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Review
Correlations Between Oxygen Affinity and Sequence Classifications of Plant Hemoglobins

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ABSTRACT:

Plants express three phylogenetic classes of hemoglobins (Hb) based on sequence analyses. Class 1 and 2 Hbs are full-length globins with the classical eight helix Mb-like fold, whereas Class 3 plant Hbs resemble the truncated globins found in bacteria. With the exception of the specialized leghemoglobins, the physiological functions of these plant hemoglobins remain unknown. We have reviewed and, in some cases, measured new oxygen binding properties of a large number of Class 1 and 2 plant nonsymbiotic Hbs and leghemoglobins. We found that sequence classification correlates with distinct extents of hexacoordination with the distal histidine and markedly different overall oxygen affinities and association and dissociation rate constants. These results suggest strong selective pressure for the evolution of distinct physiological functions. The leghemoglobins evolved from the Class 2 globins and show no hexacoordination, very high rates of O_2 binding

This article is dedicated in honor of Professor Mazzarella.

($\sim$250 μM^{-1} s^{-1}), moderately high rates of O_2 dissociation ($\sim$5–15 s^{-1}), and high oxygen affinity (K_d or $P_{50} \approx$ 50 nM). These properties both facilitate O_2 diffusion to respiring N_2 fixing bacteria and reduce O_2 tension in the root nodules of legumes. The Class 1 plant Hbs show weak hexacoordination ($K_{HisE7} \approx$ 2), moderate rates of O_2 binding ($\sim$25 μM^{-1} s^{-1}), very small rates of O_2 dissociation ($\sim$0.16 s^{-1}), and remarkably high O_2 affinities ($P_{50} \approx$ 2 nM), suggesting a function involving O_2 and nitric oxide (NO) scavenging. The Class 2 Hbs exhibit strong hexacoordination ($K_{HisE7} \approx$ 100), low rates of O_2 binding ($\sim$1 μM^{-1} s^{-1}), moderately low O_2 dissociation rate constants ($\sim$1 s^{-1}), and moderate, Mb-like O_2 affinities ($P_{50} \approx$ 340 nM), perhaps suggesting a sensing role for sustained low, micromolar levels of oxygen. © 2009 Wiley Periodicals, Inc. Biopolymers 91: 1083–1096, 2009.

INTRODUCTION

The Hb family of proteins is defined by a characteristic α-helical "globin" polypeptide fold with a bound heme prosthetic group. The most familiar globins are the "pentacoordinate" mammalian Hbs and Mbs, which give blood and striated muscle their distinctive red color. Their abundance in these tissues provided early biochemists a readily accessible and easily isolated protein for detailed biochemical and structural study. These properties have placed Hbs and Mbs at the forefront of structural and biophysical science for more than 60 years.[1,2] Characterization of the animal respiratory Hbs revealed kinetic and affinity constants well adapted for the oxygen transport and storage functions of these pentacoordinate Hbs, which have served, until recently, as a paradigm for the globin family of proteins. During the past decade, however, 100s of new globin sequences have been discovered, and these genes have been found in nearly every living organism, with functions ranging from catalytic detoxification of NO and O_2 to nematode phototaxis, as well as O_2 transport and storage.[3–5] The functions of many of these novel Hbs remain unknown, but the results of initial biophysical studies, coupled with their low intercellular concentrations, suggest strongly that most do not function in oxygen transport.

Hbs have been known to exist in plants for over 60 years. The first plant globins characterized were the leghemoglobins (Lbs), which are expressed at high (millimolar) concentrations in the root nodules of legumes. The plant-derived Lbs are a vital component of the bacterial symbiotic nitrogen fixation machinery.[6] In addition to the leghemoglobins, plants are now known to contain three other classes of Hbs, which have been called "nonsymbiotic" Hbs (nsHbs) because they are not associated with N_2 fixing bacteria (Figure 1). Whereas leghemoglobin structure and function have been examined for over six decades, the functions and mechanisms of action of the more recently discovered nsHbs are still in question. Studies of these plant proteins are rapidly expanding and indicate that the three major phylogenetically distinct nsHb genes (Classes 1, 2, and 3 in Figure 1) are individually regulated and almost certainly have distinct functions.[7–13]

Leghemoglobins

Leghemoglobins (Lbs) are monomeric oxygen transport proteins with tertiary structures that resemble Mb and the α and β subunits of adult human Hbs. The globins found inside root nodules are often a mixture of leghemoglobin "isotypes" resulting from multiple genes and post-translational modification. In soybeans, nodules contain Lba, Lbc1, Lbc2, and Lbc3, and an assortment of other electrophoretic components (Lbb, Lbd) resulting from post-translational modification of the Lba and Lbc gene products.[15] The relative concentrations of these proteins vary with the developmental stage of the nodule. Younger nodules have larger concentrations of Lbcs, and mature nodules favor Lba. Methodical analysis of O_2 and CO binding kinetics reveals little difference between the kinetic or equilibria of oxygen binding to these proteins (Table III),[16] and there is currently no clear explanation for the purpose of multiple Lb isotypes in root nodules.

Lbs must facilitate transport of oxygen across root nodules, and do so while maintaining a very low ($\leq$10 nM) concentration of free oxygen to prevent inhibition of the rhizobial nitrogenase complex.[17] The need to function at low O_2 requires a higher association equilibrium constant for oxygen binding (23 μM^{-1}) than that of muscle Mbs (Table I). This increase in affinity in soybean Lba (GLYmaGLB2Sa) was achieved by raising the association rate constant to $\sim$200 μM^{-1} s^{-1} (which approaches the diffusion limit of $\sim$1000 μM^{-1} s^{-1}) with little change in the dissociation rate
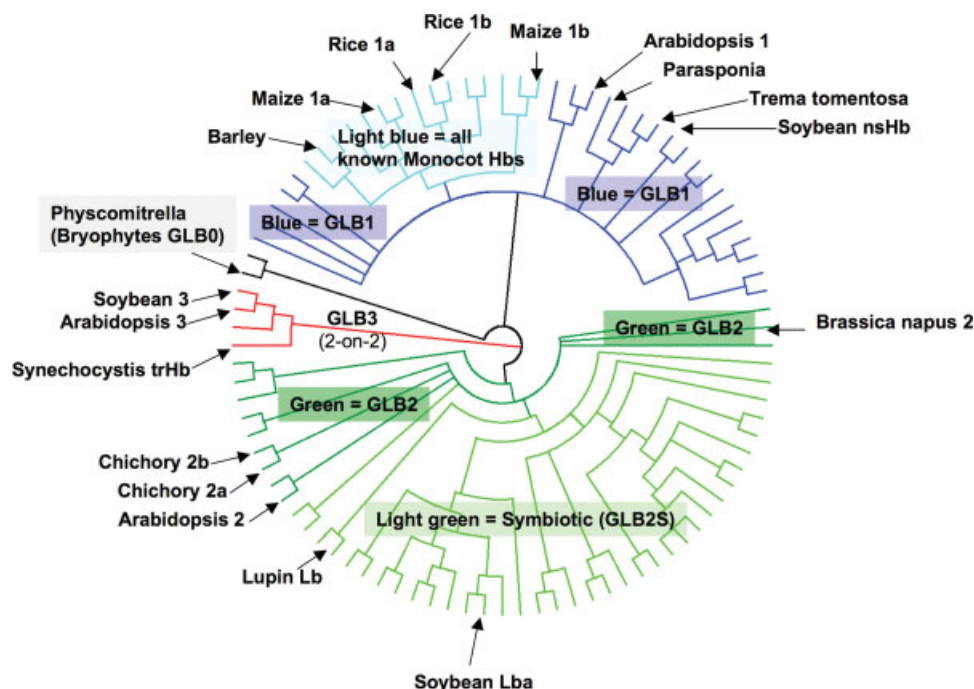
FIGURE 1 **Circular cladogram of plant hemoglobins.** Plant hemoglobin protein sequences group generally into three classes or clades and perhaps a fourth, if the Bryophyte globins are considered GLB0. The cladogram was derived from a 90% consensus tree rooted with GLB3[14]; relationships amongst major 3-on-3 globins GLB0, GLB1, and GLB2 are unresolved; and branch lengths are not to scale. Class 1 Hbs are found in all plants, with multiple representations in monocots. In a seemingly unique event in the dicot Parasponia, a Class1 Hb has given rise to a symbiotic hemoglobin. Class 2 Hbs are found only in dicots and gave rise to the leghemoglobins (Lb) in legumes and some other nodulating species. Class 3 Hbs are found in many plants and have homology to the 2-on-2 (truncated) bacterial hemoglobins.[14]

constant. Thus, Lba maintains high rates of O_2 binding and release for efficient transport. Other Lbs from kidney bean (PHAvuGB2S), cowpea (VIGunGLB2S), lupin (LUPluGLB2S), green pea (PISsaGB2S), and broad bean (VICfaGB2S)) also have oxygen dissociation rate constants similar to Mb (15 s^{-1}), and rapid association rate constants,

some greater than 200 μM^{-1} s^{-1} (Tables I, III, and IV).[18] These adjustments in O_2 binding parameters can be understood by the primary and tertiary structural differences between Mb and Lba.

In Mb, Ser$^{(F7)}$ is a key amino acid adjacent to the proximal His$^{(F8)}$, which coordinates the heme group. The hydroxy

Table I **Effects of Replacing the Distal His$^{(E7)}$ with Leu in Mb and Plant Hbs. All Rate and Equilibrium Constants Were Measured at pH 7.0, 20°C**

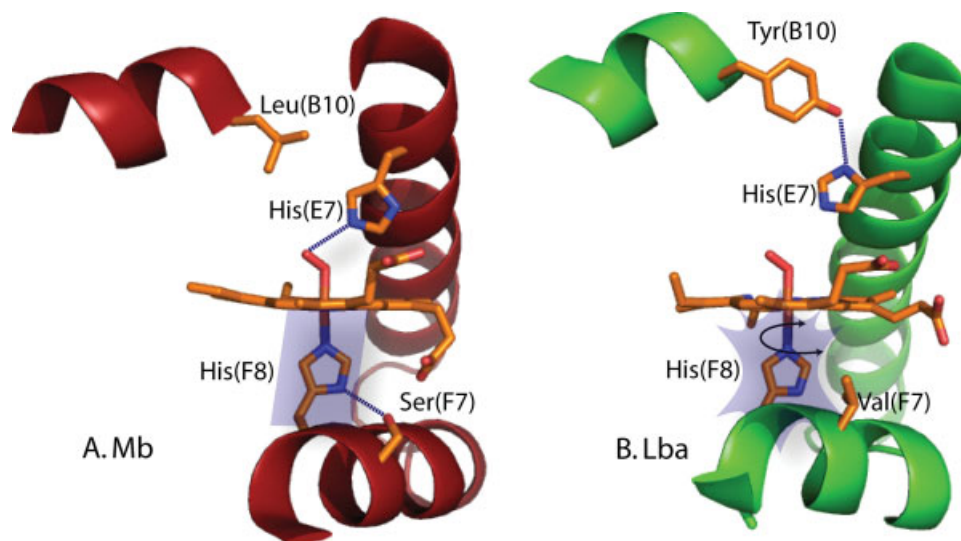| Hemoglobin	$k'_{O2,pent}$ ($\mu M^{-1}s^{-1}$)	k_{O2} (s^{-1})	$K_{O2,pent}$ (μM^{-1})	K_H	$k'_{O2}/(1+K_H)$ ($\mu M^{-1}s^{-1}$)	$K_{O2,pent}/(1+K_H)$ (μM^{-1})	Reference
SW Mb	17	15	1.1	0	17	1.1	19
SW Mb H64L	94	4,100	0.023	0	94	0.023	19
Soybean Lba	130	5.6	23	0	130	23	20
Soybean Lba H61L	400	24	17	0	400	17	20
Rice GLB1	60	0.038	1600	2	21	540	9
Rice GLB1 H73L	620	51	12	0	620	12	9
ARath GLB1	74	0.12	620	2.1	24	200	10, 21
ARath GLB1 H66L	270	94	2.9	0	270	2.9	21
ARath GLB2	86	0.14	610	67	1.3	9.0	10
ARath GLB2 H69L	550	23	24	0	550	24	21

FIGURE 2   Oxygen stabilization in Mb and Lba. A) The proximal His[(F8)] in Mb is constrained by a hydrogen bond with the side chain of Ser[(F7)] that prevents it from rotating into a trans-stabilizing conformation. The distal His[(E7)] stabilizes bound oxygen by donating a hydrogen bond to the electronegative terminal oxygen atom. B) His[(F8)] in Lba is under no such restriction, and rotates into an orientation favoring trans-stabilization of oxygen through the heme plane. A hydrogen bond with Tyr[(B10)] prevents over-stabilization of bound oxygen by His[(E7)].

methyl side chain hydrogen bonds to the Nε atom of His[(F8)] fixing the imidazole ring in a conformation that is eclipsed with the pyrrole N atoms of the porphyrin ring and inhibits in-plane movement of the iron atom and therefore ligand binding (Figure 2A).[22] To compensate for this inhibitory proximal conformation, a strong hydrogen bond is donated from His[(E7)] to stabilize the bound oxygen molecule to achieve a P_{50} of ~ 1 μM for Mb. Replacement of the distal His[(E7)] in Mb with apolar amino acids causes the rate of O_2 dissociation to increase from 15 s^{-1} to $\geq \sim 5,000$ s^{-1}, and the observed P_{50} increases from 1 to ~ 100 μM (Table I).[23–25]

In the primary structure of Lba, Val[(F7)] is found in place of Ser[(F7)].[26] Without a hydrogen bond fixing the orientation of the His[(F8)] side chain, the imidazole ring is free to occupy a staggered conformation between the pyrrole N atoms and can readily move upward toward the heme plane, greatly increasing the reactivity of the iron atom and oxygen affinity. In Lba, the distal His[(E7)] side chain is rotated away from the bound ligand by formation of a hydrogen bond with Tyr[(B10)] (Figure 2B).[27] This conformation reduces electrostatic stabilization of bound ligands as can be seen by reduced effect of the His[(E7)] to Leu mutation in soybean Lba compared with that observed for Mb and the nsHbs in Table I.

Nonsymbiotic Hbs

For many decades scientists thought that Hbs in plants were only found in legumes. A directed effort to find Hbs in other plants led to the discovery of full-length symbiotic Hbs in nodules of nonlegume plants[28] and also nonsymbiotic Hbs in non-nodulating plants.[28] The development of molecular biology techniques and genetic sequencing along with the sequence of nonlegume Hb eventually led to the identification of Hbs genes in grasses and our current perception that globins are found in all plants.[11] An additional class of Hbs with homology to bacterial "truncated" Hbs was found in *Arabidopsis thaliana* a few years later.[14] Plant Hb sequences fall into three general classes (Figure 1). However, not all plants have members of each class. All appear to have both Class 1 and Class 3 nsHbs.[12,29] Monocots lack Class 2 nsHbs, but generally have two or more members of the Class 1 nsHbs family.[30] Dicots generally have one Class 1 nsHb and one Class 2 Hb, unless (as in the case of legumes and some other nodulating dicots) the Class 2 Hb has evolved into a leghemoglobin,[17] or in the nonlegume Parasponia, a Class 1 nsHb has evolved into a globin with leghemoglobin-like functional and structural properties.[31]

Class 1 Nonsymbiotic Hbs

The physiological function of Class 1 nsHb has been investigated by immunolocalization, biochemical, and reverse genetics methods. Stress-inducible (including hypoxia) expression of these proteins occurs in many tissues ranging from the aleurone and developing embryos to a variety of vegetative tissues.[10,11,32] An intriguing function of Class 1

nsHbs during hypoxia has been proposed by Robert Hill and coworkers.[33,34] They have suggested that Class 1 nsHbs are responsible for maintaining the redox and energy status of plant cells during fermentative metabolism (which occurs during hypoxia). Under these conditions NADH and NO build up, and Class 1 nsHbs could scavenge oxygen and use it to oxidize NO to NO_3^-, with the concomitant formation of ferric nsHb. Ferric nsHb could then be rereduced by NADH, thereby replenishing the pool of NAD^+ for continued fermentation.

The arguments in favor of this function include increased survival[7,33] and increased ATP levels in hypoxic cell lines overexpressing nsHb1.[35,36] Cells with decreased nsHb1 levels also have elevated NO concentrations.[37] Consistent with this function, oxy-nsHb1 reacts with NO to yield nitrate and ferric nsHb1,[37,38] and NADH-driven reduction can be facilitated with oxido-reductase enzymes (like monodehydroascorbate reductase) found in plants.[34] This chemical reaction, NO dioxygenation, is common to many globins including Mbs, red blood cell Hbs, and microbial flavohemoglobins. Thus, the argument of a unique NO scavenging role for Class 1 nsHbs requires further proof and identification of a specific cognate reductase[38] as has been obtained in structural, kinetic, and gene knock out studies of bacterial and yeast flavohemoglobins, which contain a built-in reductase.[39,40]

Biophysical interest in Class 1 (and Class 2) nsHbs has resulted from their novel mechanism for ligand binding. When oxygen binds to traditional Hbs such as myoglobin, leghemoglobin, and animal red blood cell Hbs, it binds rapidly to a five-coordinate ferrous heme iron.[24,41] In contrast to these "traditional" pentacoordinate Hbs with their open binding sites for exogenous ligands, the distal histidines in plant nsHbs are bound to the iron atom in both the reduced and oxidized states, and the spectra of the unliganded protein resemble those of b-type cytochromes (Figure 3).[42] However, unlike most b-type cytochromes, which are unreactive toward oxygen, hexacoordination in nsHbs is reversible, and exogenous ligands (O_2, NO, and CO) can bind rapidly and with high affinities.[43–45]

Although globin hexacoordination was first observed in plant nsHbs, this property has now been identified in Hbs that are members of the "truncated hemoglobin" family and found in the photosynthetic microorganisms Synechocystis[46–48] and Chlamydomonas.[49] Discovery of hexacoordinate neuroglobin and cytoglobin in humans[50–53] and other animals,[51,54] as well as the characterization of hexacoordination in a Hb from Drosophila,[55] suggests that this feature is common to a diverse group of globins found ubiquitously in nature.[56]

Class 2 Nonsymbiotic Hbs

Class 2 nsHbs are also expressed in a variety of tissues including the developing embryo,[57] and in many vegetative and reproductive tissues.[58,59] Although induction conditions differ somewhat between Class 1 and Class 2 Hbs,[58] overexpression of both increase survival under hypoxic conditions.[60] Although a much smaller number of Class 2 plant globins have been investigated biophysically, it is clear that they are much more strongly hexacoordinated and, as result, have much smaller O_2 affinities than the Class 1 proteins.[61,62] As a potential explanation for the difference in hexacoordination between Class 1 and Class 2 nsHbs, Hargrove and coworkers have shown that the large Phe side chains at the B14, B13 and B10 positions in Rice Glb1a "push" on the coordinating distal histidine, facilitating its rapid dissociation and decreasing its extent of coordination.[63] The structural cause of the larger equilibrium constant for $His^{(E7)}$ coordination in the Class 2 nsHbs could be the lack of large side chains at the B13 and B14 positions, which allows the highly conserved $Phe^{(B10)}$ in the Class 2 proteins to move inward to accommodate the imidazole side chain above the heme iron atom. The net result of stronger hexacoordination is that the Class 2 nsHbs have P_{50} values ($\sim$1 μM) similar to mammalian Mbs, which are roughly 200-fold greater than those for the Class 1 nsHbs and $\sim$10-fold greater than the Lbs (Table III).

Class 3 Nonsymbiotic Hbs

These proteins have sequence homology to bacterial Hbs, which typically have shorter sequences and compact "2-on-2" (helix packing) structures.[64] In spite of this homology and in contrast to the bacterial Hbs, Class 3 plant Hbs have longer polypeptides than the other nsHbs, and it is unclear if the 2-on-2 structural designation will apply to these proteins because no crystal or NMR structures of this class of plant Hbs have been determined. Although these genes are present in most plant genomes, there are only two published reports of the presence of Class 3 nsHbs in root and shoot tissue and their expression is reduced during hypoxia.[14,65] Arabidopsis nsHb3 has very unusual ligand binding kinetics and transient hexacoordination upon reduction of the ferric protein,[14] but these properties have not been characterized well enough to speculate on the physiological function of the Class 3 plant nsHbs.

Hexacoordination and Regulation of Oxygen Binding in Class 1 and Class 2 nsHbs

To classify more quantitatively the differences in hexacoordination and oxygen affinity of Class 1 and Class 2 nsHbs, we characterized the O_2 binding properties of three additional
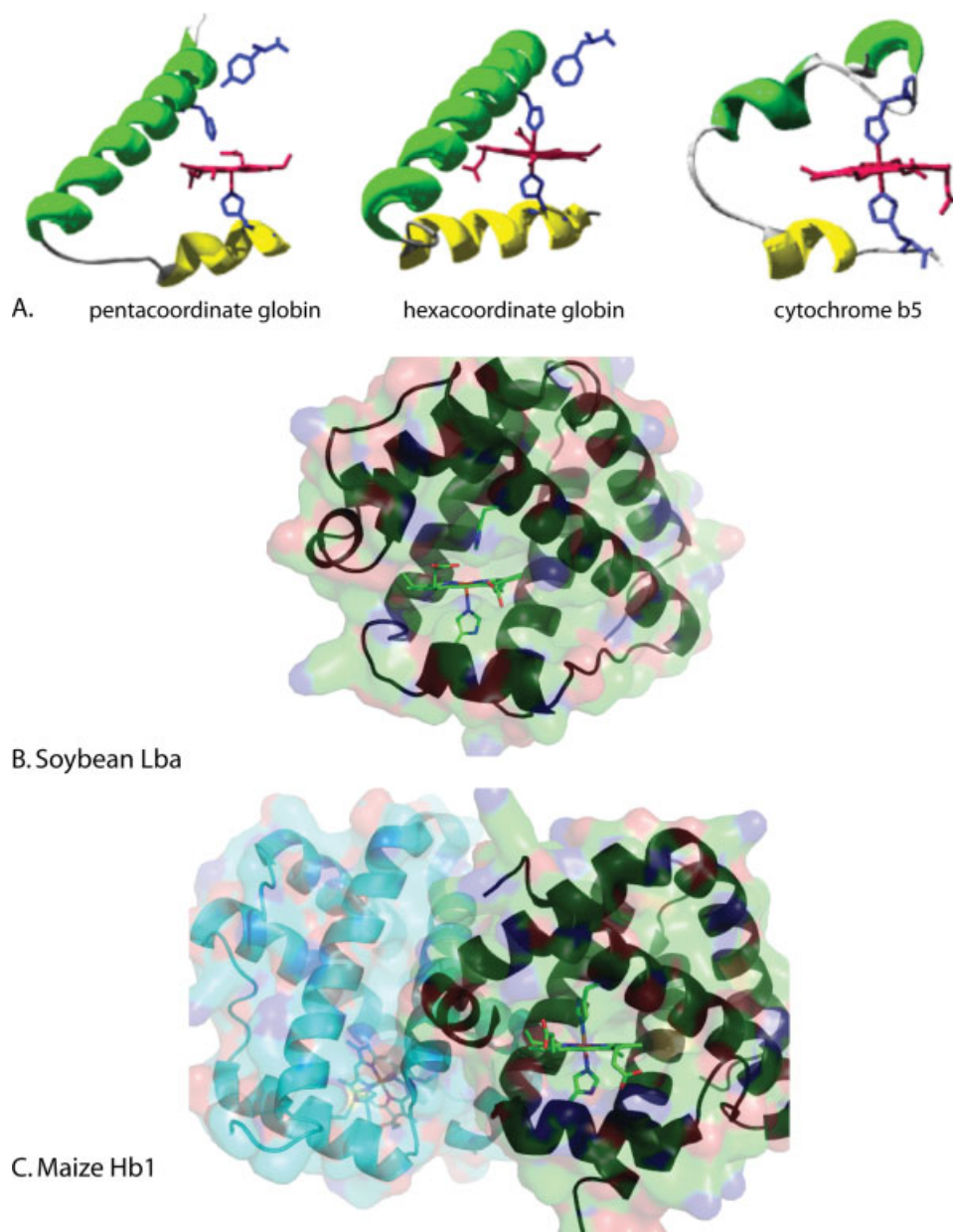
FIGURE 3   The crystal structure of Maize Hb 1 represents Class 1 nsHbs. A) Heme coordination in (from left to right) plant pentacoordinate Hbs (Lba pdb 1BIN), hexacoordinate Hbs (Rice nsHbs 1, pdb 1D8U), and cytochrome b_5 (pdb 1CYO). B) The structure of soybean Lba (pdb 1BIN) characterizes the globin fold observed in Mb, leghemoglobin, and the α and β subunits of adult human Hb. The heme group in these "pentacoordinate" Hbs is held in place by a single covalent bond between His$^{(F8)}$ and the heme iron, and multiple hydrophobic contacts with the heme pocket. C) The unit cell of the maize Hb1 crystal contains a globin dimer. Each globin has a "hexacoordinate" heme group with coordination bonds from both His$^{(F8)}$ and His$^{(E7)}$ (pdb 2r50).

Class 1 nsHbs (two from maize and one from soybean), and three new Class 2 nsHbs (two from chicory and one from tomato), and determined the crystal structure of Maize Hb1 to provide a third direct example of hexacoordination in this class of nsHbs (Figure 3). Measurements of the individual rate constants for hexacoordination were achieved by first measuring the bimolecular rate constant for CO binding to pentacoordinate deoxy-nsHb generated after photolysis and

Table II **Rate and Equilibrium Constants for the Hexacoordination by His$^{(E7)}$ at pH 7, 20°C**

Protein	$k'_{CO,pent}$ ($\mu M^{-1} s^{-1}$)	k_H (s^{-1})	k_{-H} (s^{-1})	K_H	Reference
Class 1 nsHbs					
riceHb1 ORYsaGLB1a	6.8	75	40	1.9	9
riceHb1b[a] (previously riceHb2) ORYsaGLB1b	1.8	6.7	15	0.45	67
Maize 1a ZEAmaGLB1a	1.4	22	25	0.9	67
Maize 1b[a] (previously Maize 2) ZEAmaGLB1b	44	43	19	2.3	67
Barley HORvuGLB1	2 23[1]	170 23[1]	62 14[1]	2.8 1.6[1]	68 [1]62
Arabidopsis 1 ARAthGLB1	0.55[2]	230[2]	110[2]	2.1[2]	[2]This study
Tomato 1 SOLlyGLB1	1[1] 0.8[2]	200[1] 370[2]	200[1] 185[2]	1.0[1] 2.0[2]	[1]69 [2]This study
Soybean 1 GLYmaGLB1	2.7	160	80	2	21
Average	**8.4 ± 14**	**130 ± 120**	**75 ± 70**	**1.7 ± 0.7**	
Class 2 nsHbs					
Arabidopsis 2 ARAthGLB2	77[1] 50[2] 19[3]	330[1] 1600[2] 2000[3]	12[1] 38[2] 30[3]	30[1] 50[2] 67[3]	[1]62 [2]70 [3]21
Chicory 2a CHIinGLB2a	54	2900	11	260	This study
Chicory 2b CHIinGLB2b	10	920	27	35	This study
Tomato 2 SOLlyGLB2	26	1400	30	60	This study
Average	**39 ± 30**	**1500 ± 1700**	**25 ± 27**	**84 ± 93**	

[a] Many grasses, including rice and maize, have more than one class 1 nsHb. Upon their discovery, subsequent class 1 nsHbs were originally numbered (for example "rice Hb2" in spite of the fact that they are not class 2 Hbs by the phylogenetic definition. We have changed these numberings to represent phylogenetic class, and use "a" and "b" to represent different globins within each.

then determining the individual k_H and k_{-H} parameters from the hyperbolic kinetic pattern for the observed rate of CO binding when equilibrium samples of hexacoordinate deoxy-nsHbs are mixed with increasing concentrations of ligand [9,66,45,67] (Table II, Figure 4).

Figures 4A and 4C show the dependences of the pseudo first order ligand rebinding rates on [CO] following flash photolysis. The slopes of these plots yield the bimolecular rate constants for CO rebinding to the pentacoordinate deoxyHb intermediate ($k'_{CO,pent}$). The y-intercepts of these plots also provide an estimate of rates of His$^{(E7)}$ hexacoordination ($k_H + k_{-H}$) but are difficult to define quantitatively.[45] In general, the $k'_{CO,pent}$ values for the Class 2 nsHbs are higher than those for the Class 1 nsHbs, but there are a wide range of values in each class (Table II).

Figures 4B and 4D show the dependence of the observed pseudo first order rate of binding on [CO] which was obtained after rapidly mixing equilibrium deoxy-nsHb samples with ligand in stopped-flow apparatus. These curves, along with $k'_{CO,pent}$ allow determination of k_H, k_{-H}, and K_H because the observed rate, k_{obs}, is equal to[67]:

$$k_{obs} = \frac{k_{-H}[CO]}{\frac{k_H}{k'_{CO,pent}} + [CO]} \tag{1}$$

Table I reports these values for each Class 1 and 2 nsHb that has been studied, including the six new measurements reported here for the first time. While there is variability in the values of k_H and k_{-H} across both classes, the equilibrium constants for hexacoordination, $K_H = k_H/k_{-H}$, are consistently 10–40 times larger in the Class 2 nsHbs than those for the Class 1 proteins.

The O_2 equilibrium association constant in hexacoordinate hemoglobins is governed by the product of the associa-
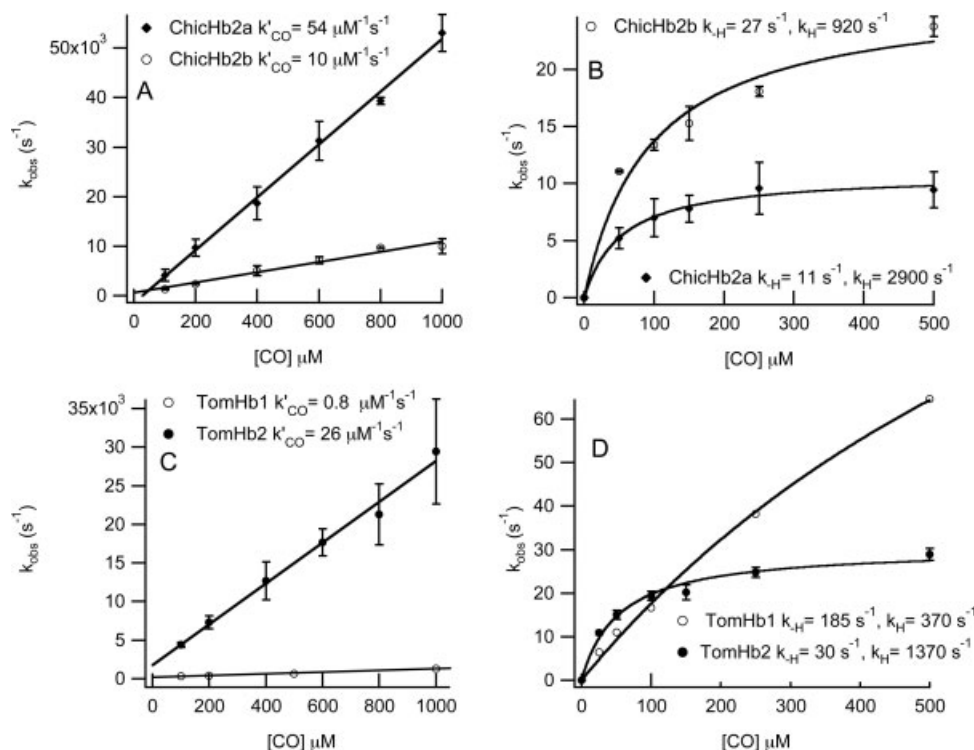
FIGURE 4 Measurement of His$^{(E7)}$ coordination and CO binding in chicory and tomato Hbs. A) Flash photolysis and CO rebinding to chicory Hbs. B) CO binding to chicory Hbs initiated by stopped-flow rapid mixing shows the asymptote characteristic of the rate constants for His$^{(E7)}$ dissociation from the heme iron. C) Flash photolysis and CO rebinding to tomato Hbs. D) CO binding to tomato Hbs initiated by stopped-flow rapid mixing.

tion equilibrium constant for oxygen binding to the penta-coordinate form of the nsHb ($k'_{O2,pent}$) times the fraction of this pentacoordinate form which occurs at equilibrium in solution ($1/(1 + K_H)$ as shown in Eq. 2).

$$K_{O_2} = \frac{K_{O2,pent}}{1 + K_H} \qquad (2)$$

The K_H values were calculated from the hexacoordination rate constants obtained from rapid mixing, CO binding experiments, and the $K_{O2,pent}$ values were calculated from the rate constants for O_2 rebinding to the pentacoordinate intermediate obtained in laser photolysis experiments and the dissociation rate constants determined in rapid mixing ligand displacement experiments.[9,67] In general, Class 1 nsHbs show somewhat higher $K_{O2,pent}$ values than the Class 2 globins. However, the variability is great, ranging from 60–4500 μM^{-1} for the Class 1 nsHbs and 20–600 μM^{-1} for the Class 2 proteins, and similar large variations are seen for the O_2 dissociation rate constants. Neither of these parameters are affected by the marked increase in K_H observed for the Class 2 nsHbs. However, internal hexacoordination does

selectively lower the bimolecular association rate constant, which is given by $k'_{O2,pent}$ times the fraction of pentacoordinate species, $1/(1+K_H)$, found at equilibrium.

When hexacoordination of the equilibrium deoxygenated state is taken into account, there is much larger separation in the O_2 affinities of the two classes of nsHbs (Table III). All of the Class 2 nsHb show overall oxygen equilibrium constants (Equation 2) in the range of 2–9 μM^{-1}, whereas the values for the Class 1 nsHbs are in the range 70–1000 μM^{-1}. Thus, it is clear that strengthening of hexacoordination in the Class 2 hemoglobins causes marked decreases in O_2 affinity and concomitant increases in P_{50} to values close to those observed for mammalian Mbs. These marked differences suggest selective pressure for different physiological functions of the two phylogenetic classes.

The Electrostatic Environment in the Heme Pockets of Class 1 and Class 2 nsHbs

It is clear that hexacoordination is the key factor regulating the differences in O_2 affinity of the Class 1 and Class 2 nsHbs. Electrostatic stabilization of bound O_2 appears to occur in

Table III   Oxygen Binding Parameters for Plant Hemoglobins at pH 7, 20°C

Protein	$k'_{O2, penta}$ ($\mu M^{-1} s^{-1}$)	k_{O2} (s^{-1})	$K_{O2,pent}$ (μM^{-1})	K_H	$K_{O2}/(1+K_H)$ (μM^{-1})	ν_{CO}[a] average (cm^{-1})	Reference
Class 1 nsHbs							
riceHb1 ORYsaGLB1a	60	0.038	1600	1.9	540	1923	63
riceHb1b ORYsaGLB1b	40	0.1	400	0.45	280	1923	63
Maize 1a ZEAmaGLB1a	44	0.054	820	0.9	430	1928	This study
Maize 1b ZEAmaGLB1b	210	0.27	780	2.3	240	1949	This study
Barley HORvuGLB1	50[1]	0.03[2]	1700[1]	2.8[1]	440[1]	1925	[1]68 [2]8
Arabidopsis 1 ARAthGLB1	74[1]	0.12[1]	620[1]	2.1[1] 1.6[2]	100[1]		[1]10 [2]62
Tomato 1 SOLlyGLB1	30[1] 32[2]	0.5[1] 0.16[2]	60[1] 200[2]	1.0[1] 2.0[2]	30[1] 67[2]	1931[2]	[1]69 [2]This study
Soybean 1 GLYmaGLB1	59	0.013	4500	~2	1,500		21
Average	**67 ± 56**	**0.14 ± 0.16**	**1200 ± 1400**	**1.7 ± 0.8**	**410 ± 440**		
Class 2 nsHbs							
Arabidopsis 2 ARAthGLB2	150 86	2 0.14	75 610	50[1] 30[2]* 67[3]	2 9.0		[1]70 [2]62 [3]10
Chicory 2a CHIinGLB2a	50	0.11	460	270	1.7	1958	This study
Chicory 2b CHIinGLB2b	50	2.7	19	35	2.1	1959	This study
Tomato 2 SOLlyGLB2	45	0.4	113	60	1.8	1934	This study
Average	**76 ± 44**	**1.1 ± 1.2**	**260 ± 260**	**96 ± 98**	**2.9 ± 3.5**		
leghemoglobins							
GLYmaGLB2Sa	130	5.6	23		23	1949	71
GLYmaGLB2Sc	124	3.6	35		35		72
PHAvuGLB2S	130	6.2	21		21		18
VIGunGLB2SII	140	5.5	25		25		18
LUPluGLB2SI	540	20	27		27		18
LUPluGLB2SII	320	25	13		13		18
PISsaGLB2SI	250	16	16		16		18
PISsaGLB2SIV	260	16	16		16		18
VICfaGLB2SV	260	19	14		14		18
PARanGLB1S	170	17	10		10		21
Average	**230 ± 130**	**13 ± 8**	**20 ± 8**		**20 ± 8**		

[a] The value for ν_{C-O} was calculated as the absorbance averaged value of the C—O stretching frequency. The FTIR spectra for soybean Lba was taken from Kundu et al.,[27] and the spectra for the other plant Hbs were obtained for this work.

both types of plant Hbs, although less comprehensive data are available. Table I compares the effects of His[(E7)] with Leu mutations in Mb, Lb, rice Glb1, AraTh Glb1, and AraTh Glb2. This mutation causes ∼1000-fold increases in the rate constants for O_2 dissociation (k_{O2}) from the two Class 1 nsHbs, ∼250-fold increases for Mb and AraTh Glb2, and

only a 5-fold increase for soybean Lba. These results imply a very strong hydrogen bond between His[(E7)] in the Class 1 nsHbs, moderately strong and favorable interactions in Class 2 nsHbs, and little stabilization in Lba.

These conclusions are supported by the FTIR spectra of CO complexes of the two classes of nsHbs and Lbs. Olson,
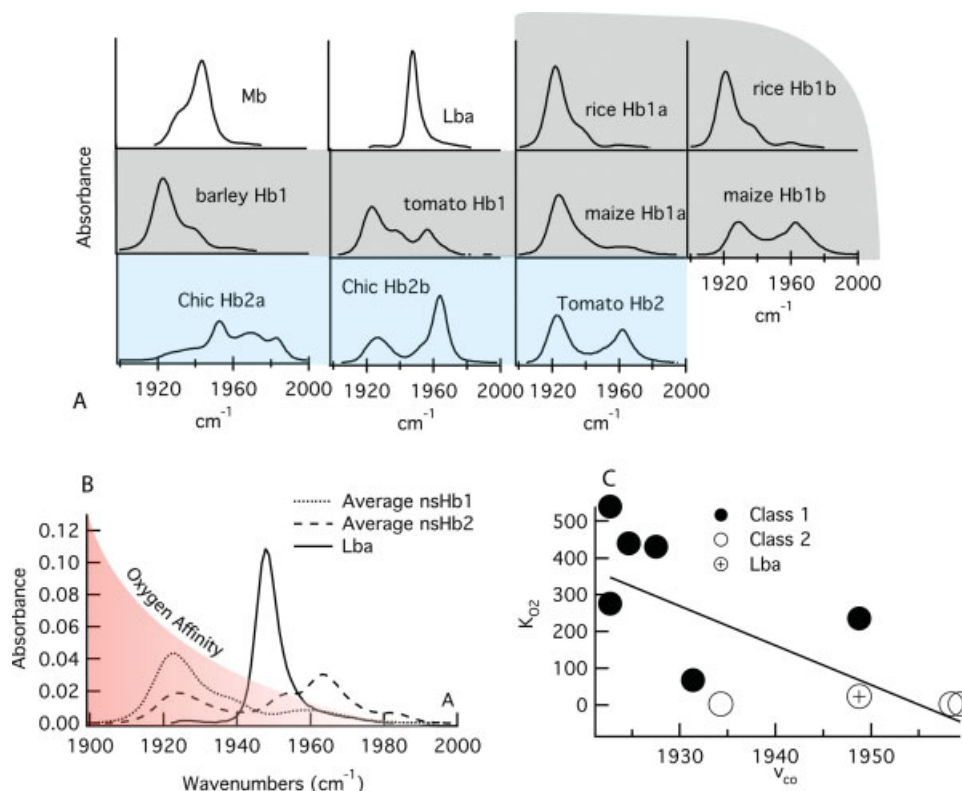
FIGURE 5 CO FTIR data for plant Hbs. A) Individual FTIR spectra for CO bound pentacoordinate (no shading), Class 1 (gray shading), and Class 2 (blue shading) Hbs. B) Averaged spectra for each group, showing the features indicative of higher oxygen affinity (red shading). C) A plot of ν_{CO} against oxygen affinity shows a weak linear correlation ($R^2 = 0.53$), indicating that hydrogen bonding with His$^{(E7)}$ does confer significant stabilization of bound oxygen.

Phillips, and others have shown that the C—O stretching frequency (ν_{C-O}) of MbCO complexes correlates strongly with the electrostatic field near the bound ligand.[25,27,73] More positive fields, indicative of strong to weak hydrogen bonding interactions, give rise to ν_{C-O} peaks in the 1910–1930 and 1940–1950 cm^{-1} regions, respectively. Apolar distal pockets and negative fields give rise to ν_{C-O} peaks in the 1960–1965 and 1970–1990 cm^{-1} regions, respectively. Representative FTIR spectra of CO bound to Mb, Lb, six Class 1 nsHbs, and three Class 2 nsHbs are shown in Fig. 5A. Mb shows two bands, one at 1930 and a larger one at 1945 cm^{-1}, whereas Lba has a predominant absorption band at 1948 cm^{-1} with a small broad band in the 1960–1970 cm^{-1} region. These peaks reflect the relatively strong hydrogen bonding potential of the His$^{(E7)}$ side chain in Mb and the weaker interaction with bound CO in Lba. The peak positions correlate with the moderately large to small increases in k_{O2} due to His$^{(E7)}$ to Leu mutations in Mb and Lba, respectively (Table I).

The nsHbs display significant conformational heterogeneity of His$^{(E7)}$ as judged by the multiple ν_{C-O} peaks in the FTIR spectra of the CO complexes. The Class 1 proteins show dominant ν_{C-O} bands in the 1920 cm^{-1} range, indicating that the His$^{(E7)}$ side chain donates a strong positive field near the bound ligand and should strongly stabilize bound O$_2$. The latter conclusion is supported by the correlations between the absorbance averaged value of ν_{C-O} (~1925 cm^{-1}, Table III), the low value of k_{O2} and the large effects of the His$^{(E7)}$ to Leu mutation for Rice Hb1 and AraTh Hb1 shown in Table I.[9,21] The Class 2 nsHbs show more heterogeneity, large bands in the apolar 1960 cm^{-1} region, and a higher absorbance averaged ν_{C-O} value (~1950 cm^{-1}) indicating a weaker net positive field due to conformations in which there is little hydrogen bonding between His$^{(E7)}$ and the bound ligand. In this case, the larger average ν_{C-O} values correlate with the larger k_{O2} values, and the more modest effects of His$^{(E7)}$ to Leu mutations (200-fold increases in k_{O2}), which are similar to those observed for Mb.

Figure 5B presents averaged FTIR spectra for the CO complexes of the five Class 1 nsHbs, the three Class 2 nsHbs and Lba shown in panel A. There is a correlation between the major

Table IV   Average Values for Physiological Rate and Equilibrium Constants for O_2 Binding to Class 1, Class 2, and leghemoglobins at pH 7, 20°C

Hb	$k'_{O2}(obs)^a \, \mu M^{-1} \, s^{-1}$	$k_{O2} \, s^{-1}$	$P_{50}(obs)^b \, nM$	K_H
Lb	230 ± 130	13 ± 8	50 ± 20	0
Class 1	24 ± 16	0.14 ± 0.16	2.4 ± 2.6	1.7 ± 0.8
Class 2	1.3 ± 1.0	1.1 ± 1.2	340 ± 410	96 ± 98

[a] The value of $k'_{O2}(obs)$ was calculated from $K_{O2,pent}(average)/(1 + K_H$ (average)) from Table III.

[b] The value of $P_{50}(obs)$ was calculated from $(1 + K_H)/K_{O2,pent}(average)$ from Table III.

peak positions and overall O_2 affinity. Panel C shows the correlation between the overall K_{O2} and the average value of ν_{C-O}. This simple spectral analysis indicates that both the O_2 binding and CO spectral properties of the Class 1 and 2 nsHbs fall into distinct categories, supporting our conclusion that the phylogenetic classification represents natural selection for specific and distinct physiological functions.

CONCLUSIONS

A summary of the key average oxygen binding properties for Class 1 nsHbs, Class 2 nsHbs, and leghemoglobins is given in Table IV. Although the standard deviations are roughly 50–100% for each parameter, the properties of these three phylogenetic classes of plant globins are very distinct and imply markedly different physiological functions. The Class 1 plant Hbs have ultra-high O_2 affinities with P_{50} values on the order of 1–10 nM due to a highly reactive iron atom, weak hexacoordination (K_H = 0.5–3.0), and very strong hydrogen bond donation by His$^{(E7)}$ (Tables I–IV). The latter structural feature leads to an extremely low rate of O_2 dissociation (0.01–0.5 s^{-1}) which, coupled with the nanomolar P_{50}, precludes a role in oxygen transport. The ultra-high affinity also appears to rule out a role in gas sensing because the protein would always be saturated with O_2 in all but completely anaerobic conditions. In contrast, the high O_2 affinity and moderately rapid bimolecular rate of O_2 binding to the equilibrium deoxy forms are consistent with Hill's proposal that the Class 1 nsHbs play a role in NO scavenging and re-oxidation of NADH under hypoxic conditions.[33] In recent reviews, Dordas[74] and Igamberdiev and Hill[72] have listed the evidence in favor of this hypothesis of a nsHb/NO cycle to enhance tolerance to hypoxic stress. The key parameter for this function is relatively rapid O_2 binding before the NO dioxygenation reaction. The weak extent of hexacoordination in the Class 1 nsHbs allows the overall $k'_{O2}(obs)$ values to be ∼10–30 μM^{-1} s^{-1}, which are similar to the k'_{O2} values for mam-

malian Mbs and Hbs that are known to rapidly detoxify NO.[75] Other possible roles of all three classes of plant nsHbs in NO metabolism are discussed in greater detail by Dordas[74] and Igamberdiev and Hill.[72]

The Class 2 Hbs exhibit strong hexacoordination ($K_{HisE7} \approx$ 100), which results in an O_2 affinity that is roughly 100-fold lower than that for the Class 1 plant globins. Strengthened hexacoordination also results in reduced overall bimolecular association rate constants (∼1 μM^{-1} s^{-1}), and the observed O_2 dissociation rate constants are still moderately low (∼1 s^{-1}). Although the overall P_{50} values are similar to mammalian Mbs, the low rates of binding and release argue against a role in O_2 transport. At high ligand concentrations, the rate of O_2 uptake is limited by the rate of His$^{(E7)}$ dissociation, k_{-H}, which ranges from 10 to 40 s^{-1}. These rates coupled with the barely detectable levels of Class 2 nsHbs in plants also argue against an O_2 storage function. The relatively low rates of uptake and release and moderate affinity would allow sensing of sustained periods of hypoxia by the generation of deoxygenated, hexacoordinate Hb forms when [O_2] decreases below micromolar levels. However, at present there is no supporting evidence for such a function other than the expression of Class 2 globins under hypoxic conditions and the observation that their over expression increases resistance to hypoxia.[60]

As described in the introduction, leghemoglobins evolved from the Class 2 globins to facilitate O_2 transport to N_2 fixing bacteria in the root nodules of legumes. Hargrove and coworkers have discussed in detail the structural changes that led to loss of hexacoordination, very high rates of O_2 binding (∼250 μM^{-1} s^{-1}), little electrostatic stabilization of bound ligands, moderately high rates of O_2 dissociation (∼5–15 s^{-1}), and high oxygen affinity ($P_{50} \approx 50$ nM).[56,68] These properties both facilitate O_2 diffusion and reduce O_2 tension in the root nodules of legumes to prevent oxygen inhibition of the bacterial nitrogenases. The Class 2 nsHb/Lb family provides a well-characterized example of convergent evolution of a Mb-like function for plant globins.

MATERIALS AND METHODS

Recombinant Protein Production and Purification

Chicory hemoglobins ChicHb 2a and ChicHb 2b (GeneBank accession number AJ007507 and AJ277797, respectively) and maize hemoglobins were expressed and purified as described by Smagghe et al.[67] RiceHb1 and riceHb2 were expressed and purified as described earlier.[9,59] For ChicHb 2a and ChicHb 2b, the media was supplemented with lactose (4 g/L) and for ChicHb2, 3μg/ml hemin chloride (solubilized in 0.1 M NaOH) was added after induction with isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma) at OD_{600} of 0.8.

For SolyGLB2 (TomHb2), RNA was extracted from ∼0.8 g fresh leaf tissue (Solanum lycopersicum) as previously described.[76] The DNA was degraded with DNase I, (amplification grade) and single stranded cDNA, suitable for PCR reaction, was synthesized using Superscript III (Invitrogen, Carlsbad CA). PCR amplification was conducted using 5′-ATC ATA CCA TAT GGG GTT CAC AGA TAA AC-3′ as the forward primer and 5′-GAG AAT TCG GCA GCA GCC TCA GCG TGC AT-3′ as the reverse primer, and the product was gel purified. The resulting fragment was then cloned in the pET28a plasmid (Novagen) between the NdeI and EcoRI restriction sites. The His-Tagged protein was expressed and purified as described earlier.[67] The purification efficiency was analyzed with SDS-polyacrylamide gel electrophoresis and spectroscopic analysis of Soret/280 nm ratios (Hewlett Packard diode array).

CO Binding Kinetics

Flash photolysis was used to measure the bimolecular CO association rate constant as described previously.[43] Rapid mixing experiments for CO binding were conducted as described by Smagghe et al.[67]

Oxygen Binding Kinetics

Flash photolysis was used to assess the bimolecular O_2 association rate constant. This method was conducted using a YAG laser apparatus described previously[43] for the flash photolysis of the ligand. To assess O_2 dissociation rate constants, a rapid mixing experiment was performed using a CO solution (1 mM + 100 μM sodium dithionite) as the displacing ligand.[53] This method was conducted using a BioLogic SFM 400 stopped-flow reactor coupled to a MOS 250 spectrophotometer. The process to prepare the oxygenated samples was previously described.[53] For Chicory Hbs, the protein was first enzymatically reduced in a N_2 sparged Eppendorf and then added to a syringe containing air-equilibrated buffer. All kinetic experiments were conducted in 0.1 M potassium phosphate buffer (pH 7.0) at 20°C. Data analysis and generation of the figures was done using the software Igor Pro (Wavemetrics, Inc).

FTIR Spectroscopy

Samples of CO bound proteins were prepared as described previously.[27] Briefly, the proteins (2–3 mM) were reduced with a dithionite solution in an Eppendorf tube equilibrated with CO. The protein sample was added to a BioCell cuvette (5 mm thickness 50 mm diameter, separated by a 40 μm spacer; BioTools, Inc.) and spectra were acquired with a Nicolet Nexus 470 FTIR spectrometer (Nicolet Instrument Corp., Wisconsin) coupled to an external liquid-nitrogen cooled MCT detector. Spectra were recorded at 1 cm^{-1} resolution in the region 1800–2100 cm^{-1}. The final IR spectra were corrected for buffer background by subtraction of the sample and control data.

Maize Hb1 Crystallization and Structure Determination

The protein was first oxidized by addition of a slight molar excess of potassium ferricyanide that was removed by using a G-25 size-exclusion column equilibrated with 10 mM potassium phosphate (pH 7.0). Hanging-drop vapor diffusion was used for crystal growth. The drop was composed by 2 μL of 2.7 mM Hbm1 and 2 μL of crystallization buffer. The mother liquor was 20% polyethylene glycol monomethylether 1900, 0.1 M sodium acetate (pH 5.6), 0.2 M ammonium sulfate. Protein crystals grew within a week at 4°C.

Diffraction data were collected at 100 K on a Rigaku/MSC home-source generator and processed using d*TREK.[77] CCP4 was used to solve the structure by molecular replacement with the riceHb1 (PDB code 1D8U). A crystallographic R-value < 50 was obtained for the initial molecular replacement solution. The structure was then refined using REFMAC,[78] a program from the CCP4 suite,[79] and by manual rebuilding using O.[80] The final tetramer model contains 584 amino acids and 584 water molecules with $R = 20.3\%$ and $R_{free} = 25.3\%$. Other details concerning this structure are filed as PDB ID 2r50.

Phylogenetics

Plant hemoglobin sequences were identified by searching the genbank database using the BLAST algorithm with various hemoglobin search strings. A structural alignment of the plant hemoglobin amino acid sequences was constructed using crystal structures as a guide,[14] and the alignment was then back translated to nucleic acid sequences. A transition-transversion rate ratio test identified saturation of third codon positions and these were consequently omitted from further analyses. Phylogenetic reconstructions were conducted using Bayesian inference (MrBayes version 3.1.2).[81,82] The data was partitioned "by codon" with third positions excluded. The general time reversible model was used, with rates specified as gamma distributed across sites. Six Monte Carlo Markov chains were run simultaneously for 3,000,000 generations with one heated chain (temp = 0.15). Other parameters were left at the default settings. Sample trees were acquired every 500 generations, and the first 2,000 trees were discarded as "burn-in". The remaining trees were used to generate a 50% consensus tree and then nodes with less than 90% posterior probabilities were collapsed to polytomies. Figure 1 was prepared in the Dendroscope application[83] with GLB3 as an outgroup.[14]

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