpressing lines and 425 in wild-type cells. Other studies[38,39] have shown that homogenates from nsHb overexpressing plant cell lines will carry out the NOD reaction 1.3–1.6 times faster than the respective wild-type lines and ~2-fold faster than an underexpressing line. In contrast, data in support of the NOD reaction by *E. coli* flavohemoglobin[40,41] include 60-fold increases in NO scavenging by overexpressing versus wild-type cell homogenates, >10-fold reduction in growth of knockout cells compared wild-type cells, and 150-fold increases in cell survival of naturally overexpressing versus wild-type cells. Thus, while the data above for nsHbs can be interpreted as modest support for a NOD function, they are not accompanied by direct evaluation of cell survival and are of a lesser magnitude than homologous experiments with *E. coli* flavohemoglobin.

Here, and in one previous publication,[18] we have demonstrated that plant nsHbs and SynHb reduce nitrite and HA at rates much faster than other Hbs under anaerobic conditions. There are two reasons why plants and cyanobacteria might benefit from nitrite and HA reduction by their Hbs during hypoxia: (1) to remove the toxic nitrogen metabolites by reducing them to ammonium and (2) to serve as an electron sink in support of anaerobic metabolism by coupling glycolytic electrons to ammonium production. In essence, the result of these activities would be the same as that proposed previously[5] but attained through a different mechanism involving reduction rather than oxidation of nitrogen metabolites and not requiring oxygen. Furthermore, this mechanism would likely cause the aforementioned phenotypes that have previously been attributed solely to the NOD reaction and could be linked to the observed nitrite-driven ATP synthesis in plant roots.[42,43] It is possible that nsHbs could carry out the NOD reaction when oxygen is present and catalyze reductive nitrogen chemistry in its absence, as they are suited exclusively for one activity or the other depending on the level of oxygenation.

Mechanism of HA Reduction by nsHbs. The experiments presented here do not provide a mechanism for HA reduction by Hbs. They only demonstrate that the reaction occurs and that ammonium is the major product. Thus, we cannot describe the rate law and can only present observed rate constants. However, the simplest reaction fitting our stoichiometry is

$$2Hb^{2+} + NH_2OH + 2H^+ \rightarrow 2Hb^{3+} + NH_4^+ + OH^- \quad (2)$$

The spectra associated with the reaction (Figure 1) show no intermediates, suggesting that reduction of HA and release of ammonium are very rapid compared to binding or that the reaction occurs in the outer sphere, with the reactants never binding the heme iron (such as the reaction between Hb and DT[44]). On the other hand, the reaction is inhibited by oxygen and CO, suggesting an inner-sphere mechanism.

An important question concerning this reaction is how Hb^{2+} delivers two electrons to a single molecule of HA. This could happen through either a geminate or a dissociative mechanism. In the geminate mechanism, the two electrons are delivered to HA while it is bound to the same Hb molecule. This would require the addition of an electron to the HA–Hb^{2+} complex followed by dissociation of ammonium. The dissociative mechanism would have a partially reduced intermediate dissociate from Hb^{3+} and find another Hb^{2+} to provide the second electron. The fact that no intermediates or products other than ammonium have been observed and that there are no known stable products resulting from the one-electron reduction of HA suggests that the reaction proceeds via the geminate reduction mechanism.

Further consideration of a geminate two-electron reduction by Hb raises the question of how an individual Hb^{2+} delivers two electrons to HA. In the presence of excess exogenous reductant (as in the catalytic reactions with DT in Figure 2), one could suppose that DT rapidly reduces the HA–Hb^{2+} complex. However, this cannot explain how the reaction proceeds with only Hb^{2+} (in the absence of DT, as in Figures 1 and 3). In these reactions, where all the electrons must come from Hb^{2+}, there must be rapid transfer of electrons from free Hb^{2+} to HA–Hb^{2+} to complete the reaction.

Such inter-Hb electron transfer has been directly measured for horse-heart myoglobin, with a rate constant of 3000 M^{-1} s^{-1}.[45] At the concentration where we measured the kinetics of HA reduction by myoglobin (~20 μM Mb), this would predict a rate of electron transfer between myoglobin molecules of 0.06 s^{-1}. Furthermore, it predicts that at higher [HA] concentrations the reaction should become limited by the rate of electron transfer, reaching (for myoglobin) an asymptote near 0.06 s^{-1}. Measurement of the observed rate constant for [HA] above 500 μM shows a deviation from linearity, but no asymptote near 0.06 s^{-1} is observed (k_{obs} for 20 mM Mb and 2.5 mM [HA] = 0.43 s^{-1}). This suggests that intermolecular electron transfer is not a limiting factor or that the presence of HA somehow facilitates electron transfer between Hbs.

Potential Roles of Hb Hexacoordination in Hydroxylamine Reduction. Rice nsHb1 and SynHb are hexacoordinate Hbs,[46] meaning that their heme irons are coordinated by six ligands in the ferric and ferrous oxidation states. The sources of coordination are the four equatorial pyrrole nitrogens of the heme porphyrin and two axial histidine side chains. One of the histidine side chains (the "distal" histidine) can dissociate from the heme iron to allow binding of exogenous ligands like oxygen, CO, NO, and (possibly) nitrite and HA. Just being hexacoordinate does not mean that Hb will rapidly reduce nitrite or HA, as neuroglobin and cytoglobin are both hexacoordinate and relatively slow at these reactions. In fact, removing the distal histidine from neuroglobin by mutation greatly increases the rate of nitrite reduction.[47]

There are three features of hexacoordinate Hbs that could contribute to catalytic HA reduction. First is the fact that heme hexacoordination increases the rate of electron transfer and heme reduction by lowering the activation energy associated with the change in oxidation state.[44] This is because the heme iron, with histidines bound in both axial positions, remains low spin in both the ferrous and ferric oxidation states. It is probably no coincidence that four of the five hemes in ccNiR are bis-histidine hexacoordinated.[31,48] If electron transfer to rereduce the Hb is rate limiting, hexacoordination could increase the limit compared to pentacoordinate Hbs such as Mb. Second, distal histidine coordination of the heme iron could provide energy for rapidly displacing the positively charged ammonium ion formed from HA reduction. The positive polarity on its side chain could also serve to attract negatively charged molecules like nitrite, while helping to displace ammonium through charge repulsion. Third, hex-

acoordination could serve to prevent HA from reacting with the ferric Hb. HA has been shown to react with ferric siroheme in NiR to form NO.[28] Coordination of the distal histidine in hexacoordinate Hbs is generally much stronger in the ferric oxidation state than in the ferrous[24] and could serve as a mechanism to block any reaction with the ferric Hb and ensure that nitrogen metabolism continues in the reductive direction.

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ABBREVIATIONS

Hb, hemoglobin; rice nsHb1, rice class 1 nonsymbiotic hemoglobin; SynHb, *Synechocystis* hemoglobin; Mb, myoglobin; NOD, nitric oxide dioxygenase; HA, hydroxylamine; NiR, nitrite reductase; ccNiR, cytochrome *c* nitrite reductase.

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■ NOTE ADDED AFTER ASAP PUBLICATION

After this paper was published online November 18, 2011, a correction was made to the caption of Figure 3. The corrected version was published November 23, 2011.