

Nitrite Reductase Activity of Nonsymbiotic Hemoglobins from *Arabidopsis thaliana*

Mauro Tiso,^{*,†,‡} Jesús Tejero,[†] Claire Kenney,[§] Sheila Frizzell,[†] and Mark T. Gladwin^{*,†,||}

[†]Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, United States

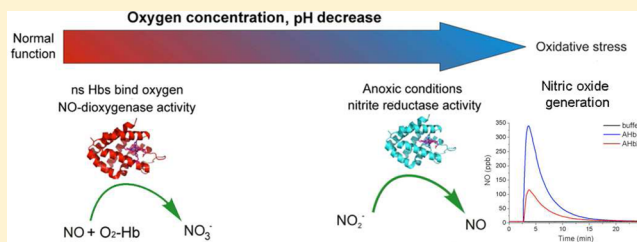
[‡]Molecular Medicine Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, United States

[§]Vascular Medicine Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, United States

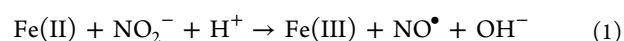
^{||}Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, United States

Supporting Information

ABSTRACT: Plant nonsymbiotic hemoglobins possess hexacoordinate heme geometry similar to that of the heme protein neuroglobin. We recently discovered that deoxygenated neuroglobin converts nitrite to nitric oxide (NO), an important signaling molecule involved in many processes in plants. We sought to determine whether *Arabidopsis thaliana* nonsymbiotic hemoglobins classes 1 and 2 (AHb1 and AHb2, respectively) might function as nitrite reductases. We found that the reaction of nitrite with deoxygenated AHb1 and AHb2 generates NO gas and iron–nitrosyl–hemoglobin species. The bimolecular rate constants for reduction of nitrite to NO are 19.8 ± 3.2 and $4.9 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$, respectively, at pH 7.4 and 25 °C. We determined the pH dependence of these bimolecular rate constants and found a linear correlation with the concentration of protons, indicating the requirement for one proton in the reaction. The release of free NO gas during the reaction under anoxic and hypoxic (2% oxygen) conditions was confirmed by chemiluminescence detection. These results demonstrate that deoxygenated AHb1 and AHb2 reduce nitrite to form NO via a mechanism analogous to that observed for hemoglobin, myoglobin, and neuroglobin. Our findings suggest that during severe hypoxia and in the anaerobic plant roots, especially in species submerged in water, nonsymbiotic hemoglobins provide a viable pathway for NO generation via nitrite reduction.



Nitric oxide (NO) is a diffusible and short-lived free radical gas with a wide range of functions in both eukaryotes and prokaryotes.¹ NO is produced in plants in response to bacterial or viral exposure and plays a role in multiple processes.^{2,3} Currently, two major pathways for NO formation in plants are accepted: one route involves enzymatic and nonenzymatic nitrate and nitrite reduction^{4–6} and the other arginine oxidation.^{7,8} However, the mechanisms of NO production in plants are not determined and controversial.^{9,10} Although the metabolic source of NO in plants remains uncertain, accumulating evidence suggests that nitrite can be a source of NO in mammals under hypoxic/ischemic conditions.^{11–13} Several hemoproteins, iron–sulfur cluster-containing proteins, and molybdenum-based reductases have recently been proposed to be nitrite reductases.¹⁴ In addition, the pentacoordinate respiratory hemoproteins hemoglobin (Hb) and myoglobin (Mb) and hexacoordinate neuronal protein neuroglobin (Ngb) have shown the ability to reduce nitrite to NO under both physiological and pathological hypoxia conditions via the reactions displayed in eqs 1 and 2.^{15–17}



Hemoglobins make up an ancient class of molecules ubiquitous in eukaryotes and uniting almost all forms of life.¹⁸ Plant hemoglobins were first found in root nodules of plants capable of symbiotic nitrogen fixation where they function to regulate oxygen delivery and were termed symbiotic Hbs. The subsequent discovery of nonsymbiotic plant hemoglobins (nsHbs) raised questions about their physiological function and renewed research attention on this field.^{19,20} Plant nsHbs have been divided into two molecular species, classes 1 and 2, which have sequences that are approximately 60% identical but differ from each other in phylogenetic characteristics, gene expression patterns, and oxygen binding properties.^{21,22} Both classes possess at least partial hexacoordinate heme geometry with proximal and distal histidines directly bound to the heme iron, similar to the mammalian neuroglobin and cytoglobin. However, despite competing with the distal histidine for the same iron binding site, both nsHbs bind oxygen with very high

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affinity at physiological pH [K_d values of ~ 2 and ~ 100 nM for *Arabidopsis thaliana* nsHb class 1 (AHb1) and class 2 (AHb2), respectively].²³ For this reason, it has been suggested that they do not participate in oxygen transport or storage but are involved in metabolic reactions,²⁴ although their physiological function remains unclear. Oxygen-bound AHb1 has been shown to have NO-dioxygenase activity using NADPH as an electron donor and producing nitrate and ferric hemoglobin.²⁵ This reaction has been proposed in NO detoxification by acting as an NO scavenger.²⁶ AHb2 is induced by low temperatures (cold stress) and ubiquitously expressed at low levels: transgenic overexpression leads to a significant improvement (40%) in the metabolic performance of the plant,²⁷ but its role in plant physiology remains elusive.

The recent finding that deoxygenated Hb, Mb, and Ngb can reduce nitrite to form NO under hypoxic and anoxic conditions^{16,17,28} suggests that this activity is inherent to the heme moiety of the globins. This hypothesis is further supported by a recent report by Sturms et al.²⁹ that extends the ability of nitrite reduction to ferrous cyanobacterial hemoglobin from *Synechocystis* and rice nonsymbiotic hemoglobin 1. However, their direct attempts to measure NO release using an NO electrode were unsuccessful, and whether these hemoglobins can produce authentic NO from nitrite has not been evaluated.

Here we tested whether *A. thaliana* nsHbs classes 1 and 2 can function as nitrite reductases and pursued kinetic characterization of the reaction with nitrite during anoxia and 2% oxygen hypoxia. We found that AHb1 and AHb2 are capable of reducing nitrite to NO and the reaction is proton-dependent in the physiological range.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich unless otherwise specified. UV–visible spectra and kinetic data were recorded on an HP8453 UV–vis spectrophotometer (Agilent, Santa Clara, CA) using 1 cm path length quartz or special optical glass cuvettes. Solutions of sodium dithionite and nitrite were prepared and kept at 25 °C with argon-degassed 0.1 M phosphate buffer (pH 7.5) under inert gas.

Cloning, Expression, and Purification of Recombinant AHb1 and AHb2. Recombinant AHb1 and AHb2 were expressed in *Escherichia coli* BL21(DE3). Restriction digestions, ligation, transformation, cloning, bacterial growth, and isolation of DNA fragments were performed using standard techniques. Purification was conducted as described previously with minor modifications.³⁰ To increase the yields of purification, we fused AHb1 and AHb2 cDNA with a six-His tag at their N-termini and cloned the cDNA into pET28a. Purification of His-tagged AHb1 and AHb2 was performed using a Ni-NTA-agarose (Qiagen) affinity column according to the manufacturer's manual, and the additional amino acids at the N-terminus were removed using a thrombin cleavage capture kit (Novagen). The eluted protein was dialyzed against PBS at 4 °C, concentrated with a 10 kDa cutoff filter, and stored in aliquots at -80 °C. The purity of each recombinant batch prepared was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and UV–visible spectroscopy.

Sample Preparation. Proteins were fully oxidized with excess potassium ferricyanide or fully reduced by incubation with 100 mM sodium dithionite; excess reagents were removed by passing the mixture through two sequential Sephadex G-25 desalting columns. Concentrations were estimated by measur-

ing the Soret peak absorbance at 425 nm of the deoxy ferrous form using an ϵ of $92 \text{ mM}^{-1} \text{ cm}^{-1}$.²⁶

Reference Spectra of Individual Species. Standard reference species of recombinant AHb1 and AHb2 were prepared following procedures previously described for other hemoglobins.^{15,17} Deconvolution of spectra into individual species was accomplished with multilinear regression analysis, using a set of pure spectra of deoxy-, iron-nitrosyl-, met-, and oxy-AHb1 and -AHb2 species as a reference basis (Figure 1 of the Supporting Information).

Anaerobic Reactions of Globins with Nitrite. Anaerobic reduced samples were prepared in a glovebox under a 2–4% H_2 atmosphere of catalyst-deoxygenated nitrogen and collected directly in cuvettes that were sealed with rubber septa inside the glovebox before use. Reaction kinetics of known amounts of AHbs with nitrite were monitored by absorption spectroscopy for the indicated time in a cuvette in the absence or presence of 3 mM sodium dithionite. All reactions were conducted at 25 °C in 0.1 M phosphate buffer of the indicated pH. Previously deoxygenated nitrite was added, using an airtight syringe, to a sealed anaerobic cuvette to initiate the reaction. Oxygen contamination was prevented by application of positive argon pressure. The time-dependent changes of single species of AHb1 and AHb2 were calculated by least-squares deconvolution of the reaction spectra. To vary the pH, deoxy species and nitrite were prepared in phosphate buffer adjusted to the target pH values. Determined values are means \pm the standard deviation (SD) from triplicate experiments representative of two independent enzyme preparations.

Measurement of NO Emission in the Gas Phase. NO gas liberated from a reaction mixture was measured with an ozone-based chemiluminescence NO analyzer (CLD88Y, Eco Physics Inc., Ann Arbor, MI, and Sievers, GE Analytical Instruments, Boulder, CO) in real time. The reactions of deoxy-AHb1 and -AHb2 (prepared by titrating stock proteins with a stoichiometric amount of dithionite in an anaerobic cuvette and then diluted to a final concentration of 25 μM ; this would contain only traces of dithionite) were conducted in 100 mM phosphate buffer (pH 7.4) at 25 °C, either in a vessel purged with helium gas in studies under anaerobic conditions or in flat flasks (surface area of 25 cm^2) purged with a 2% O_2 /98% N_2 gas mixture without bubbling and strictly regulating the flow rate (50 mL/min) for hypoxic conditions. Once a stable baseline was established, the indicated amount of nitrite was injected into the mixture as previously described.¹⁵ To test whether the release of NO into the gas phase from the solutions could be used as a continuous measurement of NO production, we built a calibration curve using amounts of NO validated by injection of sodium nitrite standards into a triiodide solution.

RESULTS

Nitrite Is Reduced to NO via Reaction with Deoxygenated AHb1 and AHb2. To examine the reaction of nitrite with plant *A. thaliana* nsHbs, we used recombinant AHb1 and AHb2. Spectrophotometric analysis of the purified proteins confirmed the presence of hexacoordinate heme in both the ferrous and ferric states with visible peaks at 529 nm (α band) and 558 nm (β band), typically observed for hexacoordinate systems (Figure 1 of the Supporting Information). However, a substantial fraction of the AHb1 molecules displayed a pentacoordinate heme geometry as previously reported (estimated to be $\sim 40\%$ by spectral deconvolution).³¹

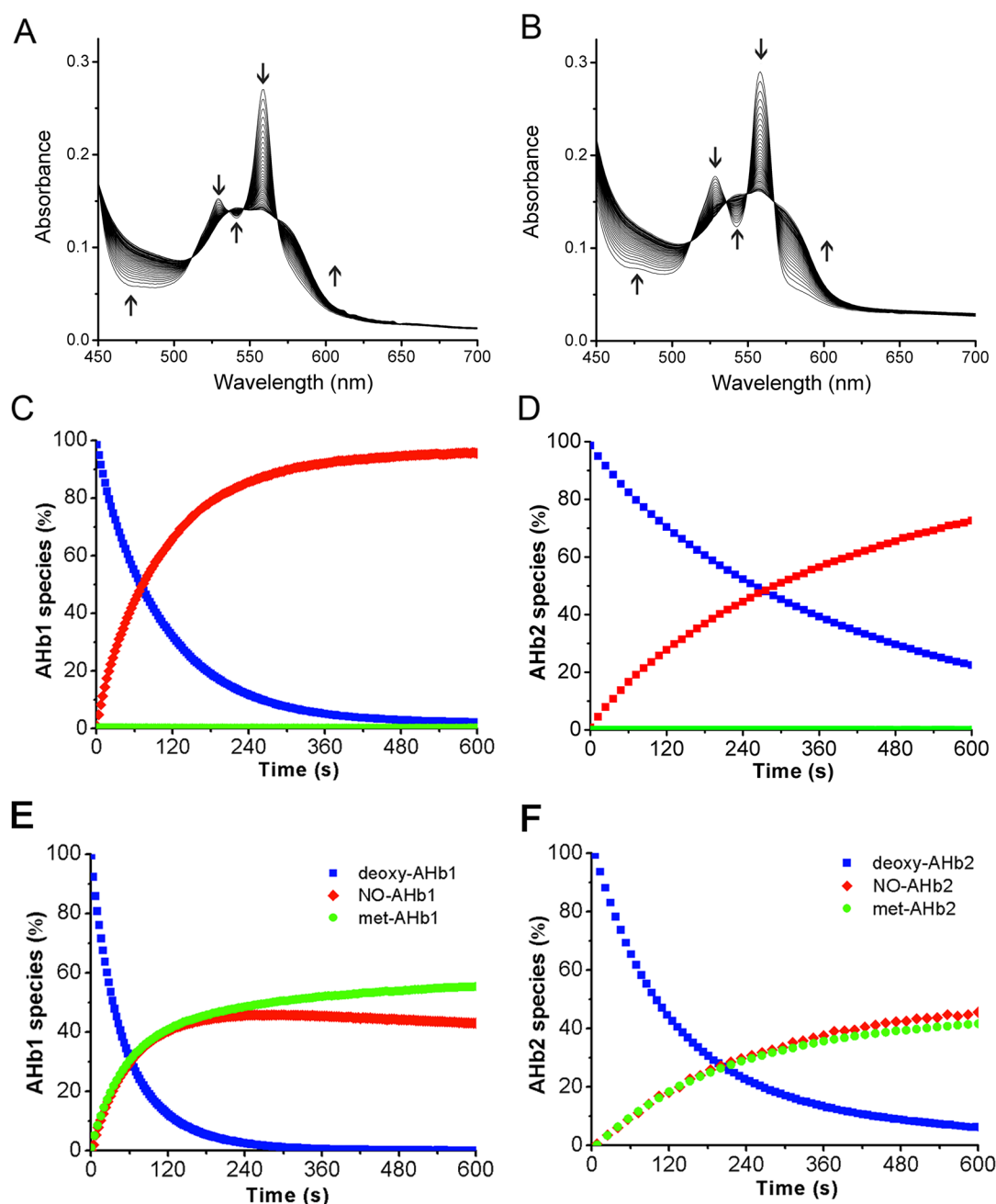


Figure 1. Anaerobic reaction of deoxygenated AHb1 and AHb2 with nitrite in the presence and absence of dithionite. (A and B) Visible spectra of the reaction between 20 μ M deoxy-AHb1 or -AHb2 and 250 μ M nitrite in the presence of 3 mM dithionite recorded at 5 and 20 s intervals, respectively. (C and D) Changes in concentration (in percentage) of deoxy (blue)-, met (green)-, and iron-nitrosyl (red)-AHb1 and AHb2 species vs time. (E and F) Anaerobic reaction as in panels C and D in the absence of dithionite. All measurements were taken in 100 mM phosphate buffer at 25 $^{\circ}$ C as described in Materials and Methods.

We prepared ferrous deoxygenated AHb1 and AHb2 in an anaerobic glovebox as detailed in Materials and Methods and performed anaerobic experiments in the presence and absence of 3 mM sodium dithionite. We recorded the visible spectra of the reaction between the proteins and deoxygenated nitrite at 25 $^{\circ}$ C in a spectrophotometer at constant intervals in a sealed airtight cuvette as exemplified in Figure 1A,B with 0.25 mM nitrite at pH 7.4 in the presence of excess dithionite. Upon addition of nitrite, the spectrum shifts from ferrous heme species to ferrous-nitrosyl species with clear isosbestic points. The traces extracted at 558 and 576 nm were fit to a single-exponential equation, and the concentrations of single species

of AHb1 and AHb2 as a function of time were calculated by least-squares deconvolution of the reaction spectra. The reaction in the presence of dithionite resulted in the full conversion of deoxygenated AHb1 and AHb2 to the respective iron-nitrosyl-heme species (Figure 1C,D), while in the absence of dithionite, we observed that two molecules of the deoxy protein form approximately one iron-nitrosyl and one ferric species both for AHb1 and for AHb2 (Figure 1E,F). This stoichiometry is consistent with previous results obtained for the anaerobic reaction of Hb, Mb, and Ngb with nitrite. The presence of dithionite in the reaction mixture helps to prevent the formation of oxygenated nsHb species and, at the same

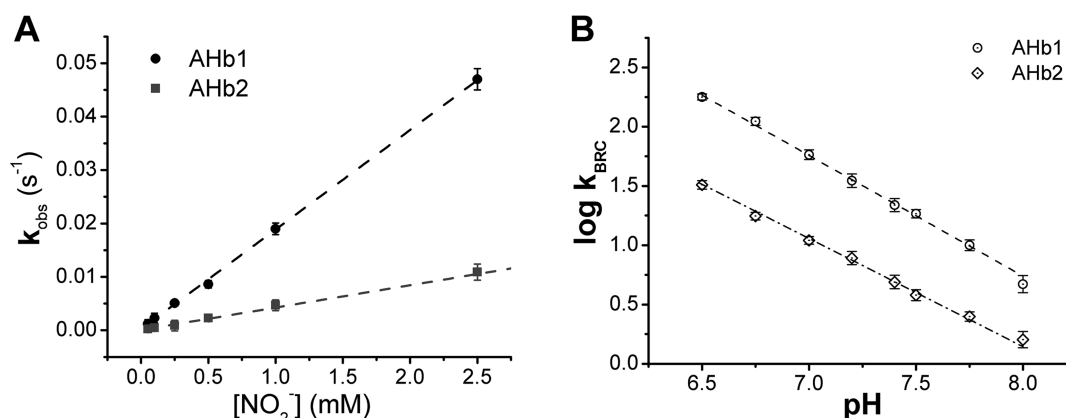


Figure 2. Kinetics of the reaction of nitrite with *A. thaliana* nsHbs. (A) Plot of observed rate constants (k_{obs}) obtained at pH 7.5 and 25 °C vs nitrite concentration. The second-order bimolecular rate constants obtained from the linear fit of the data are 18.6 ± 1.1 and $3.9 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$. (B) Effect of pH on the nitrite reductase reaction rates. The bimolecular rate constant (k_{BRC}) is linear with proton concentration (lines show linear regression analysis of the data).

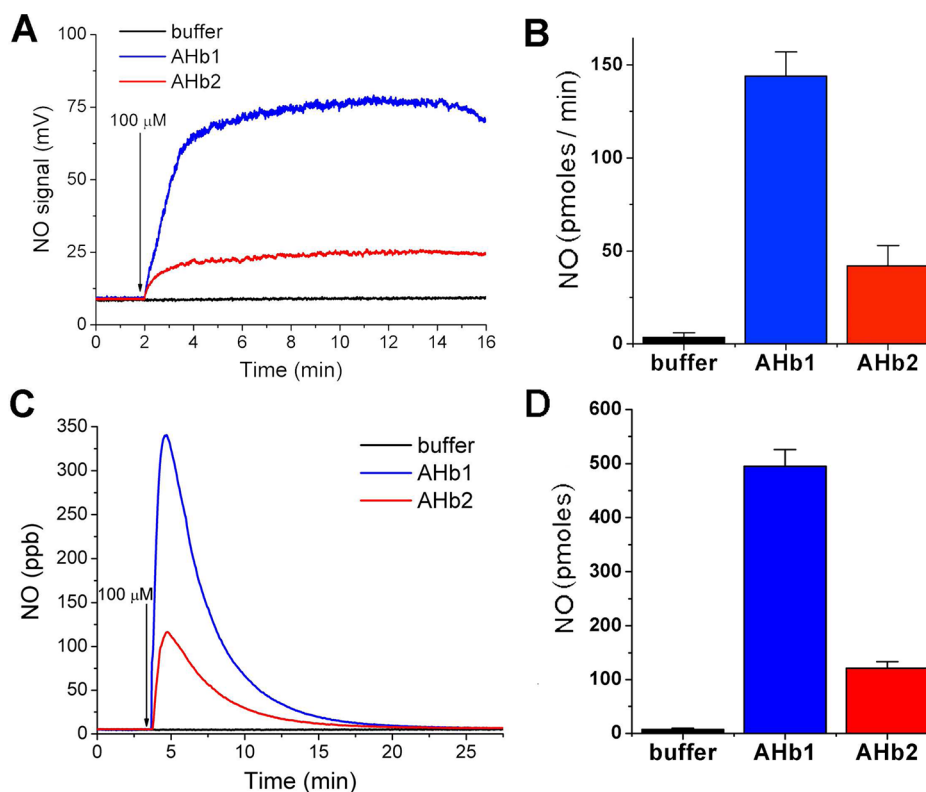


Figure 3. Reduction of nitrite by deoxy-AHb1 and -AHb2 generates NO gas during anoxia and hypoxia (2% oxygen). Chemiluminescence detection of NO gas emission during the anaerobic (A) and hypoxic (2% O_2) (C) reaction of nitrite with buffer alone (black), 25 μM deoxy-AHb1 (blue), or 25 μM deoxy-AHb2 (red). Arrows indicate the addition of nitrite in the specified amounts. Traces are representative of three or more separate experiments. (B and D) Quantification of the amount of NO detected for the reaction with 100 μM nitrite.

time, quickly reduce the resulting ferric AHb1 and AHb2 (eq 1) to the deoxy species. We performed reactions at different dithionite concentrations (1–5 mM) to verify that the dithionite reduction of ferric to ferrous heme is not the driving force of the reaction. We also had previously verified that under these conditions dithionite does not effectively reduce nitrite to NO ;³² thus, the observed formation of iron–nitrosyl Hbs results from the deoxy-Hb-mediated reduction of nitrite.

We then performed the reactions with nitrite in the concentration range of 0.05–2.5 mM at pH 7.5 and 25 °C in the presence of excess dithionite and found that the observed

rate constants depended linearly on the nitrite concentration increase (Figure 2A). Under the pseudo-first-order conditions of the assay, the observed rate constant corresponds to $k_{\text{obs}} = k_{\text{BRC}}[\text{NO}_2^-]$, where k_{BRC} is the bimolecular rate constant of the reaction between the ferrous protein and nitrite. The values of k_{BRC} obtained from the linear fit for reactions at pH 7.5 and 25 °C are 18.6 ± 1.1 and $3.9 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$ for AHb1 and AHb2, respectively.

Proton Dependence of the Nitrite Reductase Reaction with Plant Hemoglobins. The reduction of nitrite by AHb1 and AHb2 requires a proton to form the reactive nitrous acid

species according to eq 1, and therefore, increasing the concentration of protons will accelerate the nitrite reductase rate by 10-fold for each pH unit decrease. We determined the pH dependence of the observed bimolecular rate constants of the nitrite reductase reaction for AHb1 and AHb2 in the pH range of 6.5–8.0. (Figure 2B). The slopes of the linear fittings of the log of the bimolecular rate constant k_{BRC} versus pH are 1.04 ± 0.07 and 0.93 ± 0.08 for AHb1 and AHb2, respectively. These values are in agreement with the value of 1 as expected according to the reaction in eq 1. We conclude that deoxygenated plant nsHbs classes 1 and 2 reduce nitrite via an electron and proton transfer to form NO, a process analogous to the mechanism proposed for neuroglobin and other mammalian hemoglobins.^{17,33}

Reduction of Nitrite by Plant Hemoglobins Generates NO in the Gas Phase under Anaerobic and Hypoxic Conditions. Although under our in vitro conditions deoxygenated AHbs can recapture NO, we explored whether NO gas can escape ferrous heme binding at measurable rates. We used chemiluminescence to monitor NO release during the reaction of deoxy-AHb1 and -AHb2 (25 μM) and nitrite (up to 100 μM) under anoxic and hypoxic conditions. In Figure 3A, we report the detection of NO gas liberated during the anaerobic reaction in a vessel purged with helium: after injection of nitrite into the mixture, the NO level increased until it reached a plateau, and then it was stable for several minutes before decreasing (not shown). Figure 3B shows that the average rate of NO generation calculated during the plateau segment (in picomoles per minute) for AHb1 is ~ 3.5 -fold higher than the value obtained for AHb2. This result is comparable to the ratio of the relative rates of nitrite reduction obtained by spectroscopy; however, as expected, only a small but significant amount of the NO generated during the reaction was detected as gas phase NO.

Next we measured NO emission in a more physiological hypoxic state: analogous reactions were performed in a flask vented with a 2% O_2 /98% N_2 mixture (PaO_2 of approximately 14 mmHg) without bubbling and in the absence of dithionite. This method allows for the detection of the NO gas released in the headspace, which is a small fraction of the NO produced. Under these conditions, the addition of deoxy-AHb1 and -AHb2 to a buffered solution of 100 μM nitrite (or vice versa) generates in both cases an initial large increase in the level of NO that slowly decreases until it returns to the baseline (Figure 3C). Like the ratio obtained under anoxic conditions, the total amount of NO measured from the nitrite reduction of AHb1 was ~ 4 -fold larger than the amount for AHb2 (Figure 3D).

DISCUSSION

The primary finding of our study is that nonsymbiotic Hbs classes 1 and 2 from *A. thaliana* are capable of reducing nitrite to NO and the reaction rates increase linearly as the H^+ concentration increases. This reactivity is similar to that of nitrite with Hb, Mb, and Ngb^{17,33} and the bacterial nitrite reductases³⁴ in which a coupled electron and proton transfer to nitrite generates NO. A recent report by Sturms et al.²⁹ showed that hemoglobin from cyanobacterium *Synechocystis* and class 1 rice nonsymbiotic Hb are also able to convert nitrite to NO under anoxic conditions, and our work extends their findings. In an anaerobic environment, deoxygenated nsHbs reduce nitrite to NO according to eq 1 as first proposed for hemoglobin by Doyle et al.³⁵ Although the results we have obtained do not directly reveal the chemical nature of the

electron transfer process, the formation of either *N*-nitro- or *O*-nitrito heme iron-bound nitrite or alternatively the direct binding of nitrous acid to five-coordinate heme iron, as previously proposed for Ngb,¹⁷ offers an attractive pathway for the formation of end products ferric heme and NO. The NO generated has a very high affinity ($k_{\text{on}} = 10^8 \text{ M}^{-1} \text{ s}^{-1}$) for the ferrous heme, thus yielding iron–nitrosyl–heme [$\text{Fe(II)}\text{--NO}$] as a final reaction product (eq 2). The overall stoichiometry is two molecules of deoxygenated protein form one iron–nitrosyl and one ferric species. This ratio is confirmed for AHb1 and AHb2 by the spectral deconvolution results shown in panels E and F, respectively, of Figure 1 (despite AHb1 showing a late conversion of iron–nitrosyl to the ferric species). In the presence of dithionite, however, the ferric heme species formed by oxidation of the deoxygenated Hbs is reduced back to the ferrous form that reacts again according to eq 1. Thus, the stoichiometry is now one molecule of deoxy protein forming one molecule of $\text{Fe(II)}\text{--NO}$ Hb, and the latter nitrosyl species is produced at a rate that is the same as the deoxy species consumption rate. In Table 1, we compare the rate constants

Table 1. Nitrite Reductase Rate Constants of Nonsymbiotic Hemoglobins from *A. thaliana* and Related Heme–Globin Proteins

protein	nitrite reductase rate constants ($\text{M}^{-1} \text{ s}^{-1}$)	
	pH 7.4	pH 7.0
AHb1	19.8 ± 3.2^a	$\sim 58^a$
AHb2	4.9 ± 0.2^a	$\sim 11^a$
rice nsHb1	ND ^e	$\sim 83^d$
SynHb	ND ^e	$\sim 68^d$
human Hb (T-state)	$\sim 0.12^b$	ND ^e
human Hb (R-state)	$\sim 6^b$	ND ^e
sperm whale Mb	5.6 ± 0.6^c	$\sim 11^d$
human Ngb	0.12 ± 0.02^c	$\sim 0.25^c$
human Ngb, H64L	259 ± 8^c	$\sim 956^c$

^aThe k_{BRC} values were determined at 25 °C, in 100 mM sodium phosphate buffer in the presence or absence of dithionite as described in Materials and Methods. ^bValues from ref 15, in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C. ^cValues from ref 17. ^dValues from ref 29. ^eNot determined.

we obtained for AHb1 and AHb2 to those of other mammalian globins at pH 7.4 and the values reported for rice nsHb1 and cyanobacterium *Synechocystis* Hb (synHb) at pH 7.0. AHb1 has a rate constant approximately 4–5-fold higher than that of AHb2 and comparable to the values reported by Sturms et al.²⁹ for rice Hb1 and synHb when compared at the same proton concentration. However, the rate constants of both AHb1 and AHb2 are 1 order of magnitude higher than the values reported for wild-type Ngb, which also presents a hexacoordinate heme. We have shown in human Ngb that mutation of the distal histidine with either a Leu (H64L) or a Gln (H64Q) residue locks the heme iron center in the pentacoordination state and results in an ~ 2000 -fold increase in the rate of the nitrite reductase reaction.¹⁷ The distal HisE7 side chain position of AHb1 and AHb2 has been reported to modulate the equilibrium between penta- and hexacoordinate species and the differences in the binding affinity of external ligands for their heme iron.^{31,36} Therefore, the faster nitrite reductase rate of AHb1 versus that of AHb2 could be associated with the substantial fraction of AHb1 molecules in pentacoordinate heme geometry (approximately 40%), and the distal HisE7

residue position might play a major role in regulating the nitrite reductase activity of AHb1 and AHb2.

Functional and Biological Implications. NO signaling in plants involves various second messenger molecules such as cGMP, cADP ribose, and Ca^{2+} and modulates several physiological functions during the entire life of the plant. NO can have a protective or toxic effect on cells, depending on its concentration. It can alter the expression of specific genes and plays a key role in metabolism, defense, root development, and cell elongation.³⁸ Perazzolli et al.²⁶ showed that oxygen-bound AHb1 plays a role in NO detoxification by catalyzing the NADH-dependent oxidation of NO back to nitrate via the NO dioxygenase reaction. However, AHb1 expression is strongly induced in roots under conditions of hypoxia³⁹ and is required for the survival of plants after a severe hypoxic challenge,⁴⁰ suggesting that oxygenated AHb1 might not be the species exerting a function related to hypoxic stress. Nitrite levels in plants vary according to the kind of plant, the tissue localization, and the nitrogen content of the soil (nitrate and nitrite levels may change enormously because of agricultural fertilization); however, nitrite concentrations are kept in the high micromolar range by nitrate reductase enzyme activity. In particular, the exposure to nitrate or nitrite induces AHb1 expression.^{41,42} Our results show that under hypoxic and anoxic conditions AHb1 and AHb2 are able to produce NO by nitrite reduction (Figure 3). This indicates that under conditions of poor soil oxygenation and especially for species submerged in water the pH-dependent reduction of nitrite by deoxygenated nsHbs is a viable pathway for hypoxic/anoxic NO generation. Under our in vitro conditions, the resulting formation of ferric (eq 1) and nitrosyl-bound Hbs (eq 2) limits the NO turnover to the initial concentration of deoxygenated nsHbs; however, in vivo ferric nsHbs can be directly reduced by NADPH,²⁶ by a mixture of NADH and FAD, as for alfalfa nsHb,⁴³ or by a methemoglobin reductase, as for barley nsHb.⁴⁴ In mammalian cells, deoxy-hemeprotein-related NO production was observed to inhibit mitochondrial respiration (at the level of complex I and complex IV) and prevent electron leakage with subsequent formation of superoxide and peroxynitrite at the site of complex I and complex III.^{16,45} An analogous mechanism might serve to prevent the formation of reacting oxygen and nitrogen species during plant hypoxia. As suggested for other hemoglobins, it is feasible that via the nitrite reductase and NO dioxygenase reactions nsHbs control NO levels serving both as a NO scavenger and as a generator, indirectly regulating the multiple effects of NO on plant physiology based on ambient oxygen tension. The few in vivo studies on AHb1 and AHb2 cellular interactions and regulation of plant metabolism are insufficient to form a clear picture of their physiological role; further studies using transgenic plants will be helpful in elucidating the cellular function of these proteins.

Hypothesis on Primordial Hemoglobin Function. Phylogenetic analyses suggest that plant nsHbs precede evolution of more specialized symbiotic leghemoglobin^{46,47} and that a common ancient evolutionary origin of all hemoglobins can be traced to a prokaryotic era.⁴⁸ In addition, the discovery of “protoglobins” in strictly anaerobic Archaea⁴⁹ suggests that the last common globin ancestor might have arisen before oxygenic photosynthesis. In the Earth’s preaerobic atmosphere (during the first 2 billion years of its existence), nitrates and nitrites were successfully employed as electron acceptors in bacterial and plant respiration,⁵⁰ and oxygen was indeed a toxic compound for cells. The evolution of denitrification processes

and nitrite reductase enzymes may have constituted the earliest pathway for NO formation and signaling predating the evolution of NOS enzymes. On the basis of these considerations, we hypothesize that the nitrite reductase reactions of heme-containing globins, such as Hb, Mb, Ngb, and nsHbs, represent conserved biochemical processes from a preaerobic Earth and could represent a primordial form of “NO synthases” retained by modern hemoglobins during hypoxia and anoxia.

■ ASSOCIATED CONTENT

📄 Supporting Information

Supplemental Figure 1: Visible standard reference spectra of AHb1 and AHb2 proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*M.T.G.: telephone, (412) 692-2210; fax, (412) 692-2260; e-mail, gladwinmt@upmc.edu. M.T.: telephone, (301) 443-4317; e-mail, tisom@niddk.nih.gov.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

AHb1, class 1 nonsymbiotic hemoglobin; AHb2, class 2 nonsymbiotic hemoglobin; Hb, hemoglobin; Mb, myoglobin; Ngb, neuroglobin; wt, wild-type.

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