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Soybean dihydrolipoamide dehydrogenase (ferric leghemoglobin reductase 2) interacts with and reduces ferric rice non-symbiotic hemoglobin 1§

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Abstract

Ferrous oxygenated hemoglobins ($Hb^{2+}O_2$) autoxidize to ferric Hb^{3+}, but Hb^{3+} is reduced to Hb^{2+} by enzymatic and non-enzymatic mechanisms. We characterized the interaction between the soybean ferric leghemoglobin reductase 2 (FLbR2) and ferric rice non-symbiotic Hb1 ($Hb1^{3+}$). Spectroscopic analysis showed that FLbR2 reduces $Hb1^{3+}$. Analysis by tryptophan fluorescence quenching showed that FLbR2 interacts with $Hb1^{3+}$, however the use of ITC and IEF techniques revealed that this interaction is weak. *In silico* modeling showed that predicted FLbR2 and native $Hb1^{3+}$ interact at the FAD-binding domain of FLbR2 and the CD-loop and helix F of $Hb1^{3+}$.

Keywords

Fluorescence quenching; Hemoglobin; Isothermal calorimetry; Non-symbiotic; *Oryza sativa*; Protein-protein interaction

1. Introduction

Hemoglobins (Hbs) are O_2-binding proteins that have been identified in organisms from the three kingdoms of life [1–3]. In plants Hbs are classified into symbiotic, nonsymbiotic and 2/2-like Hbs [4, 5]. Symbiotic Hbs (or leghemoglobins (Lbs) when isolated from legumes)

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have a high affinity for O_2, are synthesized in the nodules of N_2- fixing plants, and their apparent function is to facilitate the diffusion of O_2 to bacteroids [6–8]. Transcripts for 2/2-like plant Hbs have been detected in organs from plants growing in normal and stressed conditions; kinetic analysis suggested that 2/2-like plant Hbs may function as O_2-carriers [9]. Non-symbiotic Hbs (nsHbs) are localized in specific tissues of plant organs [10, 11]. A distinctive characteristic of some plant nsHbs is that their affinity for O_2 is very high, mainly due to a very low O_2-dissociation rate constant [5, 12–14]. The properties of high O_2-affinity nsHbs suggest that the function of these proteins is diverse [4, 15–18].

Only ferrous (reduced) Hbs (Hbs^{2+}) bind O_2, however oxygenated Hbs may autoxidize to ferric Hb (Hb^{3+}) [19]. Ferric Hb is unable to bind and transport O_2, thus O_2-carrying Hbs should be maintained in the Hb^{2+} form. Enzymatic and non-enzymatic mechanisms exist in organisms to reduce Hb^{3+} to Hb^{2+}. In plants, mechanisms that reduce Lb^{3+} to Lb^{2+} have been described in N_2-fixing legumes [20]. Specifically, lupin, soybean and cowpea Lb^{3+} reductases were characterized and showed that these proteins reduce Lb^{3+}, that this reduction is a NADH-dependent reaction, and that resulting Lb^{2+} was able to bind O_2 [21–24]. A soybean cDNA coding for ferric Lb reductase 2 (FLbR2) was expressed in *Escherichia coli* and it was showed that recombinant FLbR2 reduces soybean $Lb a^{3+}$ [24]. The amino acid sequence of FLbR2 is 89% identical to the sequence of a pea dihydrolipoamide dehydrogenase (DLDH), thus FLbR2 was classified as a soybean DLDH with Lb^{3+} reductase activity [24].

Plant nsHbs are O_2-binding proteins that may interact with cell molecules [4, 25, 26], thus a possibility is that $nsHbs^{3+}$ interact with plant reductases to be reduced to $nsHb^{2+}$. This work reports the characterization of the interaction between soybean FLbR2 and a ferric rice nsHb1 ($Hb1^{3+}$). Results showed that FLbR2 reduces $Hb1^{3+}$ and that FLbR2 interacts weakly with $Hb1^{3+}$.

2. Experimental Details

2.1. Purification of recombinant proteins

2.1.1. Purification of recombinant soybean FLbR2—Recombinant FLbR2 was obtained by following the procedure described by Moran et al. [24] with minor modifications. Briefly, *E. coli* BL21(DE3) cells (Novagen) containing the pET-28a(+)::FLbR2 construct [24] were grown in ZYP-5052 broth containing a 1000× metals mix and kanamycin [27]. Cells were harvested, sonicated and incubated overnight at 4°C after the addition of DNase, RNase and lysozyme. The resulting solution was cleared by centrifugation, the supernatant was chromatographed on a His Trap HP Ni-chelating resin (GE Healthcare), and the yellowish fraction was collected, dialyzed and lyophilized or frozen at −80°C until used. Protein purification was verified by SDS-PAGE [28], and protein was quantitated by using a dye-binding assay (Bio-Rad) and bovine seric albumin as standard.

2.1.2. Purification of recombinant rice $Hb1^{3+}$—Recombinant Hb1 was purified from *E. coli* $pEMBL18^{+}$::rHb1 by following standard procedures [12]. Briefly, the cell paste was sonicated and incubated at 4°C as above (see subsection 2.1.1.). The resulting solution was

cleared by centrifugation and the supernatant was fractionated with ammonium sulphate (40–90% saturation), and chromatographed on a Phenyl-Sepharose (Pharmacia-Amersham) and DEAE. Cellulose (DE52, What man) resins. Recombinant Hb1 was purified to near homogeneity by isoelectric focusing (IEF, in the pH range 5–8) (see subsection 2.5.). The recombinant Hb1 was quantified on the heme basis using the dipyridine.hemochrome assay [29]. Ferric Hb1 was prepared by the addition of K ferricyanide to a final concentration of 5 mM. Recombinant soybean Lb*a* [30] was isolated following the procedure described above for the isolation of recombinant Hb1.

2.2. Visible spectroscopy analysis

The activity of FLbR2 was assayed according to the procedure described by Saari and Klucas [31]. The reduction of soybean Lba^{3+} or rice Hb1^{3+} by FLbR2 was followed spectroscopically using a reaction mixture that contained 0.6 μM FLbR2, 50 μM Lba^{3+} or Hb1^{3+} and 500 μM NADH in a final volume of 60 μl of 50 mM Na phosphate buffer (pH 6.5). All experiments were performed by triplicate.

2.3. Fluorescence spectroscopy analysis

Fluorescence measurements were performed in a 1 cm path length cuvette on an SPEX FluoroMax fluorometer (Jobin Yvon Horiba) at 30°C, and using the dM3000 software. Fluorescence data were collected with the tryptophan excitation emission setup at 295 nm. The emission spectra were recorded from 300 to 400 nm. Spectra were acquired by averaging three scans collected at the rate of 1 nm·s^{-1}. The concentration of Hb1^{3+} was 50 μM throughout the experiment, whereas the concentration of FLbR2 was increased from 0.6 to 3.6 μM. The cumulative emission, due to the tryptophan fluorescence of individual FLbR2 and Hb1^{3+} and buffer (50 mM Na phosphate (pH 6.5)), was used as the control. Final spectra were calculated from the fluorescence difference between the individual FLbR2 and Hb1^{3+} and buffer effects and the interacting FLbR2 and Hb1^{3+}. The emission intensities were corrected for dilution effects.

2.4. Isothermal titration calorimetry analysis

A Micro Calorimetry System (Microcal) was used for the ITC measurements of interaction between FLbR2 and Hb1^{3+}. All measurements were performed at 30°C. A 50 mM Na phosphate (pH 7.0) buffered solution of FLbR2 (10 μM) was injected, in 10 μl increments, into a 2.5 ml volume of buffered Hb1^{3+} (50 μM); a stirring speed of 200 rpm was maintained during the experiment and injection intervals were 5 min apart. Protein samples were exchanged in the same buffer prior to data collection and data were collected on 5 independent reproducible sample sets. Control data of FLbR2 into phosphate buffer were used for normalization against dilution effects. Data were analyzed using the ORIGIN data analysis software (Microcal Software).

2.5. Isoelectric focusing analysis

Analytical IEF gel electrophoresis was performed according to the procedure described by the supplier (Bio-Rad, Mini PROTEAN 3) and Jun et al. [32]. Four percent polyacrylamide/ 2.5% *bis*-acrylamide and 2% ampholytes (Bio-Lyte 3/10, 4/6) were used in the IEF gel. The

reaction mixture contained 0.6–1.2 μM FLbR2, 0.75–1.54 μM $Hb1^{3+}$ and 500 μM NADH. Aliquots of the reaction mixture, pure $Hb1^{3+}$ and FLbR2 and IEF standards (bovine seric albumin (pI 4.9), human carbonic anhydrase (pI 6.6) and sperm whale myoglobin (pI 7.0)) (Sigma-Aldrich) were loaded onto the gel and electrofocused at a gradient voltage of 150–400 V for about 3 h. After focusing the gel was stained with Coomassie Blue.

2.6. In silico modeling

The structure of soybean FLbR2 was predicted by using the crystal structure of pea DLDH (PDB ID **1DXL**) as template. The pea DLDH and soybean FLbR2 amino acid sequences (GenBank accession numbers **X63464** and **AF074940**, respectively) were aligned using the Homology module of the Insight II program (Accelrys, Inc.), and amino acids were automatically substituted. The best rotamer for all side chains was searched automatically using the default parameters of the Homology module. The energy of the whole structure was minimized (100 steps of the steepest descent minimization) using the Discovery force field from Insight II, and the best rotamers for all amino acid side chains were searched again. The refined FLbR2 model was submitted to the ElNémo web server (http://igs-server.cnrs-mrs.fr/elnemo, last accessed on May, 2012) to identify low frequency motions that may be associated to the binding of $Hb1^{3+}$ to FLbR2. Docking between predicted FLbR2 and native $Hb1^{3+}$ (PDB ID **1D8U**) was calculated using the ZDOCK program (http://zlab.bu.edu/zdock/, last accessed on May, 2012). Images were edited using the VMD program [33].

3. Results and Discussion

Plant non-symbiotic Hbs^{2+} bind O_2 [12–14], however $nsHb^{3+}$ is unable to bind and transport O_2, thus O_2-carrying nsHbs should be maintained in the $nsHb^{2+}$ form. In plants, reductases that reduce Lb^{3+} to Lb^{2+} and Hb^{3+} to Hb^{2+} have been described in legumes [21–24] and barley [25], respectively. The objective of this work was to characterize the interaction of soybean FLbR2 with rice $Hb1^{3+}$. However, before analyzing the reduction of $Hb1^{3+}$ we checked the functionality of the recombinant FLbR2 purified in this work by reducing soybean Lba^{3+}. Figure 1 shows that FLbR2 reduces Lba^{3+} in the presence of NADH: Lba^{3+} was reduced by NADH but the rate of reduction increased in the presence of FLbR2, this showed that the reduction of Lba^{3+} is due not only to NADH but to FLbR2. Thus, the recombinant FLbR2 used in this work was functional.

3.1. Soybean FLbR2 reduces rice $Hb1^{3+}$

Spectroscopic analysis showed that FLbR2 reduces $Hb1^{3+}$ (Figure 2). Reactions as a function of time were monitored for the reduction of $Hb1^{3+}$ in the 450–650 nm range. The absorbance at 543 and 576 nm, which was contributed by $Hb1^{2+}O_2$, increased as a function of time. This evidence showed that the resulting $Hb1^{2+}$ is oxygenated after the reduction of $Hb1^{3+}$ by FLbR2. The spectroscopic characteristics for the reduction of $Hb1^{3+}$ by FLbR2 were similar to those reported for the reduction of soybean [31] and cowpea [23] Lbs^{3+} by FLbRs.

3.2. Soybean FLbR2 interacts with rice $Hb1^{3+}$

Rice Hb1 contains Trp25, 138 and 146, whereas soybean FLbR2 contains Trp196. We used the tryptophan fluorescence quenching technique to investigate an observable interaction between FLbR2 and $Hb1^{3+}$. Preparations of 50 μM $Hb1^{3+}$ and 0.6–3.6 μM FLbR2 were used as blanks throughout the evaluations due to the possibility of fluorescence quenching from potential FLbR2- FLbR2 and $Hb1^{3+}$-$Hb1^{3+}$ interactions. Thus, the observed fluorescence quenching resulted only from the FLbR2-$Hb1^{3+}$ interaction and not from any FLbR2-FLbR2 and $Hb1^{3+}$-$Hb1^{3+}$ interactions. Results showed that tryptophan fluorescence intensity decreased about 30% after the addition of 0.6 μM FLbR2 to 50 μM $Hb1^{3+}$, and that the fluorescence quenching slightly decreased after the addition of 1.2–3.6 μM FLbR2 to the $Hb1^{3+}$ preparation (Figure 3). This observation showed that FLbR2 interacted with $Hb1^{3+}$.

3.3. Interaction of soybean FLbR2 with rice $Hb1^{3+}$ is weak

The strength of the interaction between FLbR2 and $Hb1^{3+}$ was evaluated by ITC and IEF techniques. ITC measures the amount of heat released when two molecules interact. As indicated in the subsection 3.2., preparations of FLbR2 and $Hb1^{3+}$ were used as controls throughout the evaluations because of potential FLbR2-FLbR2 and $Hb1^{3+}$-$Hb1^{3+}$ interactions, thus the detected heat release resulted only from the FLbR2-$Hb1^{3+}$ interaction. The ITC titration for the FLbR2-$Hb1^{3+}$ interaction was compared to titrations for the yeast frataxin-Fe^{2+} [34], which is a μM protein-partner interaction, and calreticulin D317A mutant-trisaccharide [35], which is a non-interacting protein-partner, interactions. Figure 4A shows that heat is released upon the addition of FLbR2 to a $Hb1^{3+}$ preparation, and that heat release increases with the FLbR2/$Hb1^{3+}$ molar ratio. This observation shows that FLbR2 interacts with $Hb1^{3+}$, which is consistent with observations from the fluorescence quenching analysis (Figure 3). However, the amount of heat release is rather low for the FLbR2-$Hb1^{3+}$ interaction (~ −4 to −3 μcal/sec for a 50 min period, or ~ −12 to −10 kcal/mol for the FLbR2/$Hb1^{3+}$ molar ratio) (Figure 4A) compared to the yeast frataxin-Fe^{2+} interaction (~ −3.5 to −2.2 μcal/sec for a 50 min period, or ~ −30 to −5 kcal/mol for the frataxin/Fe^{2+} molar ratio), but higher than the calreticulin D317A mutant-trisaccharide interaction (~ −0.3 to −0.15 μcal/sec for a 60 min period, or ~ −0.7 to −0.2 kcal/mol for the calreticulin D317A mutant/trisaccharide molar ratio). This observation suggests that the interaction of FLbR2 with $Hb1^{3+}$ is weak. The strength of the interaction between FLbR2 and $Hb1^{3+}$ was also evaluated by detecting proteins shift in IEF gels. Figure 4B shows that no gel shift occurred when FLbR2 was incubated with $Hb1^{3+}$. Thus, results from the ITC and IEF analyses are consistent and are evidence that the interaction between FLbR2 and $Hb1^{3+}$ is weak.

3.4. In silico analysis of the soybean FLbR2-rice $Hb1^{3+}$ complex

In order to identify regions for the interaction between FLbR2 and $Hb1^{3+}$ we predicted the structure of FLbR2 and performed docking analysis for the (predicted) FLbR2 - (native) $Hb1^{3+}$ interaction. The structure of FLbR2 was predicted from the template crystal structure of a pea DLDH (PDB ID **1DXL**). Figure 5A shows that the structures of predicted FLbR2 and native pea DLDH are highly similar. Docking analysis predicted that FLbR2 and $Hb1^{3+}$ interact at the FAD-binding domain of FLbR2 and the CD-loop and helix F of $Hb1^{3+}$ (Figure 5B). Also, this model predicted a closed↔ open conformation for the FAD-binding

domain of FLbR2 upon interaction with $Hb1^{3+}$, which may allow accessibility of heme to $FADH_2$ for electron transfer. An interesting observation was that Tyr187 is reoriented from FAD to heme in the closed →open conformation of FLbR2 (Figure 5C), thus a possibility is that Tyr187 mediates the transfer of electrons from FLbR2 to $Hb1^{3+}$. These observations should be experimentally verified by co-crystallization and X-ray analysis of the FLbR2-$Hb1^{3+}$ complex.

4. Conclusions

The main conclusions from the results reported in this work are (i) that soybean FLbR2 interacts with and reduces to rice $Hb1^{3+}$, (ii) the interaction between FLbR2 and $Hb1^{3+}$ is weak, and (iii) that this interaction possibly occurs at the FAD-binding domain of FLbR2 and the CD-loop and helix F of $Hb1^{3+}$.

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Abbreviations

DLDH	dihydrolipoamide dehydrogenase
FLbR2	soybean ferric leghemoglobin reductase 2
Hb	hemoglobin
IEF	isoelectric focusing
ITC	isothermal titration calorimetry
Lb	leghemoglobin

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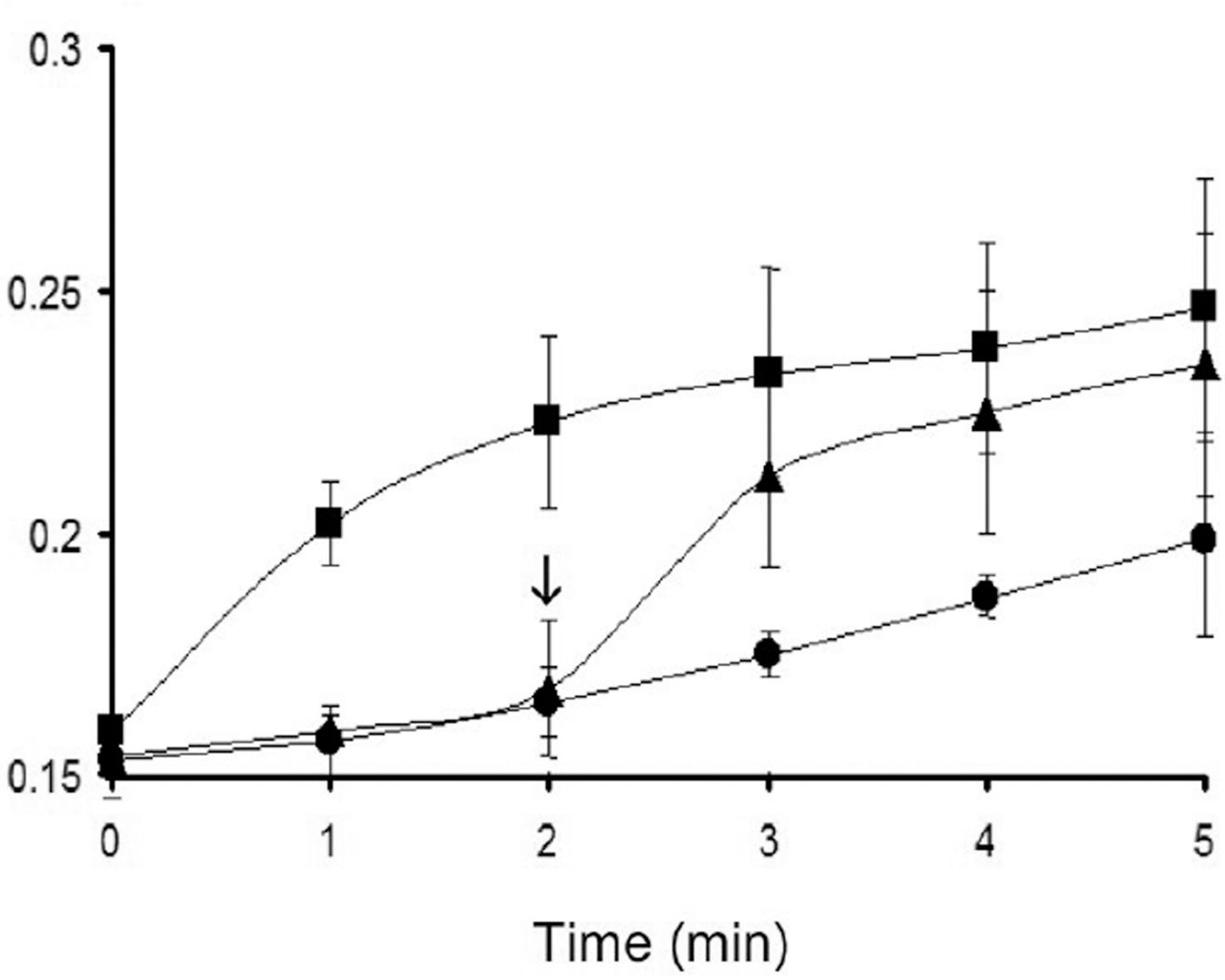

Figure 1.

Reduction of soybean Lba^{3+} by soybean FLbR2 and NADH. Reactions contained: 50 μM soybean Lba^{3+} + 500 μM NADH (●); 50 μM soybean Lba^{3+} + 500 μM NADH + 0.6 μM FLbR2 (■); and 50 μM soybean Lba^{3+} + 500 μM NADH + 0.6 μM FLbR2 added 2 min after incubation (arrow) (▲). Reduction of soybean Lba^{3+} was detected at 540 nm wavelength, which corresponds to the βpeak of oxygenated soybean Lba^{2+} [31].

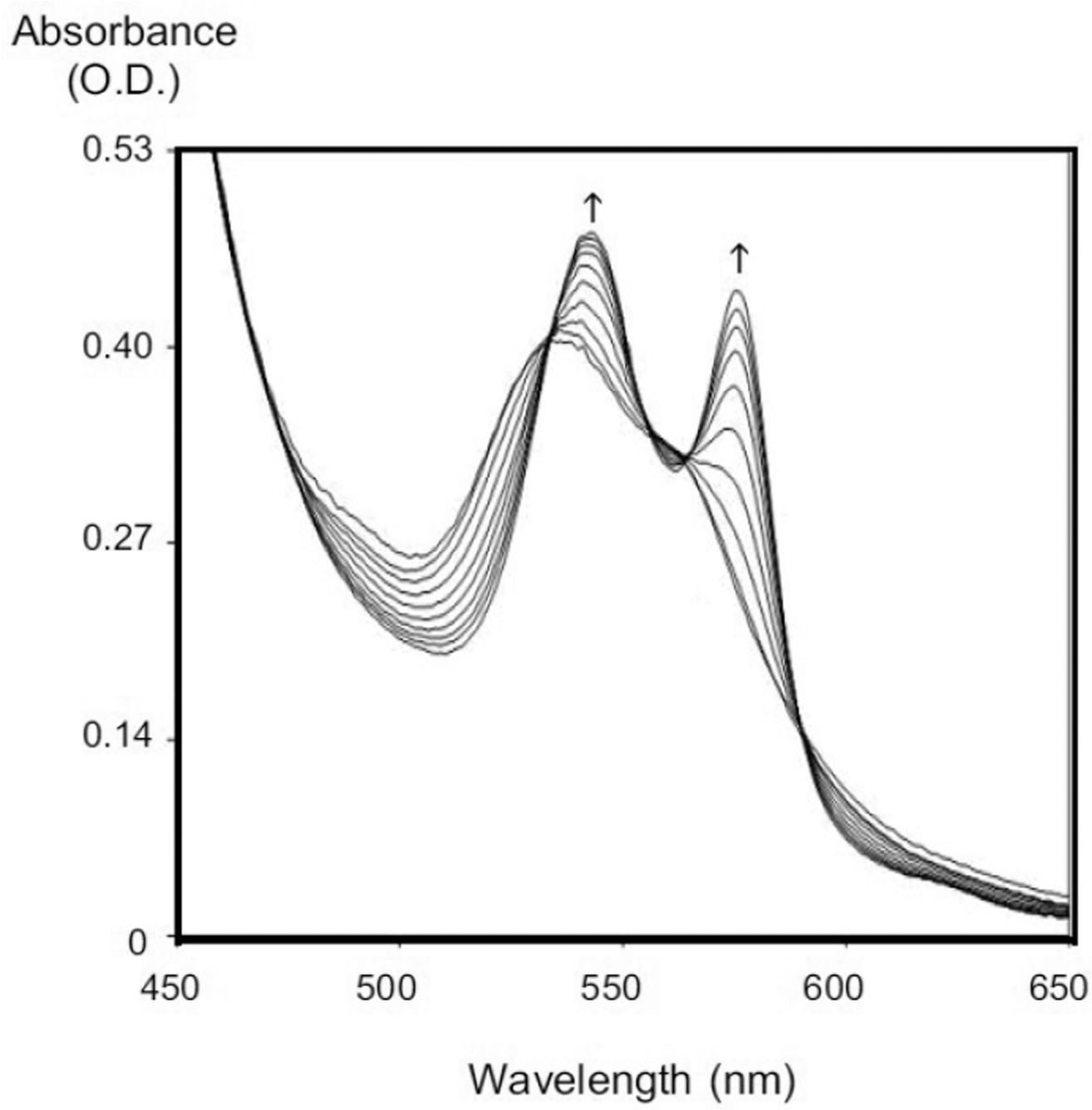

Figure 2.

Reduction of rice $Hb1^{3+}$ by soybean FLbR2. The reaction mixture contained 0.6 μM FLbR2, 50 μM $Hb1^{3+}$ and 500 μM NADH. Spectra were scanned at 2 min intervals. Upward arrows show the increase of absorbance for the αand βpeaks that occurs when $Hb1^{3+}$ is reduced and oxygenated to $Hb1^{2+}O_2$.

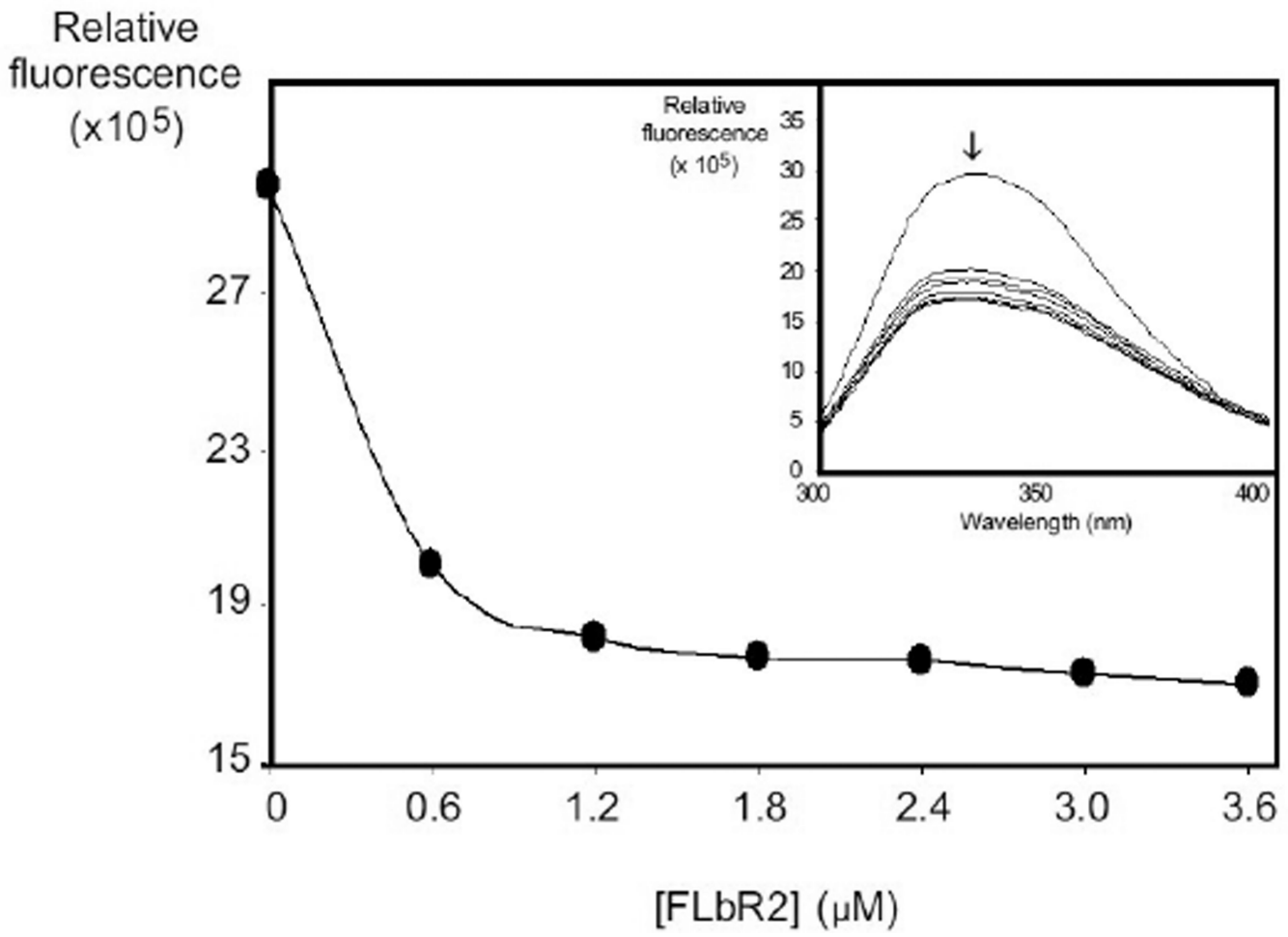

Figure 3.

Tryptophan fluorescence quenching for the soybean FLbR2-rice $Hb1^{3+}$ interaction. Excitation wavelength was 295 nm and emission of fluorescence was recorded at 340 nm. Inset, emission spectra of $Hb1^{3+}$ preparations with increasing amounts of FLbR2: upper spectrum, no addition of FLbR2 to the $Hb1^{3+}$ preparation; lower spectra, 0.6–3.6 μM FLbR2 added to the $Hb1^{3+}$ preparation; downward arrow shows the fluorescence decrease at 340 nm. The data shown here are representative of three replicates.

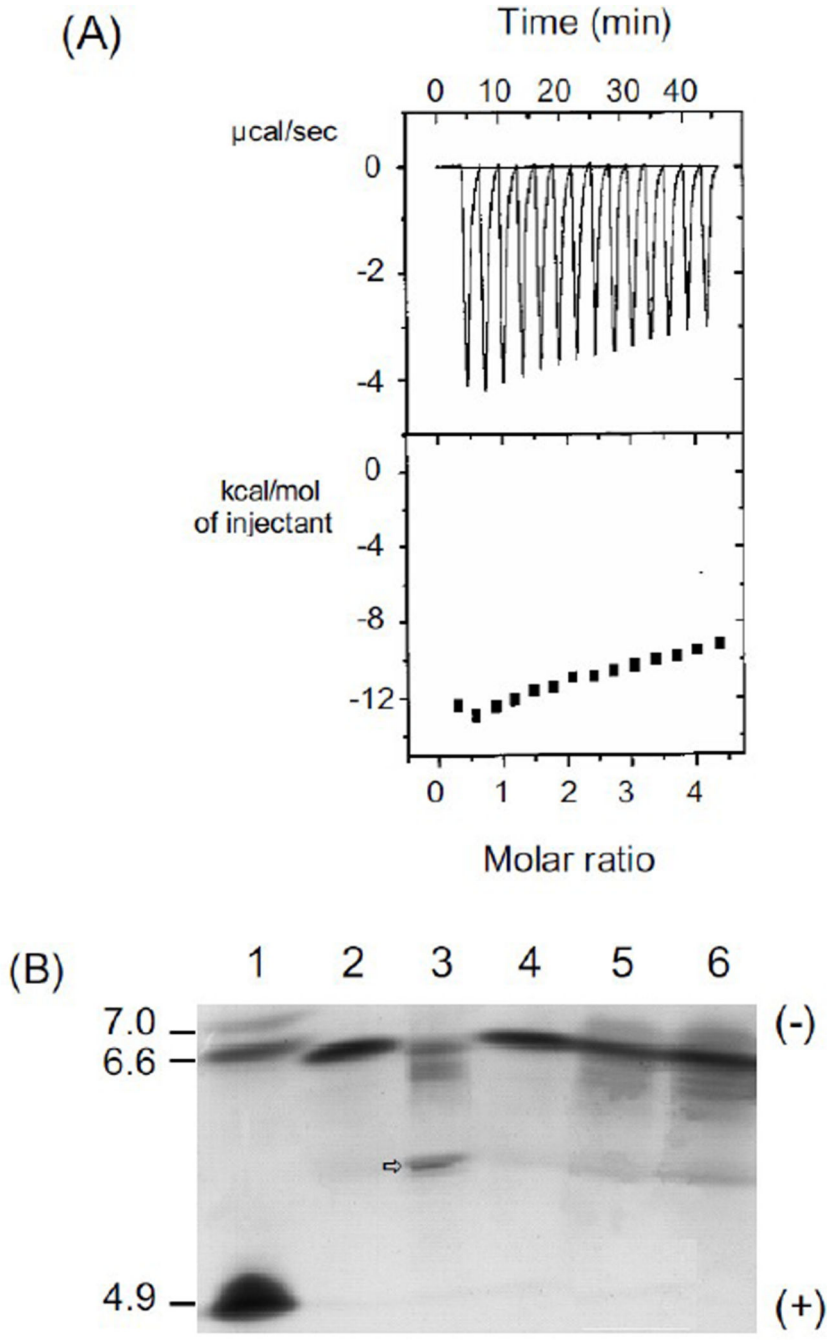

Figure 4.

(A) Isothermal titration calorimetry of the soybean FLbR2-rice $Hb1^{3+}$ interaction. Aliquots of 10 µM FLbR2 were injected into a 50 µM $Hb1^{3+}$ solution at 5 min intervals. (B) IEF analysis of the soybean FLbR2-rice $Hb1^{3+}$ interaction. Lane 1, IEF standards; lane 2, 1.54 µM $Hb1^{3+}$; lane 3, 1.2 µM FLbR2 (arrow); lane 4, 1.54 µM $Hb1^{3+}$ incubated with 500 µM NADH; lane 5, 0.75 µM $Hb1^{3+}$ incubated with 0.6 µM FLbR2 and 500 µM NADH; lane 6, 1.54 µM $Hb1^{3+}$ incubated with 1.2 µM FLbR2 and 500 µM NADH.

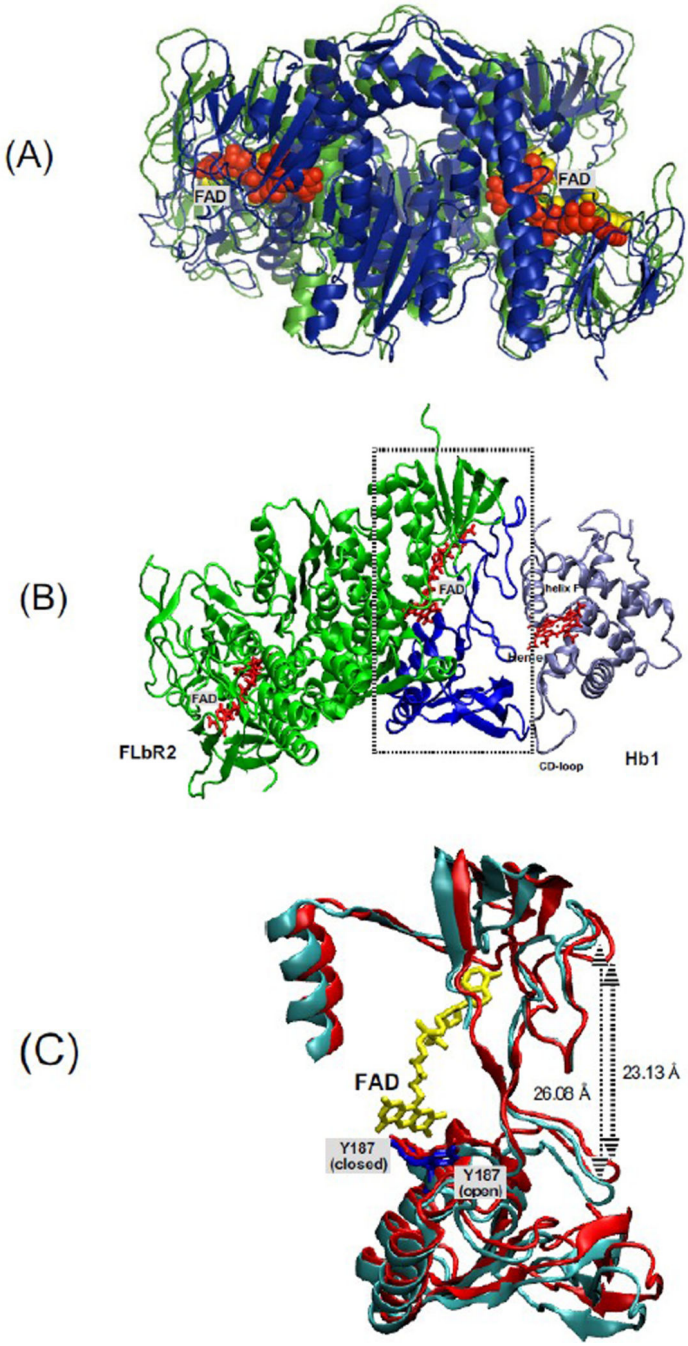

Figure 5.

(A) Overlay of the (dimeric) structure of predicted soybean FLbR2 (blue) and native pea DLDH (green); FAD is shown in red color and in the space filling mode. (B) Proposed model for the interaction between predicted soybean FLbR2 (green and blue) and native rice $Hb1^{3+}$ (gray); the FAD-binding domain of FLbR2 is shown in blue, and FAD and heme cofactors are shown in red; the dashed line box shows the FAD-binding domain of FLbR2 (enlarged in (C)) where the proposed interaction with $Hb1^{3+}$ occurs. (C) Predicted closed (red) and open (green) conformations of the FAD-binding domain from soybean FLbR2;

Tyr(Y)187 oscillates between the open ↔ closed conformations and is postulated to mediate the transfer of electrons from FLbR2 to $Hb1^{3+}$.