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Redox Potential of a Soybean Ferric Leghemoglobin Reductase

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The visible spectra of soybean ferric leghemoglobin reductase exhibited a charge transfer band at 530 nm under aerobic condition. Spectra of the oxidized enzyme show a flavin peak at 454 nm and the enzyme has three redox states associated with the active site of the enzyme. The enzyme has an active disulfide bridge and two-electron transfer may dominate in the ferric state of leghemoglobin reduction. The midpoint potentials of the enzyme were determined by spectrotitration to be −0.294 V for disulfide/dithiol and −0.318 V for $FAD/FADH_2$. Since the midpoint potentials for $NAD^+/NADH$ and the ferrous/ferric states of leghemoglobin are −0.32 V and +0.22 V, respectively, it is proposed that two electrons are transferred sequentially from NADH to FAD, to the disulfide group, and then to the ferric state of leghemoglobin in the enzyme reaction.

Keywords: Electron potential, Flavoprotein, Leghemoglobin, Oxidoreductase, Spectrotitration.

Introduction

Burris and Haas (1944) were the first to suggest that the reduced pyridine nucleotides might function as reductants of leghemoglobin (Lb^{3+}) in leguminous root nodules. Although higher oxidation states of Lb, such as Lb^{3+} and Lb^{4+}, exist, only Lb^{2+} binds to O_2. Lb^{2+} is readily oxidized *in vitro* to Lb^{3+} or Lb^{4+} by exposure to metabolites commonly found in nodules, such as superoxide, nitrite, flavins, ascorbates, or peroxide (Riguad and Puppo 1977; Moreau *et al.*, 1995).

A protein with ferric leghemoglobin reductase (FLbR) activity has been isolated from soybean nodules (Ji *et al.*, 1991; 1994). The FLbR cDNA was isolated, sequenced, and expressed with the expression vector, pTricHisC. The deduced amino acid sequence of FLbR from the gene sequence showed that the primary structure of FLbR is highly homologous to a family of pyridine nucleotide–disulfide oxidoreductases, especially dihydrolipoamide dehydrogenase (DHLipDH). Pyridine nucleotide-disulfide oxidoreductases are a family of homodimeric flavoenzymes that have an active disulfide bridge and FAD in each monomer. In this paper, the active disulfide bridge and oxido-reduction properties of FLbR were studied. The redox potentials of the FLbR reaction were determined for the E to EH_2 and EH_2 to EH_4 transitions by a spectrotitration method, which is useful for measuring the redox potential of flavoproteins (Stankovich, 1991). From the determination of the E'_m values and the spectral studies for FLbR, the direction of electron transfer was proposed.

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Materials and Methods

Materials and reagents Sepharose 6B was purchased from Pharmacia. P-6-DG column was purchased from Bio-Rad (Richmond, USA). DL-Dihydrolipoate, DL-lipoamide, DL-lipoate, potassium ferricyanide, NADH, NAD+, DCIP, methylene blue, IPTG, arsenic acid, arsenic trioxide, phenylarsine oxide, cadmium sulfate, riboflavin, dithionite, ferricyanide, horse skeletal muscle myoglobin (Mb), human hemoglobin (Hb), Torula yeast dihydrolipoamide dehydrogenase (DHLipDH), and bovine intestinal mucosa DHLipDH were purchased from Sigma (St. Louis, USA). The pTrcHisC::FLbR clone and the polyclonal antibody raised against soybean FLbR were gifts from Dr. Robert V. Klucas (University of Nebraska, Lincoln, USA).

Expression and purification of the recombinant FLbR The *E.coli* Top 10 cells carrying the pTricHisC::FLbR expression construct were cultured with SOB media including 50 μg/ml ampicillin until the A_{600} reached a value of 0.5. The overexpression of FLbR was induced by addition of IPTG at a final concentration of 1 mM, and the recombinant FLbR-containing medium was used for the purification of recombinant FLbR. The cells were collected by low speed centrifugation (5000 × *g* for 15 min). Cell pellets were resuspended in 1:20

(v/v of original culture broth) of 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA. Cells were lysed by sonication with five 30-s bursts at a medium intensity setting (Sonifier II cell disrupters, Branson, Danbury, USA) while holding the suspension on ice (Lee, 1995). The lysate was frozen in a methanol dry ice slurry or liquid nitrogen and then quickly thawed at 37°C and thereafter further subjected to three more rapid freeze thaw–sonication cycles.

The following procedures were performed at 4°C. After cell lysis, insoluble debris was removed by centrifugation at 5000 × *g* for 15 min. Ammonium sulfate was added to the supernatant to 50% (w/v) and centrifuged at 10,000 × *g* for 15 min. The supernatant was applied to a Sepharose 6B (Pharmacia, Uppsala, Sweden) column equilibrated with 50% (w/v) ammonium sulfate-saturated 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA and eluted in one step with 25% (w/v) ammonium sulfate-saturated 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA. The eluate was monitored at 280 nm with a UA-5 absorbance detector (ISCO, Lincoln, USA) and the fractions exhibiting elevated absorbance were collected. The eluate was concentrated and desalted with a centrifugal filtration at 8000 × *g* for 30 min (Centricon-30, Amicon, Beverly, USA).

The pTricHisC vector codes for six tandem histidine residues in the N-terminal peptide that have a high affinity for nickel-charged ProBond affinity resin (Invitrogen, San Diego, USA). The desalted and concentrated eluate was applied to a ProBond affinity column equilibrated with 50 mM potassium phosphate buffer, pH 7.8, including 0.5 M sodium chloride, and eluted with 50 mM potassium phosphate buffer, pH 6.5, including 200 mM imidazole. The purified protein was desalted and concentrated with a Centricon-30 as described above. The pTricHisC::FLbR recombinant protein contains the enterokinase cleavage site (Asp4-Lys) at the N-terminal leader peptide of the recombinant FLbR. Enterokinase is a specific protease that cleaves the C-terminal of lysine in its preferred cleavage site. The purified FLbR was incubated with enterokinase (1 U/100 μg protein) overnight at 37°C to remove the polyhistidine tail. The enterokinase-cleaved protein and the polyhistidine residues were applied again to a ProBond affinity column equilibrated with 50 mM potassium phosphate buffer, pH 7.8, including 0.5 M sodium chloride, and washed with the same buffer, which leaves the polyhistidine residues bound to the nickel-charged ProBond column. The eluted recombinant FLbR was collected and desalted through a P-6-DG column (Bio-Rad) with 50 mM potassium phosphate buffer, pH 6.5, including 1 mM EDTA.

The expressed recombinant FLbR was electrophoresed on 8.5% polyacrylamide gel containing 0.1% SDS and stained with Coomassie Brilliant Blue. The gel was Western blotted with polyclonal antibodies raised against soybean FLbR (Chae and Kim, 1987; Ji *et al.*, 1991). The protein concentration was measured by the protein microassay procedure (Bio-Rad) using bovine serum albumin as a standard (Kim and Kim, 1984).

Preparation of soybean nodule Lb^{31} Soybean (*Glycine max* [L.] Merr. cv. Hobbit) seeds were inoculated with *Bradyrhizobium japonicum* strain USDA110 and the nodules harvested after 30 to 35 d, washed, and stored at −80°C. Lb^{3+} was prepared using the method of Saari and Klucas (1984). The whole procedure was carried out at 4°C. Nodules were placed in three-fold excess (w/v) of 0.1 M potassium phosphate buffer, pH 6.5, with polyvinylpolypyrrolidone (0.3 g/g weight of fresh nodules) and homogenized with a Sorvall Omni-Mixer (DuPont Instruments, Boston, USA) for 1 min, cooled on ice and homogenized again for 1 min at maximum speed. The homogenate was passed through three layers of cheese cloth and the filtrate was centrifuged at 15,000 × *g* for 15 min. Proteins that precipitated between 35 to 80% (w/v) of ammonium sulfate were collected, suspended in 0.1 M potassium phosphate buffer, pH 6.5, and dialyzed overnight against the same buffer. Lb^{3+} was obtained by the addition of 10 mM of potassium ferricyanide. The dialysate was loaded onto a Sephadex G25 column equilibrated with 20 mM Tris buffer, pH 9.2, to remove ferricyanide and nicotinate, and fractions containing Lb^{3+} were collected. Fractions with A_{560}/A_{620} ratios less than 1.0 were pooled and dialyzed overnight against 20 mM potassium phosphate buffer, pH 7.0. The dialyzed fraction containing Lb were loaded onto a Sephadex G75 column equilibrated with 50 mM potassium phosphate buffer, pH 6.5, and the fractions containing Lb^{3+} were pooled and concentrated by ultrafiltration with YM 10 membrane under nitrogen gas. The Lb^{3+} fractions were stored at −80°C and used as the substrate of FLbR. Lb^{3+} was quantitated by using the extinction coefficient of 157 $mM^{-1}cm^{-1}$ at 403 nm.

Enzyme assays of FLbR FLbR activity was assayed by measuring the formation of $Lb^{2+}O_2$ from Lb^{3+}. The initial rates of $Lb^{2+}O_2$ were determined by measuring the increase of absorbance at 574 nm. Lipoamide and ferricyanide reduction activities were then measured by the decrease in absorbance at 340 nm and 420 nm, respectively.

Disulfide inhibitors of FLbR Three microliters of FLbR in 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA, was incubated with 100 mM NADH and 1 mM dithiol binding compounds, arsenic acid, arsenic trioxide, phenylarsine oxide, cadmium sulfate, cupric nitrate, p-chloromercuric benzoate, or 5,5′-dithio-bis-(2-nitrobenzoic acid) on ice. After 30 min, aliquots of the samples were assayed for Lb^{3+} reduction, lipoamide reduction, and ferricyanide reduction activities. Solutions containing all of the components, except for dithiol-binding compounds, were used as the control. For the DHLipDH study, 3 μg of bovine DHLipDH replaced FLbR.

Aerobic titration of FLbR spectrum The concentration of FLbR was determined at 448 nm using an extinction coefficient of 11.1 $mM^{-1}cm^{-1}$ on the basis of the FAD content in the enzyme (Thorpe and Williams, 1976). The spectrophotometer system used in these studies was a Spectronic 3000 diode array spectrophotometer (Milton Roy, Chicago, USA) equipped with MRCO computer programs for spectrum manipulation and conversion of spectra into ASCII (American Standard Communication) file. The ASCII file was graphed using Microsoft Excel and Cricket Graph programs on a Power PC (Macintosh). FLbR solutions (20 mM) in 50 mM potassium phosphate, pH 7.0, were scanned from 650 to 300 nm at 25°C. The purified FLbR was titrated with NAD^+ under aerobic conditions. Aliquots of NAD^+ were added and the change in the flavin peak at 454 nm and charge transfer at 530 nm were observed. The equilibration time between additions was 5 min.

Measurement of the redox potentials for FLbR *Analysis of the anaerobic apparatus* Oxygen leakage into the system affects the redox potential and, thus, a strictly anaerobic system must be maintained during the titration. A hemoprotein reduction experiment was carried out on the anaerobic apparatus to verify that anaerobiosis was maintained (Becana *et al.*, 1991). The reaction mixture contained 50 mM potassium phosphate, pH 6.5, 30 mM soybean Lb^{3+}, 10 mM riboflavin, and 200 mM NADH. For the anaerobic assay, reaction mixtures without NADH solution were placed in a 1-ml cuvette, which was capped with a serum stopper, and sparged with ultra-pure N_2 for 30 min on ice. An aliquot of N_2-sparged NADH solution was added to start the reaction.

Anaerobic titration (E/EH_2) of FLbR Redox potentials of FLbR (E/EH_2) were measured spectrophotometrically by reductive titration using NADH as the reducing agent under anaerobic conditions as described above. The purified FLbR was oxidized with 100 μM ferricyanide and the oxidized FLbR was separated from the small molecules by passage through a P-6-DG column (Bio-Rad) equilibrated with N_2-sparged 50 mM potassium phosphate buffer, pH 6.5. The oxidized FLbR eluate from the P-6-DG column was sparged with N_2 for 30 min on ice. The N_2-sparged NADH solution was injected into a serum-stopped cuvette containing the N_2-sparged FLbR (20 mM) using a 10 μl air-tight syringe (Hamilton, Reno, USA). The decrease in the absorbance of the flavin peak (454 nm) and the increase in the absorbance of the charge transfer band (530 nm) indicated that reduction to the EH_2 state had occurred. The conversion to the EH_2 state was complete when the absorption band at 530 nm was at a maximum. The equilibration time needed between additions was 5 min.

Anaerobic titration (EH_2/EH_4) of FLbR Redox potentials of FLbR were measured spectrophotometrically by oxidative titration of the EH_4 state using NAD^+ as the oxidizing agent under anaerobic conditions as described above. The purified FLbR was reduced with 100 μM dithionite to the EH_4 state and then passed through a P-6-DG column (Bio-Rad) with N_2-sparged 50 mM potassium phosphate buffer, pH 6.5. The eluate from P-6-DG column was sparged with N_2 for 30 min on ice. The N_2-sparged NAD^+ solution was injected into a cuvette containing the N_2-sparged FLbR (20 mM) with a 10 μl air-tight syringe (Hamilton). The increase of the flavin peak (454 nm) and the increase at the charge transfer band (530 nm) indicated oxidation to the EH_2 state. The conversion to the EH_2 state was complete when the absorption band at 530 nm was a maximum. The equilibration time needed between additions was 5 min.

Calculation of concentrations of the E, EH_2, and EH_4 forms Difference extinction coefficients, $\Delta\varepsilon(EH_2\text{-}E)$ and $\Delta\varepsilon(EH_2\text{-}EH_4)$, for FLbR were calculated from the titration spectra (Matthews and Williams, 1976). Absorption data from the titrations of FLbR with NAD^+/NADH were used to examine the extinction coefficients, $\Delta\varepsilon(EH_2\text{-}E)$ and $\Delta\varepsilon(EH_2\text{-}EH_4)$, at 530 nm. EH_2 is the only enzyme species which absorbs light at 530 nm, since the concentration of EH_2 was determined by measuring changes in absorbance at 530 nm. The concentrations of E and EH_4 were determined by subtraction. For the E/EH_2 system, the concentrations of E was determined from the equation; $[E] = [E_{total}] - [EH_2]$. For the EH_2/EH_4 system, likewise for EH_4, the equation was; $[EH_4] = [E_{total}] - [EH_2]$.

Calculation of the FLbR redox potential The determined concentrations of enzymes ([E], $[EH_2]$, and $[EH_4]$) were used to calculate the redox potential (E'_h) using the Nernst equation for the equilibrium between the FLbR enzyme system and the known NAD^+/NAD system (Matthews and Williams, 1976; Maeda-Yorita and Aki, 1984). The resulting values for E'_h (E/EH_2 or EH_2/EH_4) were averaged. The midpoint potential (E'_m) for the reduction of E to EH_2 (or EH_2 to EH_4) was determined from the titration curve (E'_h versus percent of the enzyme reduction).

Results

Expression of FLbR from the pTrcHisC::FLbR clone The properties of the constructed protein were determined to establish that the purified recombinant protein is FLbR. The purified preparation of recombinant FLbR is a protein of 54 kDa which is the same size as soybean FLbR on SDS PAGE (lanes 2 and 5 in Fig.1). Western blot analysis with polyclonal antibodies against the purified FLbR from soybean nodules was performed (Fig. 2). The crude extracts of the *E.coli* host cell (Top 10) showed few, if any, bands on Western blots, implying that anti-soybean FLbR antibody does not cross-react with the host *E.coli* proteins (lanes 3–6 in Fig. 2).

Two-electron transfer of the FLbR reaction The effects of the disulfide-binding compounds [arsenic acid, arsenic trioxide, phenylarsine oxide, cadmium sulfate, cupric nitrate, p-chloromercuric benzoate, or 5,5′-dithio-bis-(2-nitrobenzoic acid)] were measured for FLbR and DHLipDHs. The disulfide-binding compounds inhibited the Lb^{3+} reduction activity of the FLbR and lipoamide

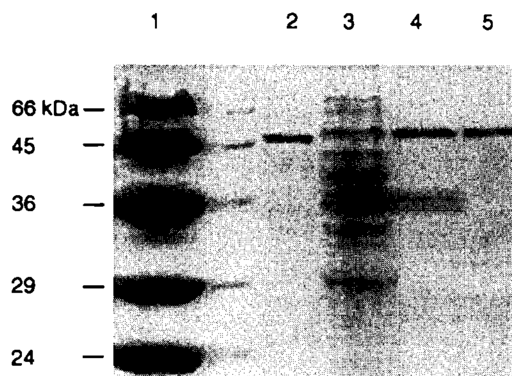

Fig. 1. Electrophoretic analysis of recombinant FLbR fractions. Electrophoresis was performed in 8.5% SDS-PAGE gel and the gel was stained with Coomassie Brilliant Blue to detect proteins. Samples were: lane 1, molecular weight markers (bovine serum albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde 3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa); lane 2, purified soybean FLbR (2 μg); lane 3, crude extracts of *E. coli* (10 μg) after induced with IPTG; lane 4, partially purified recombinant FLbR (2 μg) after the Sepharose 6B step; lane 5, purified recombinant FLbR (2 μg) after the nickel-charged affinity column step.

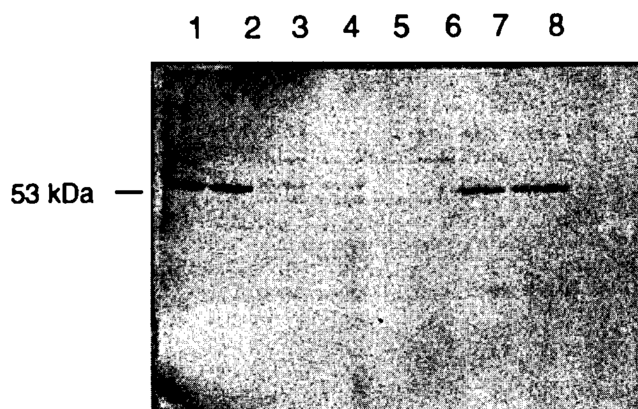

Fig. 2. Immunodetection of FLbR proteins with anti-soybean FLbR polyclonal antibody on Western blots. Sample loads were: lanes 1 and 2, 2 μg purified recombinant FLbR; lanes 3, 4, 5 and 6, 10 μg crude extracts of *E. coli* Top 10; lanes 7 and 8, 10 μg crude extracts of the expressed construct (pTrcHisC::FLbR) in *E. coli* Top10.

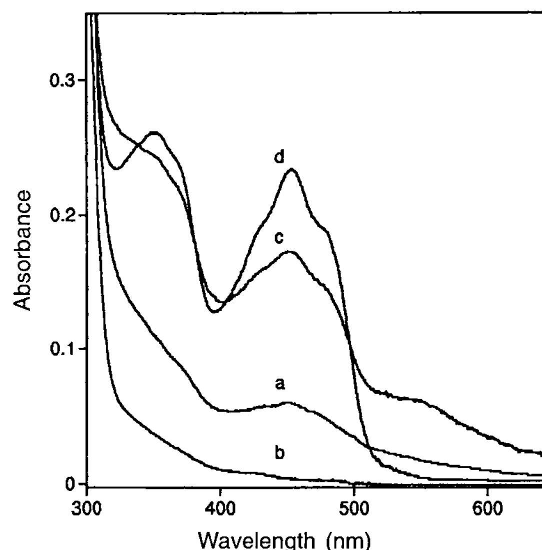

Fig. 3. Aerobic oxido-reduction of FLbR. a, 20 μM FLbR in 50 mM KPi, pH 7.0; b, EH_4 state of FLbR reduced with 100 μM dithionite; c, EH_2 state of FLbR oxidized with 53 μM NAD^+; d, E state of FLbR oxidized with 92 μM NAD^+.

reduction activity of bovine DHLipDH. FLbRs were inhibited by incubation with disulfide-binding compounds, indicating the involvement of a disulfide group in the active site of FLbR.

Redox states of FLbR DHLipDHs are always isolated and purified in the oxidized forms (Williams, 1991). The native and recombinant FLbR are isolated and purified in a partly reduced form (line a in Fig. 3), which is a unique property compared to DHLipDHs. Addition of NAD^+ under aerobic condition results in the formation of the EH_2 state of FLbR with a charge transfer band at 530 nm (line c in Fig. 3), which is also unique for FLbR, because the DHLipDHs studied so far exhibit a charge transfer band at 530 nm but only under anaerobic conditions (Williams, 1991). Upon addition of more NAD^+, FLbR exhibited an absorption peak at 454 nm and the charge transfer band was diminished (line d in Fig. 3), but exhibited distinct shoulders around 440 and 470 nm, indicating that there was covalent association of flavin with the protein. The addition of sodium dithionite produced the EH_4 state of FLbR (line b in Fig. 3). The data showed that FLbR is isolated in a partly reduced form and that it could be oxidized by NAD^+ and reduced by dithionite.

Redox potentials of FLbR

Anaerobic apparatus Anaerobic titrations require an apparatus in which strictly anaerobic conditions can be maintained. To verify such conditions, the reduction of Lb^{3+} by NADH and riboflavin were used to detect trace amounts of O_2 during 20 min (data now shown). In this system, the initial substrate was $metLb^{3+}$ and the final product was $deoxyLb^{2+}$ with an absorption band at 555 nm, confirming that the apparatus maintained anaerobiosis.

Anaerobic titration of FLbR Spectrotitration is a useful technique for measuring the redox potential of flavoproteins because these are not directly reactive at the electrode surfaces as the flavin is buried inside the protein. The fully oxidized state (E) of FLbR was produced by adding ferricyanide, followed by gel filtration, and then aliquots of NADH were added to produce the EH_2 state (Fig. 4). Isobestic points were observed at 405 and 497 nm for the transition from E to EH_2 states. The fully reduced state of FLbR was produced by the addition of sodium dithionite (Fig. 5) and then reoxidized to the EH_2 state by the addition of NAD^+. FLbR was reversibly reduced and oxidized demonstrating that FLbR can be changed to the E, EH_2, and EH_4 states.

Concentrations of the E, EH_2, and EH_4 states of FLbR The concentrations of E, EH_2, and EH_4 of FLbR were measured as described in Materials and Methods. The results are shown in Table 1 for the E to EH_2 state and in Table 2 for the EH_2 to EH_4 state. The redox potentials for $[E]/[EH_2]$ and $[EH_2/EH_4]$ were calculated as described in the appendix.

The midpoint potential of FLbR For redox equations, n is 2 electron transfer (equivalents) per molecule, R (gas constant) is 1.98717 calorie/degree/mol, F (Faraday constant) is 23,063 calorie/V/equivalent, and T is the absolute temperature. The redox potentials ($E'_{h,E/EH_2}$ or $E'_{h,EH_2/EH_4}$) according to the variable concentrations of oxidant (or reductant) were calculated using Eq. (4.8) for E/EH_2 (Table 1) and Eq. (4.16) for EH_2/EH_4 (Table 2). The calculated values of E'_h were plotted against reduction

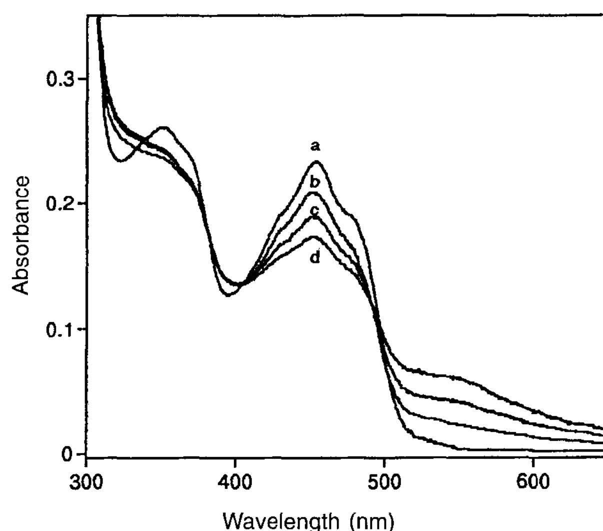

Fig. 4. Anaerobic titration (E/EH_2) of FLbR (from a to d). The fully oxidized FLbR (20 μM FLbR) was titrated with NADH under anaerobic conditions. a, E state of FLbR; b, after addition of 12 μM NADH; c, after addition of 41 μM NADH; d, after addition of 62 μM NADH.

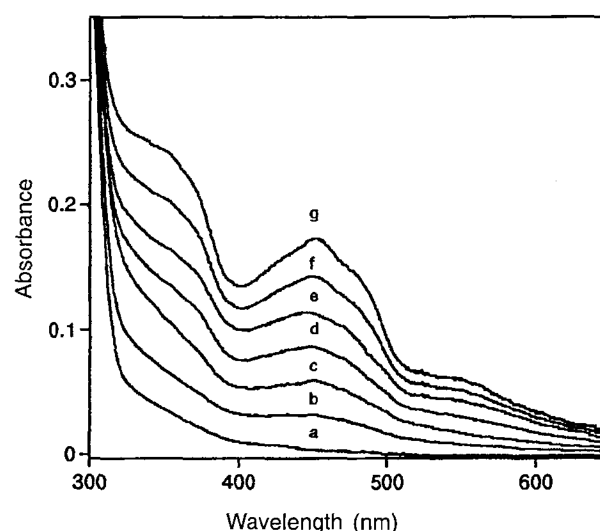

Fig. 5. Anaerobic titration (EH_2/EH_4) of FLbR (from a to g). The fully reduced FLbR (20 μM FLbR) was titrated with NAD^+ under anaerobic conditions. a, EH_4 state of FLbR; b, after addition of 3 μM of NAD^+; c, after addition of 12 μM of NAD^+; d, after addition of 32 μM of NAD^+; e, after addition of 65 μM of NAD^+; f, after addition of 84 μM of NAD^+; g, after addition of 89 μM of NAD^+, EH_2 state of FLbR.

Table 1. Redox potential of the oxidized/two-electron reduced couple of FLbR.

% reduction[a]	[E] (υM)[b]	$[EH_2]$ (υM)[c]	$[NAD^+]$ (υM)[d]	[NADH] (υM)[e]	$E'_{h,E/EH_2}$ (V)[f]
15	17.0	3.0	3.0	0.04	−0.287
21	15.8	4.2	4.2	0.10	−0.289
31	13.8	6.2	6.2	0.30	−0.291
40	12.0	8.0	8.0	0.60	−0.292
49	10.2	9.8	9.8	1.20	−0.294
61	7.8	12.2	12.2	2.80	−0.295
71	5.8	14.2	14.2	5.80	−0.297
78	4.4	15.6	15.6	10.40	−0.299
83	3.4	16.6	16.6	19.40	−0.302
88	2.4	17.6	17.6	33.40	−0.303

[a] The percent of reduction was calculated from the equation, % reduction of the enzyme = $[EH_2]/\{[E] + [EH_2]\} \times 100$.
[b] The concentrations of E were determined from the equation, $[E] = [E_{total}]\ 2\ [EH_2]$.
[c] The concentrations of EH_2 were determined from measurements of absorbance at 530 nm using the extinction coefficients, $\Delta\varepsilon(EH_2\text{-}E)$.
[d] The concentrations of NAD^+ formed is equivalent to the concentrations of EH_2 formed.
[e] The concentrations of NADH formed is determined from the equation, $[NADH] = [NADH_{added}] - [NAD^+]$.
[f] The redox potential (E'_h) of the system was calculated from the equation, $E'_h = E'_m + RT/nF \ln\{[EH_2][NAD^+]/[E][NADH]\}$, where E'_m is 20.32 V, the midpoint potential for NAD/NADH at pH 7.0 (Massey and Veeger, 1960); *R* is the gas constant; *T*, the absolute temperature; *F*, the Faraday constant; and *n*, the number of electrochemical equivalents.

Table 2. Redox potential of two-electron/four-electron reduced couple of FLbR.

% reduction[a]	$[EH_4]$ (υM)[b]	$[EH_2]$ (υM)[c]	[NADH] (υM)[d]	$[NAD^+]$ (υM)[e]	$E'_{h,EH_2/EH_4}$ (V)[f]
25	5.0	15.0	15.0	70.0	−0.314
35	7.0	13.0	13.0	35.0	−0.315
40	8.0	12.0	12.0	24.0	−0.316
44	8.8	11.2	11.2	17.8	−0.317
47	9.4	10.6	10.6	14.4	−0.318
55	11.0	9.0	9.0	7.9	−0.319
63	12.6	7.4	7.4	3.6	−0.322
71	14.2	5.8	5.8	1.7	−0.324
77	15.4	4.6	4.6	0.9	−0.325
85	17.0	3.0	3.0	0.3	−0.327

[a, b, c, d, e, f] All calculations were as described in Table 1.

percentage of the enzyme (Fig. 6) and the midpoint potentials of FLbR were determined.

The disulfide/dithiol redox potential is the E/EH_2 redox potential and the $FAD/FADH_2$ redox potential is the EH_2/EH_4 redox potential (Maeda-Yorita *et al.*, 1994; Hopkins and Williams, 1995a). For FLbR, the E'_m of the E/EH_2 system (disulfide/dithiol) was measured to be −0.294 V, and that of EH_2/EH_4 ($FAD/FADH_2$) was measured to be −0.318 V.

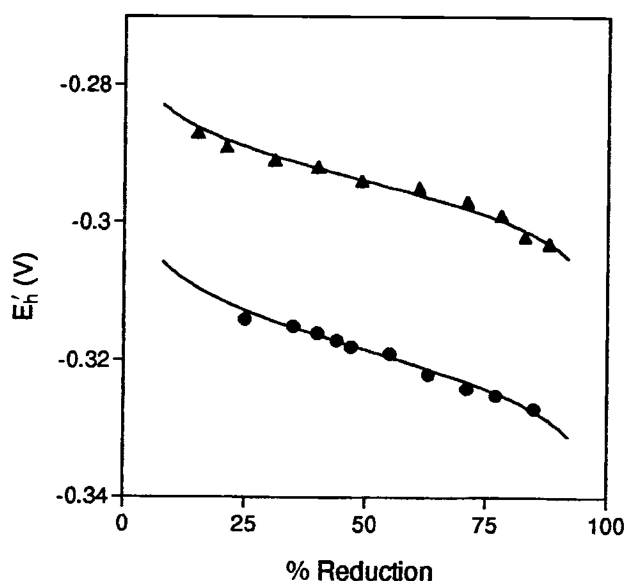

Fig. 6. Redox-potential for disulfide/dithiol and $FAD/FADH_2$ couples of FLbR. E'_h is plotted against percent enzyme reduction data from anaerobic NAD^+/NADH titration. The triangles represent data obtained for the disulfide/dithiol couple and the circles represent data obtained for the $FAD/FADH_2$ couple. The solid lines represent the fitted curves for each redox couple.

Discussion

The Western blot of crude extracts and purified recombinant FLbR showed a distinct single band of around 54 kDa (lanes 1 and 2, purified recombinant FLbR; lanes 7 and 8, cell extracts in Fig. 1) showing that the expressed protein is FLbR. The recombinant FLbR exhibited enzymatic activities that were consistent to those reported for soybean FLbR (Saari and Klucas, 1984). The purified recombinant protein reduced Lb^{3+} to Lb^{2+} and the values of K_m and k_{cat} for Lb^{3+} were 9.5 mM and 6.8 s^{-1}, respectively, which were consistent to those reported for soybean FLbR. The other approach is to measure the enzyme activity of FLbR from the expressed protein. The values of k_{cat}/k_m were 716 $mM^{-1}s^{-1}$, 190 $mM^{-1}s^{-1}$, and 76 $mM^{-1}s^{-1}$ for the reduction of Lb^{3+}, Met Mb, and Met Hb, respectively, with FLbR. These values suggest that the expressed FLbR prefers Lb^{3+} as a substrate and has a higher catalytic rate and substrate specificity for Lb^{3+} than Mb^{3+} or Hb^{3+}.

In most pyridine nucleotide-disulfide oxidoreductase reactions, flavohydroquinone easily reduces substrates in a two-electron reaction producing reduced substrates and oxidized flavin (Stankovich, 1991). Also, flavoquinone easily oxidizes substrates in a two-electron reaction producing oxidized substrates and the reduced flavohydroquinone. The physiological function of DHLipDH is to catalyze a reversible NADH-linked oxido-reduction of dihydrolipoamide via a two-electron transfer (Matthews and Williams, 1976; Thorpe and Williams, 1976; Wilkinson and Williams, 1979; Patel *et al.*, 1995). Disulfide-binding reagents and divalent metal ions inhibited certain reactions involved in the flavoquinone or dihydroflavoquinone (two-electron transfer) production of DHLipDH (Williams 1976; 1991). Treatment of DHLipDHs with arsenite, p-chloromercuric benzoate, 5,5′-dithio-*bis*-(2-nitrobenzoic acid), and Cd^{2+} showed a

significant decrease of DHLipDH activity, but the diaphorase and electron transferase activities were increased, showing that these activities are independent of the disulfide group but DHLipDH activity is dependent on the disulfide (Williams, 1965). Ferricyanide, a typical one-electron acceptor, is a substrate in the electron transferase reaction and in the production of the flavosemiquinone radical (Tsai *et al.*, 1983). The flavosemiquinone radical can be stabilized by chelation with divalent metal ions or disulfide-binding reagents via O(4a) and N(5) of the isoalloxazine ring among the flavoquinone derivatives (Müller *et al.*, 1970). The electron transferase reaction in DHLipDH is stabilized by divalent metal ions and disulfide-binding reagents.

Disulfide-binding compounds significantly inhibited the lipoamide reduction activity in FLbR and DHLipDHs showing that two-electron transfer reactions occurred in the lipoamide reduction. Furthermore, the disulfide-binding compounds stimulated the electron transferase activities of FLbR (up to 138%) and bovine DHLipDH (165%), showing that the one-electron transfer reaction is stabilized with disulfide-binding compounds and that flavosemiquinone might be involved in the electron transferase reaction. However, the disulfide-binding compounds drastically inhibited the heme reduction activity of FLbR, showing that two-electron transfer might dominate in heme reduction and semiquinone might not be produced during a reaction.

The spectrum of the E state has an absorption peak at 454 nm and a minimum absorption of the charge transfer peak at 530 nm. The E state of the enzyme was evident and the flavin peak at 454 nm was a maximum when excess oxidant was added to the enzyme. The EH_2 state was produced when the charge transfer peak at 530 nm was a maximum upon the addition of oxidant or reductant to the reduced or oxidized enzyme. The EH_4 state of the enzyme exhibited no flavin peak at 454 nm, nor charge transfer peak at 530 nm. The EH_4 state was evident when the flavin peak at 454 nm reached a minimum upon addition of excess reductant to enzyme. Absorption spectra from the titrations of FLbR with NAD^+/NADH were used to determine the different extinction coefficients, $\Delta\varepsilon(EH_2\text{-}E)$ and $\Delta\varepsilon(EH_2\text{-}EH_4)$, at 530 nm, and revealed to be 2700 and 2950 $M^{-1}cm^{-1}$, respectively.

The E'_m of DHLipDH from pig heart was reported to be −0.28 V for disulfide/dithiol and −0.314 V for FAD/FADH2 (Matthews and Williams, 1976), and from *E. coli* was found to be −0.264 V for the disulfide/dithiol and −0.314 V for the FAD/$FADH_2$ (Wilkinson and Williams, 1979). FLbR has a flavin that is reversibly oxidized or reduced with the oxidant or reductant (Fig. 3). FLbR has three redox states associated with the active state of the enzyme (E, EH_2, and EH_4). The EH_2 state of the enzyme had the typical charge transfer peak at 530 nm showing that a charge transfer exists between the oxidized FAD and thiol (line c in Fig. 3). The disulfide-binding reagent data showed that a two-electron transfer might be involved in the reduction of Lb^{3+} to Lb^{2+} by FLbR. The midpoint potential of FAD/$FADH_2$ in FLbR was measured as 20.318 V and that of disulfide/dithiol to be 20.294 V at pH 7.0 (Table 1, Table 2 and Fig. 6). The midpoint potential of the soybean Lb^{3+}/Lb^{2+} couple is +0.22 V at pH 7.0 (Henderson and Appleby, 1972). From the measured midpoint potentials in this work, it is proposed that two electrons might be transferred from NADH to the FAD, to the disulfide group with formation of a charge transfer complex between an active site thiol and the oxidized flavin, and then finally to the substrate, Lb^{3+}.

Appendix

The redox potential of the E/EH_2 system in FLbR The redox potentials of FLbR were calculated from the titration data for the E/EH_2 at equilibrium after each addition of NADH using the Nernst equation. The midpoint redox potential (E'_m) for the NAD^+/NADH is −0.32 V at pH 7.0 (Massey and Veeger, 1960). The concentration of NAD^+ formed is equal to the concentration of the EH_2 formed and the concentration of NADH is calculated by subtraction (Matthews and Williams, 1976).

For the titration of E to EH_2 for FLbR, the two reactions are:

$$E + 2H^+ + 2e^- = EH_2 \quad (4.1)$$

$$NAD^+ + 2H^+ + 2e^- = NADH + H^+ \quad (4.2)$$

The equation for the redox potential of E/EH_2 [reaction (4.1)] using the Nernst equation is:

$$E'_{h,E/EH_2} = E'_{m,E/EH_2} + [RT/nF]\ \ln\{[E][H^+]^2/[EH_2]\} \quad (4.3)$$

The equation for the redox potential of NAD^+/NADH [reaction (4.2)] using the Nernst equation is:

$$E'_{h,NAD+/NADH} = E'_{m,NAD+/NADH} + [RT/nF] \times \ln\{[NAD^+][H^+]^2/[NADH]\} \quad (4.4)$$

In a buffered system which maintains the pH value in solution, the produced $[H^+]$ [from Eq. (4.2)] should not be counted on the determination of the redox potential (Mansfield, 1962; Maeda-Yorita and Aki, 1984). At equilibrium, the values of $E'_{h,NAD+/NADH}$ and $E'_{h,E/EH_2}$ are equal (Mansfield, 1962) and the equations (4.5), (4.6), (4.7) and 4.8) are derived from Eqs. (4.3) and (4.4):

$$E'_{h,NAD+/NADH} = E'_{h,E/EH_2} \quad (4.5)$$

$$E'_{m,NAD+/NADH} + [RT/nF]\ \ln\{[NAD^+][H^+]^2/[NADH]\}$$

$$= E'_{m,E/EH_2} + [RT/nF]\ \ln\{[E][H^+]^2/[EH_2]\} \quad (4.6)$$

$$E'_{m,E/EH_2} = E'_{m,NAD+/NADH} + [RT/nF]\ \ln\{[NAD^+][H^+]^2$$

$$\times [NADH]\} - [RT/nF]\ \ln\{[E][H^+]^2/[EH_2]\} \quad (4.7)$$

$$= E'_{m,NAD+/NADH} + [RT/nF]\ \ln\{[EH_2][NAD^+] \times [E][NADH]\} \quad (4.8)$$

The redox potential of the EH_2/EH_4 system in FLbR
The redox potential of the EH_2/EH_4 system at equilibrium after each addition of NAD^+ was calculated using the Nernst equation and the midpoint redox potential of -0.32 V for the $NAD^+/NADH$ couple (Massey and Veeger, 1960). The concentration of EH_2 formed is equivalent to the concentration of NADH formed and the concentration of NAD^+ is determined by subtraction (Matthews and Williams, 1976). For the titration of EH_4 to EH_2 for FLbR, the two reactions are:

$$EH_2 + 2H^+ + 2e^- = EH_4 \quad (4.9)$$

$$NAD^+ + 2H^+ + 2e^- = NADH + H^+ \quad (4.10)$$

The equation for redox potential of EH_2/EH_4 [reaction (4.9)] using the Nernst equation is:

$$E'_{h,EH_2/EH_4} = E'_{m,EH_2/EH_4} + [RT/nF]\ \ln\{[EH^2][H^+]^2/[EH_4]\} \quad (4.11)$$

The equation for redox potential of NAD+/NADH [reaction (4.10)] using a Nernst equation is:

$$E'_{h,NAD+/NADH} = E'_{m,NAD+/NADH} + [RT/nF] \times \ln\{[NAD^+][H^+]^2/[NADH]\} \quad (4.12)$$

In a buffered system which maintains the pH value in solution, the produced $[H^+]$ [from Eq. (4.10)] should not be counted on the determination of the redox potential (Mansfield, 1962; Maeda-Yorita and Aki, 1984). At equilibrium, the values of $E'_{h,NAD+/NADH}$ and $E'_{h,EH_2/EH_4}$ are equal (Mansfield, 1962) and the equations (4.13), (4.14), (4.15), and (4.16) are derived from Eqs. (4.11) and (4.12):

$$E'_{h,NAD+/NADH} = E'_{h,EH_2/EH_4} \quad (4.13)$$

$$E'_{m,NAD+/NADH} + [RT/nF]\ \ln\{[NAD^+][H^+]^2/[NADH]\} = E'_{m,EH_2/EH_4} + [RT/nF]\ \ln\{[EH2][H^+]^2/[EH^4]\} \quad (4.14)$$

$$E'_{m,EH_2/EH_4} = E'_{m,NAD+/NADH} + [RT/nF]\ \ln\{[NAD+][H^+]^2 /[NADH]\} - [RT/nF]\ \ln\{[EH_2][H^+]^2/[EH_4]\} \quad (4.15)$$

$$= E'_{m,NAD+/NADH} + [RT/nF] \times \ln\{[EH_4][NAD^+]/[EH_2][NADH]\} \quad (4.16)$$

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