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Characterization of monomeric soybean leghemoglobin using Mössbauer spectroscopy with a high velocity resolution

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Abstract A preliminary investigation of monomeric soybean leghemoglobin *a* in oxy- and deoxy-forms was carried out for the first time using Mössbauer spectroscopy with a high velocity resolution at 90 K. Mössbauer parameters of measured spectra were evaluated and compared with that for the results of the well-known monomeric heme protein, myoglobin.

Keywords Mössbauer spectroscopy; Soybean leghemoglobin; Heme iron stereochemistry

1 Introduction

Leghemoglobins are a class of monomeric plant hemoglobins which transports and scavenges molecular oxygen in rood nodules for their function in nitrogen fixation [1–3]. Such function requires that leghemoglobins have almost 20-fold higher affinity for oxygen than myoglobin [4], in spite of sharing a similar overall structure and globin fold to the paradigm globin (Fig. 1). In general, monomeric hemoglobins involved in oxygen transport and storage exhibit higher oxygen affinity than their tetrameric hemoglobin counterparts.

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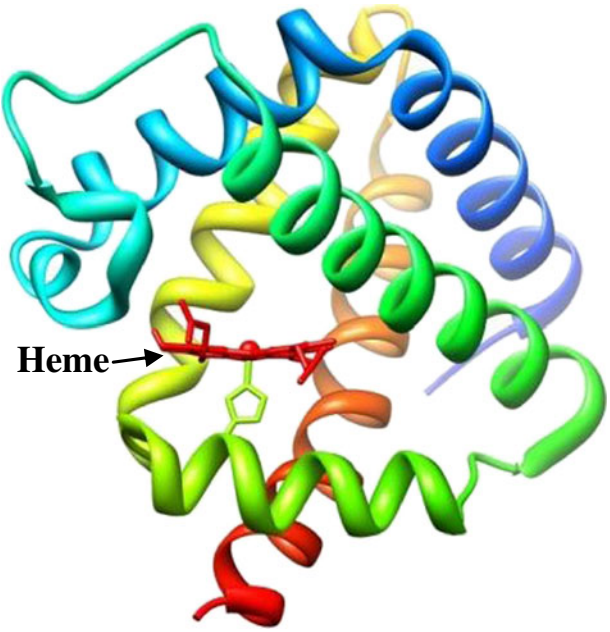

Fig. 1 Structure of soybean leghemoglobin *a* (PDB ID used: 1BIN)

Tetrameric hemoglobins with typical three-dimensional conformation and different molecular structure demonstrate different oxygen affinity related in part to the features of the heme iron electronic structure and stereochemistry [5–8]. Similarly, different monomeric hemoglobins demonstrate variations in oxygen affinity due to their heme pocket architecture [4, 9]. It is expected that subtle differences in oxygen affinity in monomeric hemoglobins would also arise due to differences in their heme iron electronic structure and stereochemistry. Such subtle differences are best probed by Mössbauer spectroscopy. Till date, however, Mössbauer spectroscopy was applied mostly for tetrameric hemoglobins and only for myoglobin as a case for monomeric mammalian hemoglobin (see, for instance, [10–16]). Therefore, it is essential and imperative that investigation of the other monomeric hemoglobin be performed for a better understanding of heme iron stereochemistry in this class of globins. In this work we present the first results of the study of monomeric soybean leghemoglobin *a* (Lba) using Mössbauer spectroscopy with a high velocity resolution.

2 Experimental

Soybean leghemoglobin *a* gene was expressed in *E. coli* and purified by an assortment of standard chromatographic methods in large scale in the ferric form [17]. For Mössbauer measurements, purified protein was concentrated and both oxygenated (oxy-Lba) and deoxygenated (deoxy-Lba) forms were used. The deoxy-form was produced by addition of stoichiometric concentrations of sodium dithionite to the ferric form of Lba. For oxy-Lba, the sodium dithionite reduced ferrous protein was desalted on a Sephadex G-25 column under positive oxygen pressure and the resulting oxy-Lba solution was further saturated with oxygen. Both leghemoglobin solutions were prepared using 20 mM potassium phosphate

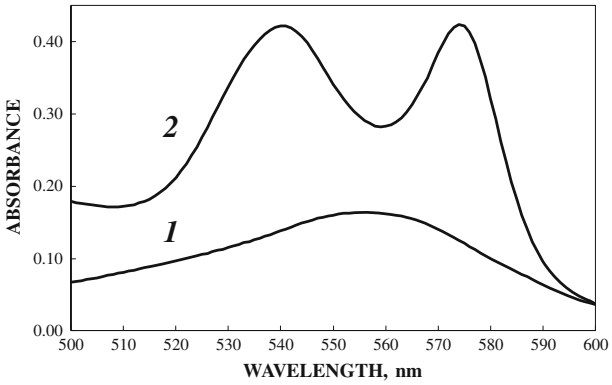

Fig. 2 A part of absorbance spectra of deoxy-form (***1***) and oxy-form (***2***) of soybean leghemoglobin prepared for Mössbauer measurements demonstrated difference in Q-bands

buffer with pH = 7.0. Concentrated solutions were placed in Plexiglas holders with a volume of about 9 ml and immediately frozen with liquid nitrogen. Additionally, these protein solutions were analyzed using absorbance spectroscopy. Absorbance spectra demonstrated well-known bands characteristic for both deoxy-Lba and oxy-Lba (see Q-band region in Fig. 2). Soret peaks for deoxy-Lba and oxy-Lba were 426 and 411 nm, respectively, while Q-band 1 was 555 nm for deoxy-Lba and Q-bands 1 and 2 were 540 and 574 nm for oxy-Lba.

Mössbauer spectra were measured using an automated precision Mössbauer spectrometric system built on the base of the SM-2201 spectrometer with a saw-tooth shape velocity reference signal formed using quantification with 4096 steps. Details and characteristics of this spectrometer and the system were given elsewhere [18–20]. The 1.8×10^9 Bq ^{57}Co in rhodium matrixes (Ritverc GmbH, St. Petersburg) was used at room temperature. The Mössbauer spectra were measured in transmission geometry with moving absorber in the cryostat at 90 K and recorded in 4096 channels. Spectra of Lba samples were measured in velocity ranges of about $\pm$2.5 mm/s for oxy-Lba and of about $\pm$3.5 mm/s for deoxy-Lba. For their analysis, spectra were converted into 1024 channels by a consequent summation of four neighboring channels. Statistical count rate in the deoxy-Lba and oxy-Lba spectra presented in 1024 channels was $\sim 6.0 \times 10^6$ and $\sim 7.2 \times 10^6$ counts per channel and the signal-to-noise ratio was 19 and 24, respectively. The spectra were computer fitted with the least squares procedure using UNIVEM-MS program with a Lorentzian line shape. The spectral parameters such as: isomer shift, δ, quadrupole splitting, ΔE_Q, line width, Γ, relative subspectrum area, S, and statistical quality of the fit, χ^2, were determined. An instrumental (systematic) error for each spectrum point was $\pm$0.5 channel (the velocity scale), the instrumental (systematic) error for the hyperfine parameters was $\pm$1 channel. If an error calculated with the fitting procedure (fitting error) for these parameters exceeded the instrumental (systematic) error we used the larger error instead. Values of δ are given relative to α–Fe at 295 K.

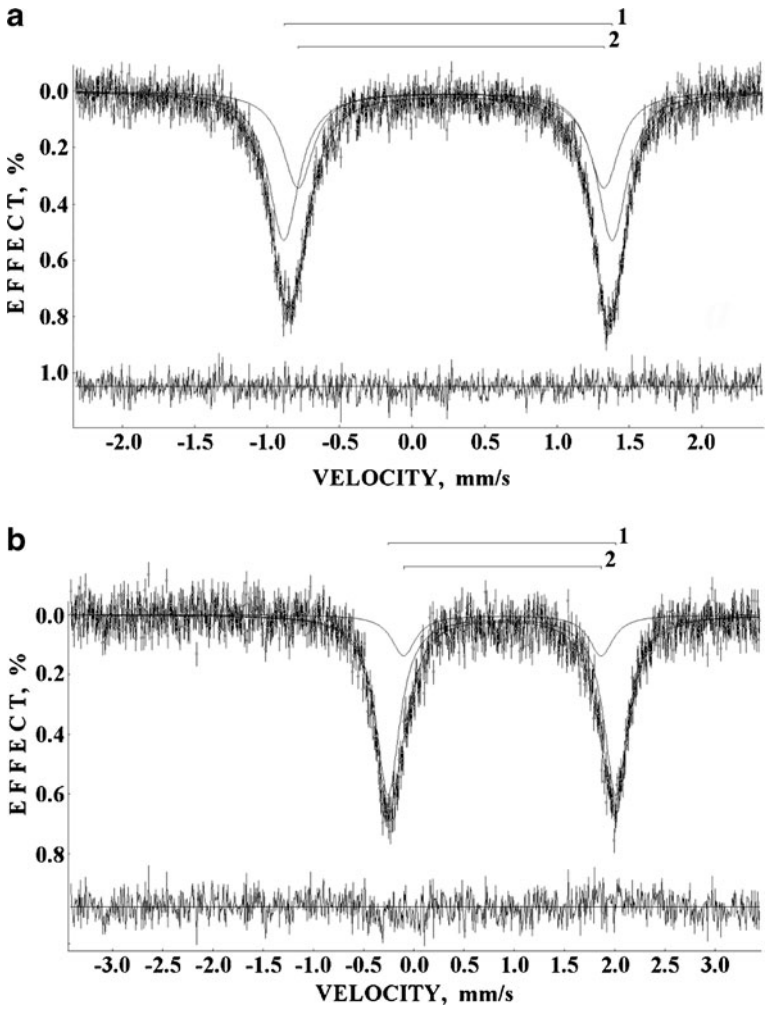

Fig. 3 Mössbauer spectra of soybean leghemoglobin in the oxy-form (**a**) and deoxy-form (**b**) measured at T = 90 K and presented in 1024 channels. 1 and 2 are components obtained from the best fit. Differential spectra are shown below

3 Results and discussion

Soybean leghemoglobin *a* consists of one polypeptide chain (Fig. 1) which binds heme, an iron (II)–porphyrin complex, similar to myoglobin. Mössbauer spectra of oxy- and deoxy-forms of Lba are shown in Fig. 3. These spectra demonstrate two absorption peaks and are similar to well-known spectra of oxy- and deoxy-myoglobin [11, 13], as expected for monomeric hemoglobins belonging to the class of oxygen transporters. However, the best fits of both Lba spectra were performed using two quadrupole doublets on the basis of differential spectra instead of one doublet fit used for myoglobin spectra fits previously (see, for instance, [11, 13]). Two quadrupole doublets for the oxy-Lba Mössbauer spectrum were obtained with supposition of equal line widths. Mössbauer hyperfine parameters for these

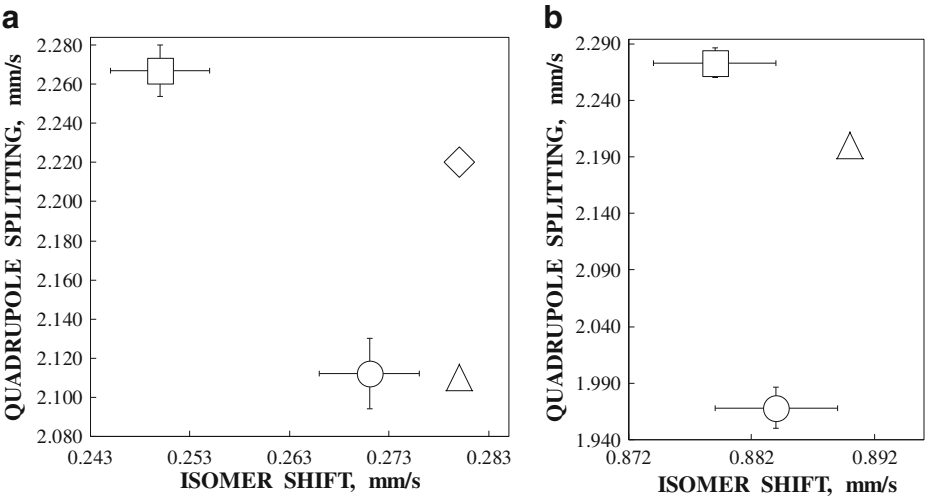

Fig. 4 Comparison of Mössbauer hyperfine parameters for oxy- and deoxy-forms of monomeric hemoglobins (**a**): □ – component 1 for oxyleghemoglobin, ◯ – component 2 for oxyleghemoglobin, △ – sperm whale oxymyoglobin, ◊ – horse oxymyoglobin; and (**b**): □ – component 1 for deoxyleghemoglobin, ◯ – component 2 for deoxyleghemoglobin, △ – deoxymyoglobin. Data for myoglobins were taken from [11, 13]

quadrupole doublets are shown in the plot of quadrupole splitting and isomer shift (Fig. 4a) in comparison with parameters obtained for sperm whale and horse oxymyoglobins at 100 K in [11] (it is relevant to mention here that: a) these data were obtained in applied magnetic field; b) temperature dependences of isomer shift and quadrupole splitting in the temperature range of 100–90 K did not change the values of these parameters beyond the instrumental errors). It is clearly seen that hyperfine parameters for both quadrupole doublets of soybean oxy-leghemoglobin are different from those of sperm whale and horse oxymyoglobins. Mössbauer hyperfine parameters for deoxy-Lba are also presented in the plot of quadrupole splitting and isomer shift (Fig. 4b) in comparison with parameters obtained for deoxymyoglobin in [13] with unknown protein origin and temperature of measurement. These parameters are also different for deoxy-Lba and deoxy-myoglobin.

Taking into account relationship of Mössbauer parameters and heme iron electronic structure and stereochemistry we can conclude that heme iron environments in soybean leghemoglobin *a* and in two myoglobins are different. A comparison of structural and functional differences of soybean leghemoglobin and myoglobin was considered previously in [17, 21] and the heme pocket architectural differences in these two proteins in both proximal and distal regions is shown in Fig. 5.

As for two different components in oxy-Lba and deoxy-Lba, it should be noted that there were no contaminations in the samples or Lba oxidation in the oxy-form with metform or hemichromes formation as investigated from their absorbance spectra (see Fig. 2). Nevertheless, it was interesting that relative areas of components 1 and 2 were different for oxy-Lba and deoxy-Lba (Fig. 6). On the basis of different hyperfine parameters for the components 1 and 2 for the oxy-Lba Mössbauer spectrum we can suppose some differences in the heme iron – molecular oxygen bond. It is possible that there are two different orientations of distal His E7 that result in different strength of hydrogen bonding with oxygen molecule in the heme pocket leading to two spectral components with relative area ratio of

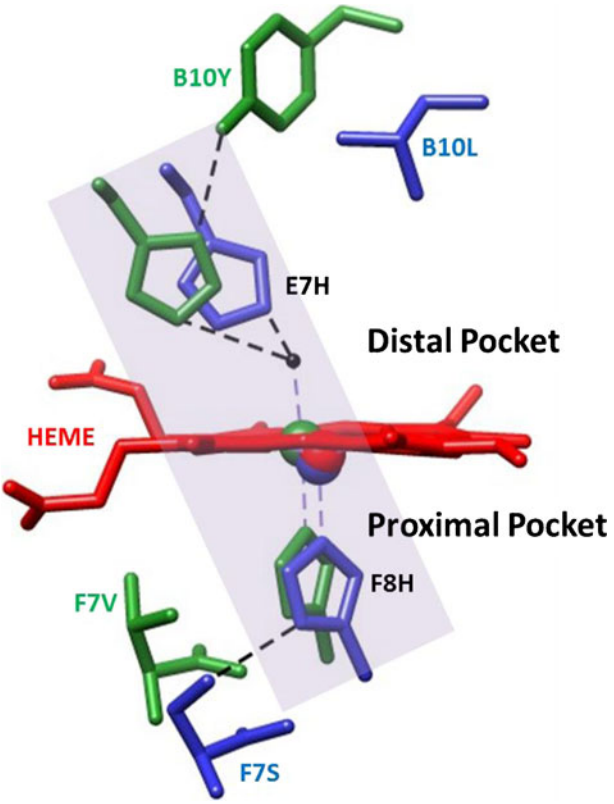

Fig. 5 Structural differences in heme iron environment in myoglobin (*blue*) and soybean leghemoglobin (*green*)

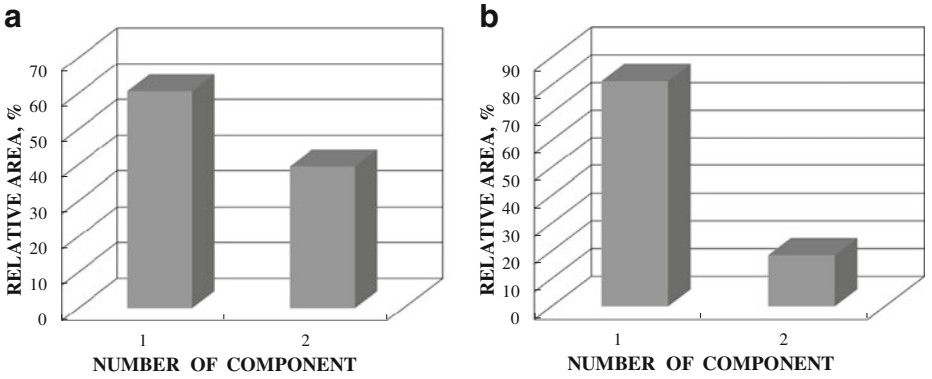

Fig. 6 Comparison of relative areas for the components 1 and 2 obtained from the fits of Mössbauer spectra of soybean oxyleghemoglobin (**a**) and deoxyleghemoglobin (**b**)

about 60 %:40 % that corresponds to probabilities of these orientations. Recently, it was shown that two conformations for oxy-Lba were possible for the distal His E7 [22] that confirmed our supposition. Similarly, different hyperfine parameters obtained for two components in the deoxy-Lba Mössbauer spectrum may indicate some differences in the heme iron – proximal His F8 bond. It is possible that two different orientations of imidazole ring of the proximal His F8 with different probabilities led to two spectral components with relative area ratio of about 80 %:20 %.

4 Conclusion

The first study of soybean leghemoglobin in the oxy- and deoxy-forms using Mössbauer spectroscopy with a high velocity resolution was successfully carried out. The results showed that Mössbauer hyperfine parameters for soybean leghemoglobin were slightly different from those obtained for myoglobins by other authors that correlated with structural differences of these proteins in the heme region. It is also suggested that soybean leghemoglobin may have two different conformations in the heme regions in both oxy- and deoxy-forms with different probabilities.

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