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Spectroscopic analysis of moss (*Ceratodon purpureus* and *Physcomitrella patens*) recombinant non-symbiotic hemoglobins

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Abbreviations: CerpurnsHb, *Ceratodon purpureus* non-symbiotic hemoglobin; Hb, hemoglobin; Lb, leghemoglobin; nsHb, non-symbiotic hemoglobin; PhypatnsHb, *Physcomitrella patens* non-symbiotic hemoglobin

Non-symbiotic hemoglobins (nsHbs) are O_2-binding proteins widely distributed in land plants, including primitive bryophytes. Little is known about the properties of bryophyte nsHbs. Here, we report the spectroscopic characterization of two moss recombinant nsHbs, CerpurnsHb of *Ceratodon purpureus* and PhypatnsHb of *Physcomitrella patens*. Spectra showed that the absorption maxima of the ferrous and ferric forms of recombinant CerpurnsHb are located at 418, 531 and 557 nm and 407, 537, 569 (shoulder) and 632 (shoulder) nm, respectively, and of PhypatnsHb are located at 422, 529 and 557 nm and 407, 531, 571 (shoulder) and 647 (shoulder) nm, respectively. These absorption maxima are similar to those of rice Hb1. Also, the absorption maxima of the oxygenated ferrous form of recombinant CerpurnsHb and PhypatnsHb are located at 412, 541 and 575 nm and 414, 541 and 574 nm, respectively, similar to those of oxygenated rice Hb1 and cowpea leghemoglobin II. This evidence indicates that CerpurnsHb and PhypatnsHb are mostly hexacoordinate and that they bind O_2.

Non-symbiotic hemoglobins (nsHbs) are O_2-binding proteins widely distributed in land plants, from primitive bryophytes to evolved monocots and dicots.[1] Based on O_2-affinity and sequence similarity nsHbs are classified into type 1 and type 2 (nsHbs-1 and nsHbs-2, respectively).[2,3] The O_2-affinity of nsHbs-1 is very high because of an extremely low O_2-dissociation rate constant.[3-5] Analyses by X-ray crystallography, site-directed mutagenesis and visible spectroscopy revealed that the extremely low O_2-dissociation rate constant of nsHbs-1 primarily results from Fe-heme hexacoordination by distal His.[4,6] Absorption spectra of nsHbs-1 are similar to those of other Hbs, however nsHbs-1 exhibit distinctive absorption maxima in the deoxyferrous (Hb^{2+}) form. Specifically, nsHb-1^{2+} exhibits peaks at ~526 and ~556 nm, which is characteristic of hexacoordinate Hbs.[4,7] In contrast, pentacoordinate Hbs exhibit a broad peak centered at ~556 nm in their Hb^{2+} form.[7,8]

Little is known about the biophysical properties of primeval land plant nsHbs (e.g., the postulated ancestors of nsHbs-1 and nsHbs-2 [1]), such as bryophyte nsHbs. To better understand these nsHbs, the structure of a moss (*Ceratodon purpureus*) nsHb (CerpurnsHb) was modeled.[9] The predicted structure suggested that Fe-heme in this protein is hexacoordinate, however this observation has not been verified experimentally. Here, we report the generation and spectroscopic characterizations of recombinant CerpurnsHb and *Physcomitrella patens* nsHb (PhypatnsHb).

We generated recombinant CerpurnsHb and PhypatnsHb from plasmids pCR2.1::CerHb[9] and pCRII::B46,[10] essentially as described by Arredondo-Peter et al.[4,8] Inserts were subcloned into the plasmid pET28b (Novagen), generating constructs pET28b::CerpurnsHb and pET28b::PhypatnsHb, then transformed into *E. coli* Tuner(DE3)pLacI. The inserts within plasmids pET28b::CerpurnsHb and pET28b::PhypatnsHb were fully sequenced. Soluble extracts were obtained from recombinant *E. coli* pET28b::CerpurnsHb and pET28b::PhypatnsHb, and recombinant CerpurnsHb and PhypatnsHb were detected by SDS-PAGE. Soluble extracts were subjected to spectroscopic analysis using soluble extracts from untransformed *E. coli* Tuner(DE3)pLacI as blanks. Ferrous Hb was oxidized to ferric (Hb^{3+}) Hb by adding potassium ferricyanide; Hb^{2+} was formed by adding sodium dithionite; and air was bubbled through the Hb^{2+} solution to generate the O_2-ligated ($Hb^{2+}O_2$) form of Hb.

DNA sequencing detected no mutations within the inserts of plasmids pET28b::CerpurnsHb and pET28b::PhypatnsHb. Thus, the sequences of recombinant CerpurnsHb and PhypatnsHb were identical to that predicted by the pCR2.1::CerHb and pCRII::B46 plasmids. SDS-PAGE analysis

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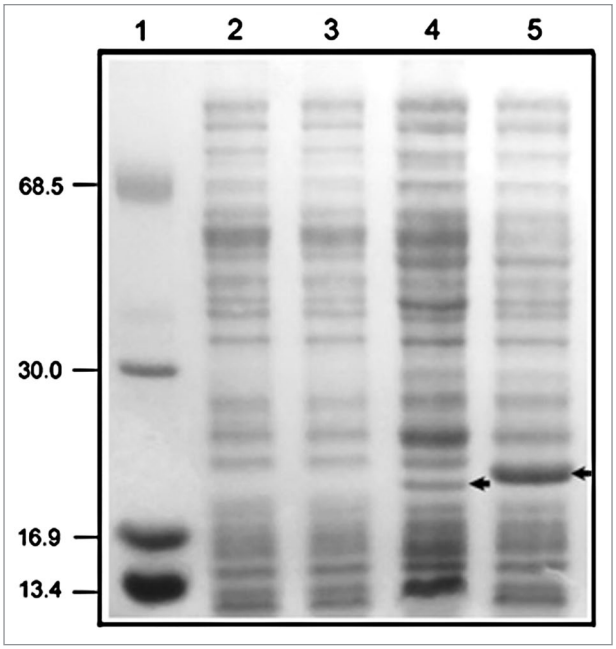

Figure 1. Detection of recombinant CerpurnsHb and PhypatnsHb synthesized by *E. coli* Tuner(DE3)pLacI. Aliquots (~30 μg of total protein) were separated by SDS-PAGE in a 15% gel. Line 1, molecular mass markers; lines 2 and 3, soluble extract of the untransformed *E. coli* Tuner(DE3) pLacI; line 4, soluble extract of the *E. coli* Tuner(DE3)pLacI transformed with the pET28b::CerpurnsHb construct; line 5, soluble extract of the *E. coli* Tuner(DE3)pLacI transformed with the pET28b::PhypatnsHb construct. Arrows indicate the recombinant CerpurnsHb and PhypatnsHb. Markers are shown in kD.

showed that recombinant CerpurnsHb and PhypatnsHb of the expected molecular masses (19.6 and 19.9 KD, respectively) were synthesized by *E. coli* Tuner(DE3)pLacI (**Fig. 1**). Spectra of recombinant CerpurnsHb and PhypatnsHb were highly similar to those of other Hbs (**Fig. 2**). The absorption maxima of Hb^{2+} and Hb^{3+} forms of recombinant CerpurnsHb are located at 418, 531 and 557 nm and 407, 537, 569 (shoulder) and 632 (shoulder) nm, respectively, and in PhypatnsHb at 422, 529 and 557 nm and 407, 531, 571 (shoulder) and 647 (shoulder) nm, respectively, similar to those of rice Hb1. Also, the absorption maxima of the $Hb^{2+}O_2$ form of recombinant CerpurnsHb and PhypatnsHb were located at 412, 541 and 575 nm and 414, 541 and 574 nm, respectively, similar to those of oxygenated rice Hb1 and cowpea LbII (**Table 1**). This evidence indicates that CerpurnsHb and PhypatnsHb are hexacoordinate and that they bind O_2. However, the 531 nm maximum of $CerpurnsHb^{2+}$ and 529 nm maximum of $PhypatnsHb^{2+}$ are weak compared with the maximum of rice Hb1.[4] This observation suggests that hexacoordination is partial in both $CerpuprnsHb^{2+}$ and $PyspatnsHb^{2+}$ and that these proteins may exist in a mixture of hexa- and pentacoordinate forms. Thus, it is likely that the O_2-affinities of CerpurnsHb and PhypatnsHb are higher than those reported for other hexacoordinate land plant nsHbs. An unusual characteristic of the oxygenated $CerpurnsHb^{2+}$ and $PhypatnsHb^{2+}$ spectra was the existence of absorption peaks at 645 nm (**Fig. 2** and **Table 1**). These spectra are similar to that from recombinant human histoglobin obtained from *E. coli* grown in a fermentation apparatus aerated using pure oxygen.[11] This observation suggests that Fe-heme is in the high-spin form in oxygenated $CerpurnsHb^{2+}$ and $PhypatnsHb^{2+}$, however the origin of peaks at 645 nm is not known. The results reported here provide knowledge of the spectroscopic properties of bryophyte nsHbs and corroborate the Fe-heme hexacoordination predicted for modeled CerpurnsHb.[9]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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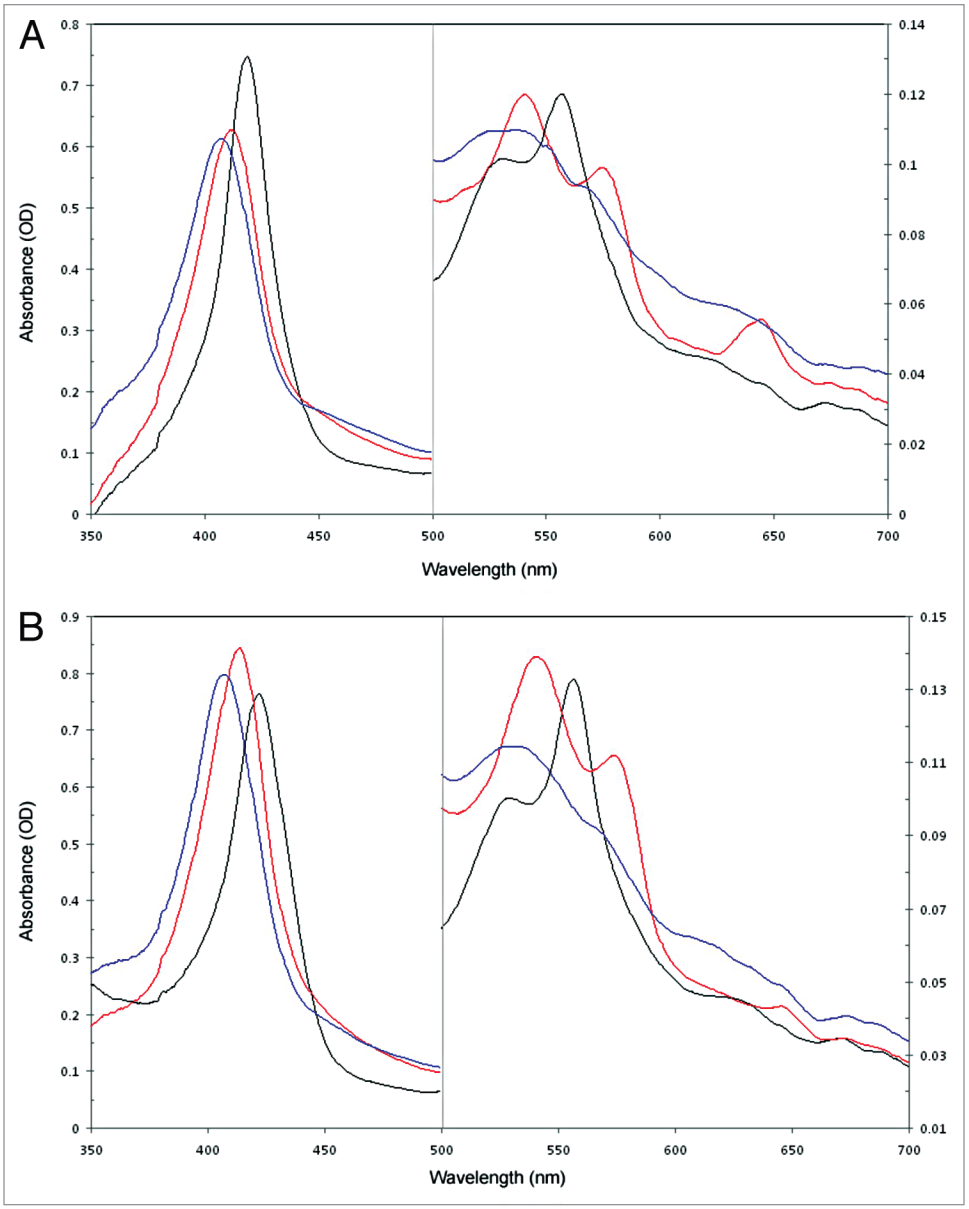

Figure 2. Absorption spectra of *E. coli* Tuner(DE3)pLacI soluble extracts containing the recombinant CerpurnsHb (A) and PhypatnsHb (B). Blue lines, Hb^{3+} form; red lines, $Hb^{2+}O_2$ form; and black lines, Hb^{2+} form.

Table 1. Spectral characteristics of *C. purpureus* and *P. patens* recombinant nsHbs and hexacoordinate rice Hb1[4] and human neuroglobin,[12] and penta-coordinate cowpea LbII[8]

	Absorption maxima (nm)					
State/ligand	**Søret region**		**Q region**			
CerpurnsHb						
Ferrous deoxygenated	418	531		557		
Ferrous oxygenated	412		541		575	645
Ferric	407		537		569 (shoulder)	632 (shoulder)
PhypatnsHb						
Ferrous deoxygenated	422	529		557		
Ferrous oxygenated	414		541		574	645
Ferric	407		531		571 (shoulder)	647 (shoulder)
Rice Hb1						
Ferrous deoxygenated	424	529		557		
Ferrous oxygenated	412		540		576	
Ferric	410		540	556 (shoulder)		
Human neuroglobin						
Ferrous deoxygenated	425	527		563		
Ferrous oxygenated	413		542		579	
Ferric	417		538		567 (shoulder)	
Cowpea LbII						
Ferrous deoxygenated	428			556		
Ferrous oxygenated	411		540		574	
Ferric	404		534	560 (shoulder)		620 (shoulder)