Quaternary Structure of Rice Nonsymbiotic Hemoglobin*

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Plant nonsymbiotic hemoglobins are hexacoordinate hemoproteins found in all plants. Although expression is linked with hypoxic environmental conditions (Taylor, E. R., Nie, X. Z., Alexander, W. M., and Hill, R. D. (1994) Plant Mol. Biol. 24, 853–862), no discrete physiological function has yet been attributed to this family of proteins. The crystal structure of a nonsymbiotic hemoglobin from rice has recently been determined. The crystalline protein is homodimeric and hexacoordinate with two histidine side chains coordinating the heme iron atom. Despite the fact that the amino acids responsible for the subunit interface are relatively conserved among the nonsymbiotic hemoglobins, previous work suggests that this group of proteins might display variability in quaternary structure (Duff, S. M. G., Wittenberg, J. B., and Hill, R. D. (1997) J. Biol. Chem. 272, 16746–16752; Arredondo-Peter, R., Hargrove, M. S., Sarath, G., Moran, J. F., Lohrman, J., Olson, J. S., and Klucas, R. V. (1997) Plant Physiol. 115, 1259–1266). Analytical ultracentrifugation and size exclusion high pressure liquid chromatography were used to investigate the quaternary structure of rice nonsymbiotic hemoglobin at various states of ligation and oxidation. Additionally, site-directed mutagenesis was used to test the role of several interface amino acids in dimer formation and ligand binding. Results were analyzed in light of possible physiological functions and indicate that the plant nonsymbiotic hemoglobins are not oxygen transport proteins but more closely resemble known oxygen sensors.

Two classes of hemoglobins have been identified in plants. The leghemoglobins were discovered many years ago in the root nodules of legumes, where they play an important role in symbiotic nitrogen fixation (1). An effort to identify the evolutionary origin of leghemoglobins led to the discovery of plant nonsymbiotic hemoglobins (nsHbs) in several plant species (2, 3). nsHbs have now been identified in many mono- and dicotyledonous plants, including barley, soybean, rice, Arabidopsis, chicory, and corn (NCBI accession number AAF44664) (4–7). Homologous hemoglobins have also recently been discovered in bryophytes, which leads to the conclusion that these proteins are ubiquitous in the plant kingdom (8). The discovery of nonsymbiotic hemoglobins was slowed by the fact that they are present in very low concentrations inside plants. The details of their in vivo expression and function are currently under investigation (9, 10).

The biophysical properties of several nsHbs have been characterized and have provided important clues about possible physiological functions for this family of proteins (4, 5, 11). nsHbs are unusual because their heme prosthetic groups are hexacoordinate in the ferric and deoxyferrous states. Hexacoordination results from two His residues that bind the heme iron at the fifth and sixth coordination sites; one coordinates the proximal side of the heme iron, which is characteristic of all hemoglobins, and a second coordinates the distal side, which is traditionally the binding site for oxygen and other ligands. Hexacoordination has been observed in hemoglobins from bacteria; protozoa; several animal species, including humans; and an oxygen-sensing heme protein from Escherichia coli (12–16). Therefore, the mechanism of using displaceable coordination for regulating heme protein function has been established. Despite this potential inhibition of ligand binding, nsHbs reversibly bind oxygen and other ligands with very high affinities (9). For this reason, it has been suggested that they do not function as oxygen storage or transport proteins but might be involved in plant metabolism under oxygen-limiting conditions (2, 10).

The crystal structure of rice nsHb (rHb1) has recently been solved and is currently the only structure of a nsHb (17). This structure reveals a homo-dimeric protein in the asymmetric unit, indicating that it can form a specific dimer independent of crystal packing. A ribbon model and details of the rHb1 dimer interface are shown in Fig. 1. The interface is composed of the amino acid residues at the beginning of the CD helix region and the G helix. The interaction between the two subunits consists of a hydrogen bond between the side chain of Glu<sup>119</sup> of one subunit and Ser<sup>49</sup> on the other. These two polar interactions surround a hydrophobic core consisting of the side chains of Val<sup>106</sup>, Val<sup>120</sup>, and Phe<sup>125</sup> of each subunit. The amino acid residues that make up this interface region are relatively conserved among the nsHbs (9, 17), indicating that the quaternary structures of other members of this family could be similar to rHb1.

Quaternary structure plays an important role in the function of many hemoglobins by facilitating allosteric and cooperativity for regulation of ligand binding. However, the solution quaternary structures of nsHbs have not yet been characterized in detail. barley nsHb was reported by Duff et al. (11) to be dimeric, whereas rHb1 was initially reported to be monomeric (4). The quaternary structures of other nsHbs have not yet been investigated. The discrepancy between the quaternary structure of rHb1 in crystals and in solution and between the solution quaternary structures of the nsHbs from barley and rice are the rationale for the characterization of rHb1 described here.

In this work, the solution quaternary structure of rHb1 was investigated along with its effects on the rate and affinity constants for ligand binding and the rate constants characterizing hexacoordination by the distal His. In addition, site-

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1 The abbreviations used are: nsHb, plant nonsymbiotic hemoglobin; Hb, hemoglobin; rHb1, rice nsHb1; HPLC, high pressure liquid chromatography.
directed mutagenesis of several conserved amino acids that make up the subunit interface was used to determine their effect on quaternary structure and ligand binding. These structural and biophysical characteristics of nsHbs are important for developing an understanding of the function of these proteins in vivo.

EXPERIMENTAL PROCEDURES

Protein Preparation—All proteins were expressed and purified using the methods described by Arredondo-Peter et al. (4). Proteins were expressed in BL21(DE3) E. coli cells using the pET system from Novagen without amino- or carboxyl-terminal tagging. This procedure results in bright red cell pellets and a red supernatant following cell lysis. All mutant proteins were generated using the Quickchange mutagenesis procedure available from Stratagene. Extinction coefficients for different ligated forms of wild type rHb1 were determined using the pyridine hemochromogen method described by Riggs (18).

Quaternary Structure Determination—Size exclusion analysis was performed at room temperature with a TSK-GEL G2000SWXL HPLC column from TOSOHAAS and a Varian Prostar HPLC system. 20-μl protein samples at concentrations ranging from 1 to 800 μM were analyzed at a flow rate of 1 ml/min, and retention time was defined as the midpoint of the absorbance chromatogram observed at 410 nm. The buffer used for all analyses was 100 mM potassium phosphate, pH 7.0, and 100 mM NaCl. The ligated states were prepared using previously described methods (19, 20), except for the Fe(CN)2 sample that was in the above buffer and 5 mM NaCN.

Analytical ultracentrifugation was performed at 25 °C with a Beckman XLA analytical ultracentrifuge using absorption optics. Sedimentation profiles were collected for samples at 25,000 and 30,000 rpm, and over protein concentrations ranging from 10 to 50 μM. Each sample was loaded into all three sectors of an equilibrium centerpiece, and all three sedimentation profiles contributed to data analysis. Apparent molecular mass at each concentration was determined by combining data from each sector of the centerpiece and each angular velocity using the Multi FIG. 1. The crystal structure of rHb1. A, dimeric protein showing the interface region. B, stereo picture of the amino acid side chains in the dimer interface. The residues with gray bonds are in one subunit, and those with black bonds are in the other. The symmetric dimer interface is a hydrophobic core of Val and Phe amino acids that is capped at either end by an electrostatic interaction between Glu119 of one subunit and Ser49 of the other. These figures were prepared from Protein Data Bank entry 1d8u.
Fit global analysis software available from Beckman. Solvent density and partial specific volume were calculated using previously described methods that have been summarized by Beckman (21, 22). These values for our buffer system and rHb1 were 1.0126 g/ml and 0.7488 ml/g, respectively. All other graphic analyses, fitting of data, and figure preparation were carried out with the program Igor Pro (Wavemetrics, Inc.). Estimates of error for final equilibrium dissociation constants were obtained from the absolute percent variation in at least three independent experiments.

**RESULTS**

**Kinetic Measurements**—Oxygen and carbon monoxide association rate constants and oxygen dissociation rate constants were determined using previously described methods (19, 20). Measurement of the rate constants for the association and dissociation of the distal His and the flash photolysis apparatus used in all experiments have been described by Hargrove (23).

**TABLE I**

Extinction coefficients for different forms of rHb1

| Oxidation State | Soret Region (nm) | Visible Region (nm) |
|----------------|-------------------|---------------------|
| (III)met       | λ410 (117)        | λ557 (21)           |
| (II)deoxy      | λ423 (157)        | λ528 (14)           |
| (II)O₂         | λ410 (120)        | λ540 (15)           |
| (II)CO         | λ416 (153)        | λ557 (16)           |

**FIG. 2.** Extinction coefficients for different ligated states of rHb1. A, the Soret region of the absorption spectrum of the ferric (solid line), oxy (dashed and dotted line), carbonmonoxy (dotted line), and deoxy (dashed line) forms of rHb1. B, the same spectra are shown in the visible region. These values were used for concentration determination in the experiments shown in Fig. 3.

**FIG. 3.** Effects of protein concentration on rHb1 association. A, size exclusion HPLC retention times. The concentration reported on the x axis has not been corrected for dilution of the protein on the column. B, apparent molecular masses (in daltons) determined by analytical ultracentrifugation. The solid line is a nonlinear least squares fit to Equation 2. C, the same data as shown in B, but with the fitted curve extrapolated to the protein concentrations shown in A.

CONCENTRATION DEPENDENCE OF THE APPARENT MOLECULAR MASS OF rHb1—Accurate analysis of the concentration dependence of association requires absorption extinction coefficients specific to each ligated state of rHb1. Fig. 2 shows these absorbance spectra with the extinction coefficients plotted on the ordinate axis, and Table I lists specific extinction coefficients used for concentration determination at different wavelengths. The extinction coefficients for the ferric and deoxy proteins were identical to those previously reported (4). These extinction coefficients are slightly smaller than other ferric hemoglobins but comparable in the reduced forms (24).

Concentration Dependence of the Apparent Molecular Mass of rHb1—Accurate analysis of the concentration dependence of association requires absorption extinction coefficients specific to each ligated state of rHb1. Fig. 2 shows these absorbance spectra with the extinction coefficients plotted on the ordinate axis, and Table I lists specific extinction coefficients used for
equilibrium dissociation constant, $P_i$ is the total protein concentration, and $f_m$ is the fraction of protein that is monomeric.

$$ f_m = \frac{P_i - K_d + \sqrt{P_i^2 + 4 K_d P_i f_m^2}}{2 K_d} \tag{1}$$

Assigning a retention time to the monomer and dimer and assuming rapid exchange in the reaction, the following equation can be used to describe the size exclusion HPLC results in Fig. 3A, $S_{obs}$ is the observed retention time, $S_m$ is the retention time of the monomer, $S_d$ is the dimer retention time, and $f_m$ is the fraction of monomers as defined in Equation 1.

$$ S_{obs} = f_m S_m + (1 - f_m) S_d \tag{2}$$

The HPLC data were fit to Equation 2, yielding a retention time of 8.42 min for the monomeric protein and 7.66 min for dimeric protein. Although this analysis confirms dimerization, the equilibrium dissociation constant extracted from this fit (900 $\mu$M) is not accurate because the protein concentrations on the abscissa of Fig. 3A cannot easily be corrected for dilution of the samples on the HPLC column.

Having established that rHb1 is a partially associated homodimer at micromolar concentrations, analytical ultracentrifugation was used to determine the concentration dependence of the fraction of monomeric protein. Using absorption to detect equilbrium sedimentation profiles limits the absorbance of the sample to ~0.1–1.2 A. For nsHbs, if both the Soret and visible absorption bands are exploited, experiments can be carried out between ~1 and 50 $\mu$M in the Beckman equilibrium centerpiece.

Fig. 3B shows the dependence of apparent molecular mass on protein concentration for ferric rHb1. At very low concentrations, the protein appears monomeric, but the apparent molecular mass increases throughout the concentration range accessible in this experiment. The fitted curve in Fig. 3B is a nonlinear least squares fit to Equation 2 with molecular mass substituted for retention time. From these data, fitted values for the equilibrium dissociation constant and the molecular masses for the monomeric and dimeric proteins were 80 $\mu$M ($\pm$10%), 17,700 Da, and 35,200 Da, respectively. Fig. 3C shows the same data as Fig. 3B, but with the fitted curve extrapolated to higher protein concentrations, indicating the concentration range over which fractional subunit association occurs for ferric rHb1.

**Analysis of the Ferric rHb1 Dimer Interface**

In an effort to test the importance of the amino acid side chains in the dimer interface, several of these interface residues were replaced by different amino acids using site-directed mutagenesis. The Ser$^{49}$ to Ala$^{49}$ (S49A), Glu$^{119}$ to Val$^{119}$ (E119V), and double substitution (S49A/E119V) mutant proteins were used to determine the role in dimerization of the symmetric electrostatic interactions at each end of the subunit interface. The substitutions V46N, V120N, and V46NV120N were used to determine the importance of the hydrophobic core in dimerization. The quaternary structures of the resulting mutant proteins were analyzed, and rate constants for oxygen and carbon monoxide binding were measured to test the impact of any quaternary structural changes on ligand binding.

Table III lists the observed molecular masses for the mutant proteins as determined by analytical ultracentrifugation. Each protein was assayed over a total protein concentration range of ~10 to ~50 $\mu$M. At these concentrations, the wild type protein exhibits a molecular mass significantly larger than that expected for monomeric protein. However, each of the interface disruption mutant proteins has the molecular mass expected for its respective monomers at all measured protein concentrations (Table III).

Rate constants for O$_2$ and CO binding are given in Table III for each of the dimer interface mutant proteins. There are no substitutions resulting in rate constants that deviate more than 2-fold from those of the wild type protein for any of the reactions measured. In addition, the reactions with wild type rHb1 were measured over a concentration range of 1–50 $\mu$M with no effect on rate constants for oxygen or carbon monoxide binding (data not shown).

Laser flash photolysis as a function of CO concentration can be used to determine the rate constants for binding and dissociation of the hexacoordinating His$^{73}$ side chain (23). The rate constants extracted from a two-exponent fit of CO rebinding after photolysis were used to extract these values using the following equations (where $\gamma_1$ and $\gamma_2$ are the two rate constants associated with rebinding).

$$ \gamma_1 + \gamma_2 = k_{-H} + k_H + k_{-CO}[CO] \tag{3} $$

$$ \gamma_1 \gamma_2 = k_{-H}[CO] \tag{4} $$

Plots of the sum and product of $\gamma_1$ and $\gamma_2$ versus CO concentration are shown in Fig. 4 for the S49A mutant protein, which, as indicated in Table III, is completely monomeric at the protein concentration used in this experiment (30 $\mu$M). The solid lines in Fig. 4 are linear least squares fits to these data. The slope of Fig. 4A (obeying Equation 3) is the bimolecular rate constant for CO binding. Dividing this value into the slope of Fig. 4B (obeying Equation 4) yields $k_{-H}$. Subtracting $k_{-H}$ from the y intercept of Fig. 4A returns $k_H$ (as indicated in Equation 3). These values for the S49A mutant protein are 5.8 ($\pm$6%) $\mu$M$^{-1}$ s$^{-1}$ ($k_{-CO}$), 590 ($\pm$10%) s$^{-1}$ ($k_H$), and 1950 ($\pm$10%) s$^{-1}$ ($k_{-H}$). The values for wild type rHb1 are 6.0 $\mu$M$^{-1}$ s$^{-1}$, 520 s$^{-1}$, and 1910 s$^{-1}$, respectively (23).

**Discussion**

**Quaternary Structure Determination**

rHb1 is a partially associated homodimer in the concentration range in which absorption spectroscopy is used for experimentation (~1–1000 $\mu$M; Fig. 3C). At a concentration of 0.8 mM, the protein is 90% associated, but at concentrations between 0.8 mM and ~5 $\mu$M, significant variation in apparent molecular mass is observed. If the nsHb from barley has a similar equilibrium dissociation constant, the data presented here explain the difference between the quaternary states initially assigned to it and rHb1. It is likely that the higher concentrations of barley nsHb used for
Apparent molecular masses and rate and equilibrium constants for ligand binding to rHb1 and several dimer interface mutant proteins

| Protein                | mw_{obs} | k_{CO}^{\text{m}} | k_{CO}^{\text{M}} | K_{O2}^{\text{m}} | K_{O2}^{\text{M}} |
|------------------------|----------|-------------------|-------------------|-------------------|-------------------|
| Wild type rice nsHb    | 24.2     | 68                | 6.0               | 0.038             | 1800              |
| S49A                   | 18.7     | 79                | 5.8               | 0.056             | 1400              |
| E119V                  | 18.7     | 82                | 5.7               | 0.055             | 1500              |
| S49A/E119V             | 18.7     | 105               | 6.4               | 0.065             | 1600              |
| V46N                   | 18.9     | 62                | 7.9               | 0.044             | 1400              |
| V120N                  | 18.7     | 72                | 6.9               | 0.028             | 2500              |
| V46N/V120N             | 17.7     | 65                | 5.7               | 0.044             | 1500              |

Determination of rate constants for hexacoordination in the monomeric mutant protein S49A. The concentration dependence of biphasic CO rebinding to rice nsHb following flash photolysis is used to determine rate constants for hexacoordination (25). The products and sums of the two fitted rate constants, \( k_{CO}^{\text{M}} \) and \( k_{CO}^{\text{m}} \), were analyzed according to Equations 3 and 4. A, the concentration dependence of the sum of \( k_{CO}^{\text{M}} \) and \( k_{CO}^{\text{m}} \) yields a slope of 5.8 and a \( y \) intercept of 2552. B, the concentration dependence of the product of \( k_{CO}^{\text{M}} \) and \( k_{CO}^{\text{m}} \) yields a slope of 11404 and a \( y \) intercept near zero. The rate constants for hexacoordination extracted from these data are similar to those of the wild type protein.

The plant nsHbs must undergo conformational change upon ligand binding (17), and are probably capable of binding a partner molecule via the interface region investigated in this work. Therefore, it is possible that nsHbs are molecular sensors that use heme ligation to trigger other physiological events.

Implications for Possible Physiological Function—The phylogenetic conservation of the dimer interface in nsHbs indicates that it is monomeric when present in micromolar concentrations (26). These experiments have important implications with respect to cooperative ligand binding and potential protein function. The results presented here for rHb1 indicate that it could not possibly bind ligands cooperatively unless it is present at concentrations >1 mM inside plant tissue. It is unlikely that this is the case, as very little nsHb has ever been extracted from plant tissue (9). However, it is difficult to completely rule out the possibility of high local concentrations of protein compartmentalized in small intracellular areas.

The Roles of Quaternary Structure and the Dimer Interface—Understanding the physiological function of the nsHbs has been the focus of much research over the past several years. Current hypotheses result from the roles of hemoglobins in other systems in combination with biophysical and structural data for nsHbs that limit some of these possibilities. It has been suggested that extremely high oxygen affinities and low dissociation rate constants preclude the nsHbs from facilitating the diffusion of oxygen in the absence of some factor that could increase the dissociation rate constant at least 100-fold (5, 17). The results of the work presented here indicate that quaternary structural changes do not significantly affect ligand binding kinetics or rate constants associated with hexacoordination. Therefore, a change in quaternary structure is not a factor that could implicate the nsHbs as oxygen transport proteins.

Table IV is a sequence alignment of the interface region of rHb1 with several other plant hemoglobins, including Parasponia hemoglobin, which is dimeric (27, 28), and soybean leghemoglobin (Lba), which is monomeric. The residues which create the dimer interface in rHb1 are also found in the nsHbs from soybean, barley, corn, Arabidopsis nsHb1, and Parasponia Hb. However, Arabidopsis contains a second nsHb that is predicted to be incapable of dimerization with respect to the criteria established here for rHb1 because it contains an Ala residue at position 49. The mutation creating a similar interface in rHb1 (S49A) results in a monomeric protein. Presumably Lba dimerization is prevented by the presence of hydrophobic residues at positions 49 and 119.

Size exclusion chromatography indicated a molecular mass closer to that expected for the dimeric protein (11). For example, if a protein concentration of 1 mM was used in these experiments, the apparent molecular mass would be ~ 32,000 Da. The first examination of rHb1 quaternary structure used analytical ultracentrifugation, which requires much lower protein concentrations (~ 3 \( \mu \)M). This experiment provided an apparent molecular mass of ~ 19,000 Da, suggesting a monomeric protein (4).
There are many examples of heme proteins that use coordination and redox properties to affect the behavior of partner molecules. Many of these proteins are heme-based sensors of small diatomic ligands like oxygen, carbon monoxide, and nitric oxide (29). FixL, CooA, soluble guanylate cyclase, and ECDos are examples of heme proteins that undergo a distal ligand displacement that produces conformational changes that affect the Fe(II) deoxy protein compared with the ferric form. This could indicate that the interface region of deoxy rHb1 is less stable in the Fe(II)deoxy protein than in the ferric form. There is little change in association equilibrium for Fe(II)-CN and O₂, but there is a 6-fold increase in the Kₐ of the Fe(II)deoxy protein compared with the ferric form. This could indicate that the interface region of deoxy rHb1 is less likely to interact with another protein and that ligation of the deoxy protein might trigger binding of a partner molecule. A similar redox and ligand-dependent quaternary structure has also been observed in the hemoglobin from Caudina arvicola, one form of which exhibits reversible hexacoordination (32, 33).

The possibility of forming heterodimeric nsHbs has not yet been investigated but is a potentially very important direction of research. If the α and β subunits of human hemoglobin had first been identified genetically and studied individually as recombinant proteins, quaternary structure and cooperativity would not have been observed until the subunits were studied together. This limitation of working with recombinant proteins must be considered when trying to identify physiological roles of the nsHbs.

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