Appendix VI

Structural Comparison of Plant Hemoglobins and Animal Myoglobins

The globin structural superfamily is a large, well studied family of globular proteins, present in all domains of life: archae, bacteria, and eukaryotes (PFAM PF00042). All members of the globin structural superfamily are thought to share a common ancestor (Punta et al. 2012). The globin structural fold is comprised of eight alpha helical segments and a heme co-factor, which coordinates binding and/or transfer of oxygen. Structural comparisons of animal myoglobin, plant leghemoglobin, and plant non-symbiotic hemoglobin monomers are shown in Figure 1A-H. The crystal structure for cow myoglobin does not exist, so we have included myoglobin structures from tuna, pig, and horse in this analysis. Based on their similarity to eachother (Figure 1F-H), we expect that they are highly similar to cow myoglobin. The crystal structures were superimposed over all backbone atoms using the Super algorithm in PyMOL (Delano, 2007) (Figure 1I-L) and the corresponding root mean square deviations (RMSDs) are shown in Table 1 . Comparison of proteins folds (Figure 1) and RMSD values (Table 1) illustrates that animal myoglobins, plant non-symbiotic hemoglobins, and plant leghemoglobins all adopt the same globin fold and are structurally very similar. Furthermore, animal myoglobins, plant non-symbiotic hemoglobins, and plant leghemoglobins all bind the identical heme prosthetic group, heme B (Figure 1M).

The minimum temperature of denaturation for soy leghemoglobin, determined by Impossible Foods using dynamic light scattering, is 64 degrees Celsius (Figure 2A). Dynamic light scattering measures the mean effective diameter (Stokes radius) of a protein as a function of temperature. Increased Stokes radius indicates protein denaturation and aggregation. Protein denaturation leads to dissociation of the protein polypeptide from the heme co-factor. The denaturation temperature for soy leghemoglobin is similar to equine myoglobin, which Impossible Foods determined to be 70 degrees Celsius using dynamic light scattering. Lysozyme was included as a control and displayed the expected denaturation temperature of 72 degrees Celsius. The denaturation temperature for bovine myoglobin is 74 degrees Celsius (Sepe et al. 2005). The USDA recommended cooking temperature for ground beef is 160 degrees Fahrenheit (71 degrees Celsius). Impossible Foods' meat analogue is cooked at a similar temperature. Therefore, both mammalian myoglobins and leghemoglobin are denatured when consumed in a cooked meat or meat analogue product, respectively.

Proteins within the globin family typically denature and dissociate from their heme co-factor at pH <4. For example, at pH 3.2, human myoglobin dissociates from its heme co-factor in 45 seconds (Konermann et al 1997). To monitor heme-binding of the leghemoglobin polypeptide as a function of pH, Impossible Foods monitored the absorption spectra of the Soret region (Figure 2B). At pH 7, the heme co-factor is bound to the folded leghemoglobin polypeptide, as indicated by the narrow Soret peak at ~415 nm. At pH 2, the heme co-factor has dissociated from the denatured polypeptide, as indicated by the broad Soret peak at ~380 nm. Therefore, even if consumed in a raw meat analogue product, leghemoglobin will denature and release the heme co-factor upon exposure to the low pH environment of gastric fluid.

Leghemoglobins, non-symbiotic hemoglobins, and myoglobins each contain the identical heme b co-factor (Figure 1M). Soybean leghemoglobin does not contain peptide sequences that are associated with allergenicity (Annex 8), denatures at 64 degrees Celsius (Figure 2A) and pH 2 (Figure 2B), and is completely digested by pepsin (Annex 10), leaving only the heme cofactor. Therefore, the health effects of ingesting soybean leghemoglobin should be equivalent to non-symbiotic plant hemoglobins and mammalian myoglobins, which are readily consumed in the diet.

References:

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Figure 1. Plant hemoglobins and animal myoglobins adopt the same structural fold. Individual plant leghemoglobins (A-B), plant non-symbiotic hemoglobins (C-E), and animal myoglobins (F-H), are shown in ribbon representation colored in gray, heme porphyrin ring is shown in red stick representation, and iron in blue CPK representation. Superposition of individual proteins shows that the 3D structure of soybean leghemoglobin is highly similar leghemoglobins, non-symbiotic hemoglobins, and myoglobins from different species (I-L).

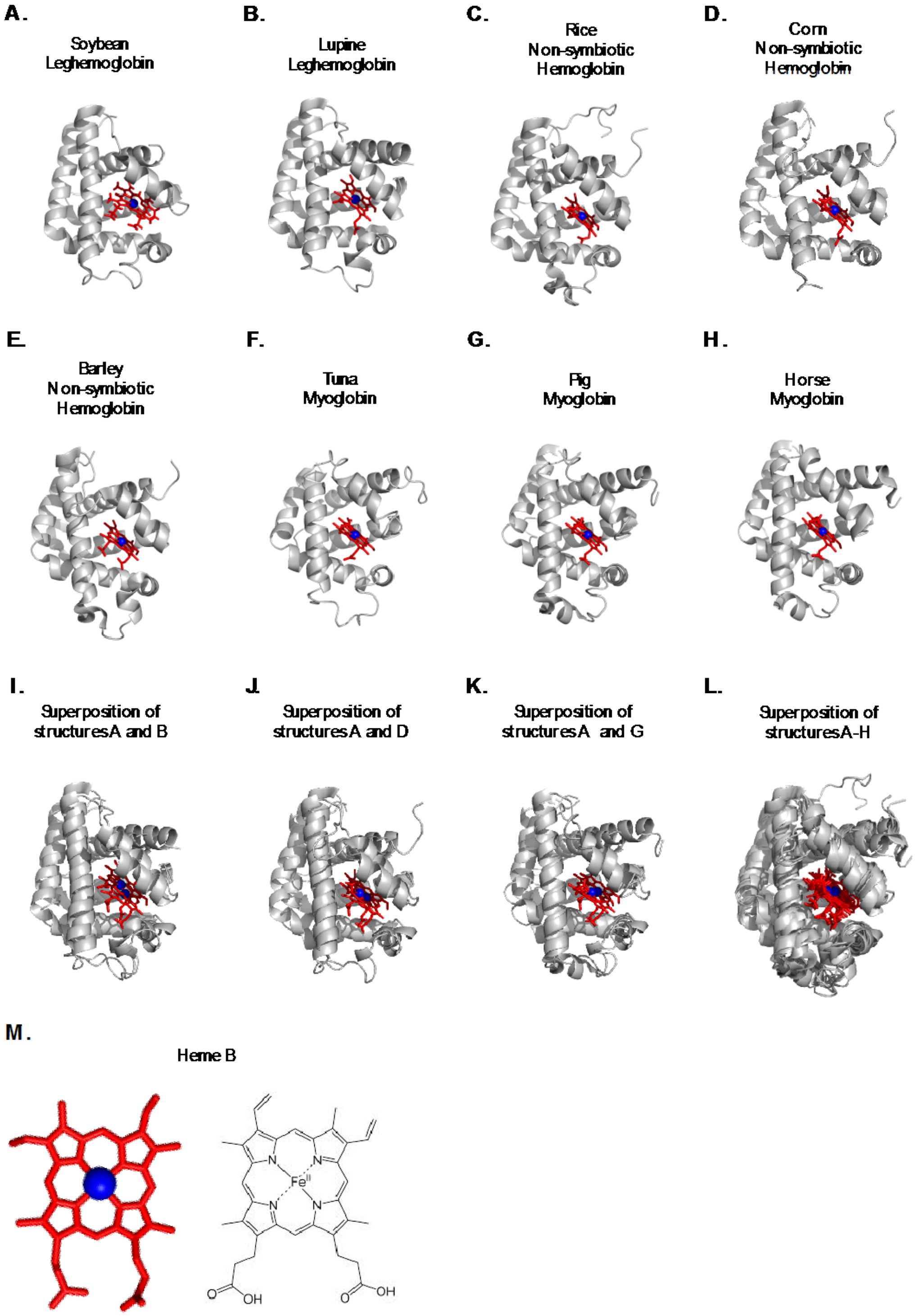

Table 1. Structural comparison between plant hemoglobins and animal myoglobins. Root-mean-square-deviation (RMSD) between all backbone atoms of superimposed X-ray crystallography protein structures (respective PDB codes are shown in parenthesis).

Species		RMSD (Å)
Soybean leghemoglobin (1BIN)	Horse myoglobin (1YMB)	4.5
Soybean leghemoglobin (1BIN)	Pig myoglobin (1PMB)	4.4
Soybean leghemoglobin (1BIN)	Tuna myoglobin (1MYT)	3.6
Soybean leghemoglobin (1BIN)	Barley non-symbiotic hemoglobin (2OIF)	2.5
Soybean leghemoglobin (1BIN)	Corn non-symbiotic hemoglobin (2R50)	1.0
Soybean leghemoglobin (1BIN)	Rice non-symbiotic hemoglobin (1D8U)	1.0
Soybean leghemoglobin (1BIN)	Lupine leghemoglobin (2GDM)	0.8
Soybean leghemoglobin (1BIN)	Soybean leghemoglobin (1FSL)	0.5

Figure 2. Leghemoglobin sensitivity to temperature and pH. (A) Impossible Foods measured the melting temperature of leghemoglobin using dynamic light scattering. Equine myoglobin (Sigma, cat# M0360) and Lysozyme (Sigma, cat# L4919) were included as controls. (B) Impossible Foods monitored heme dissociation from leghemoglobin at low pH by measuring the absorption spectra of the Soret region.