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

Consuelo Vázquez-Limón, Saraí Castro-Bustos and Raúl Arredondo-Peter*

Laboratorio de Biofísica y Biología Molecular; Departamento de Bioquímica y Biología Molecular; Facultad de Ciencias; Universidad Autónoma del Estado de Morelos; Cuernavaca, Morelos México

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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 O₂-binding proteins widely distributed in land plants, including primitive bryophytes to evolved monocots and dicots. Based on O₂-affinity and sequence similarity nsHbs are classified into type 1 and type 2 (nsHbs-1 and nsHbs-2, respectively). The O₂-affinity of nsHbs-1 is very high because of an extremely low O₂-dissociation rate constant. Analyses by X-ray crystallography, site-directed mutagenesis and visible spectroscopy revealed that the extremely low O₂-dissociation rate constant of nsHbs-1 primarily results from Fe-heme hexacoordination by distal His. In contrast, pentacoordinate Hbs exhibit a broad peak centered at ~556 nm in their Hb²⁺ form. Little is known about the biophysical properties of primitive land plant nsHbs (e.g., the postulated ancestors of nsHbs-1 and nsHbs-2), such as bryophyte nsHbs. To better understand these nsHbs, the structure of a moss (Ceratodon purpureus) nsHb (CerpurnsHb) was modeled. 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 PhypatnsHb.

We generated recombinant CerpurnsHb and PhypatnsHb from plasmids pCR2.1::CerHb and pCRII::B46, essentially as described by Arredondo-Peter et al. 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 (Hb³⁺) Hb by adding potassium ferricyanide; Hb³⁺ was formed by adding sodium dithionite; and air was bubbled through the Hb³⁺ solution to generate the O₂-ligated (Hb³⁺O₂⁻) 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...
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 Hb2+ and Hb3+ 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 Hb2+O2 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 LhII (Table 1). This evidence indicates that CerpurnsHb and PhypatnsHb are hexacoordinate and that they bind O2. However, the 531 nm maximum of CerpurnsHb2+ and 529 nm maximum of PhypatnsHb2+ are weak compared with the maximum of rice Hb1. This observation suggests that hexacoordination is partial in both CerpurnsHb2+ and PsypatnsHb2+ and that these proteins may exist in a mixture of hexa- and pentacoordinate forms. Thus, it is likely that the O2-affinities of CerpurnsHb and PhypatnsHb are higher than those reported for other hexacoordinate land plant nsHbs. An unusual characteristic of the oxygenated CerpurnsHb2+ and PhypatnsHb2+ 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. This observation suggests that Fe-heme is in the high-spin form in oxygenated CerpurnsHb2+ and PhypatnsHb2+, 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.

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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 CerpurmsHb (A) and PhypanHb (B). Blue lines, \( \text{Hb}^{3+} \) form; red lines, \( \text{Hb}^{2+} \text{O}_2 \) form; and black lines, \( \text{Hb}^{2+} \) form.
Table 1. Spectral characteristics of *C. purpureus* and *P. patens* recombinant nsHbs and hexacoordinate rice Hb1 and human neuroglobin, and penta-coordinate cowpea LbII.

| State/ligand       | Absorption maxima (nm) |
|-------------------|------------------------|
|                   | Soret 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)        |