Biosci. Biotechnol. Biochem., **72** (4), 982–988, 2008

Characterization of a Dihydrolipoyl Dehydrogenase Having Diaphorase Activity of *Clostridium kluyveri*

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Received November 6, 2007; Accepted January 8, 2008; Online Publication, April 7, 2008
[doi:10.1271/bbb.70724]

The *Clostridium kluyveri bfmBC* gene encoding a putative dihydrolipoyl dehydrogenase (DLD; EC 1.8.1.4) was expressed in *Escherichia coli*, and the recombinant enzyme rBfmBC was characterized. UV-visible absorption spectrum and thin layer chromatography analysis of rBfmBC indicated that the enzyme contained a noncovalently but tightly attached FAD molecule. rBfmBC catalyzed the oxidation of dihydrolipoamide (DLA) with NAD^+ as a specific electron acceptor, and the apparent K_m values for DLA and NAD^+ were 0.3 and 0.5 mM respectively. In the reverse reaction, the apparent K_m values for lipoamide and NADH were 0.42 and 0.038 mM respectively. Like other DLDs, this enzyme showed NADH dehydrogenase (diaphorase) activity with some synthetic dyes, such as 2,6-dichlorophenolindophenol and nitro blue tetrazolium. rBfmBC was optimally active at 40 °C at pH 7.0, and the enzyme maintained some activity after a 30-min incubation at 60 °C.

Key words: *Clostridium kluyveri*; dihydrolipoyl dehydrogenase; diaphorase

Dihydrolipoyl dehydrogenase (DLD; EC 1.8.1.4) is a component of the multi-enzyme 2-oxo-acid dehydrogenase complexes, and it catalyzes the oxidation of a dihydrolipoyl residue covalently bound to a component protein and the reverse reaction as follows (homepage of Enzyme Nomenclature of IUBMB: http://www.chem.qmul.ac.uk/iubmb/enzyme/):

$$\text{Protein } N^6\text{-(dihydrolipoyl)lysine} + NAD^+ \leftrightarrow \text{protein } N^6\text{-(lipoyl)lysine} + NADH$$

DLD can also use free dihydrolipoate, dihydrolipoamide (DLA), or dihydrolipoyllysine as substrate. It is often termed "diaphorase" for its characteristic flavin moiety and NADH oxidase activity, where the electron acceptor is generically an oxidized dye such as methylene blue, nitro blue tetrazolium (NBT), or 2,6-dichlorophenolindophenol (DCPIP).[1] DLD has been extensively characterized from a variety of organisms belonging to bacteria, archaea, and eucarya. Enzyme properties are similar among the members of these groups.[2,3] DLDs normally function as integral components of the pyruvate, 2-oxoglutarate, and branched chain 2-oxoacid dehydrogenase multienzyme complexes[4,5] and of the glycine cleavage system.[6] Therefore, DLDs play a significant role in metabolism and energy production. DLDs might be homodimers with a subunit size of about 50 kDa. Each monomer contains a tightly but noncovalently attached flavin nucleotide molecule, a redox disulfide, and one NAD^+-binding site. Upon reoxidation of the dihydrolipoyl moiety, electrons are transferred from the dihydrolipoyl moiety to redox disulfide, then to the FAD cofactor, and finally to the NAD^+ cofactor.[2]

We are interested in enzymes with diaphorase activity, since they are useful in the colorimetric determination of NAD(P)H, and hence various dehydrogenase activities can be assayed when coupled with various dyes that act as hydrogen acceptors from NAD(P)H. NADH diaphorase also has been used extensively in detecting nitric oxide synthases activity in various cell types, including neuronal cell bodies, vascular endothelium, cells of the immune system, and epithelial cells, and thus have gained considerable importance as a diagnostic chemical.[7] In fact, several kinds of diaphorases are commercially available, and their origins are bacteria such as *Clostridium kluyveri*.[8] In an earlier study, an enzyme from *C. kluyveri* was purified and characterized by Kaplan *et al.*,[8] who used starting material equivalent to the currently available commercial product (Worthington Biochemical, Lakewood, NJ). The molecular size of the purified enzyme was determined to be about 24,000, and the enzyme was expected to be a proteolysis product of a parental DLD judging by its small size. However, we recently cloned the gene encoding the small diaphorase from *C. kluyveri*, and found that the encoded diaphorase consisted of

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Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DLA, dihydrolipoamide; DLD, dihydrolipoyl dehydrogenase; LA, lipoamide; NBT, nitro blue tetrazolium; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TLC, thin layer chromatography; Tris, Tris(hydroxymethyl)aminomethane

an N-terminal flavin reductase-like domain and a C-terminal Fe-containing rubredoxin-like domain. This domain organization was completely different from those of well-known DLDs, which consist of a Pyr_redox_2 domain and a Pyr_redox_dim domain.[9] Furthermore, we found that a gene highly homologous with authentic DLD genes existed in the unpublicized genomic DNA sequence database of *C. kluyveri*, constructed by the Research Institute of Innovative Technology for the Earth (RITE).[10] We were interested in comparing the enzymatic properties of this DLD enzyme with the small diaphorase.

In this paper, we report the expression of a putative DLD gene encoding 455 amino acid residues from *C. kluyveri* on the basis of the genome database of RITE. Recently, we found that the DLD gene described in this paper is identical to *bfmBC* (DDBJ accession no. CP000673; protein id EDK33811.1) reported by a German group. Hence we use *bfmBC* and BfmBC as names of the DLD gene and the gene product respectively. We also deal with the purification and characterization of BfmBC from recombinant *Escherichia coli*.

Materials and Methods

Bacterial strains, plasmids, and cultivation. C. kluyveri ATCC 8527 was cultivated in an ATCC medium (broth 1120) at 37 °C for 5 d. *E. coli* strains DH5α and BL21(DE3) were used in the cloning and expression respectively of the *C. kluyveri bfmBC* gene encoding a DLD. The plasmid pCR2.1-TOPO with single 3′-thymidine overhangs was used in TA cloning. The plasmid pET-28a (+) was obtained from Novagen (Madison, WI). Recombinant *E. coli* stains were routinely grown in Lenox Broth (LB) medium supplemented with ampicillin (50 μg/ml) or kanamycin sulfate (40 μg/ml). For high expression of the recombinant protein, LB Broth EZMix (Sigma-Aldrich, St. Louis, MO.) was also used.

Chemicals. All chemicals were obtained from Nacalai Tesque (Kyoto, Japan). DCPIP was purchased from merck (Darmstadt, Germany). Restriction endonucleases *Bam*HI and *Sal*I were from Takara Bio (Shiga, Japan). DL-DLA was prepared by reduction of DL-lipoamide (LA) (Sigma-Aldrich) with $NaBH_4$.[11]

Cloning of the bfmBC gene. C. kluyveri chromosomal DNA was isolated using a QIAamp mini spin column (Qiagen, Valencia, CA), and used as a template for PCR amplification. Oligonucleotide primers 5′-CCCGGATCCATGGCTTATAAATATGATCTGATTG-3′ and 5′-CCCGTCGACTTACTCCCTCATCAATTCCC-3′, including *Bam*HI and *Sal*I sites (underlined), were designed to amplify a 1,368-bp fragment of the *bfmBC* gene (DDBJ accession no. CP000673;protein id EDK33811.1). PCR amplification was carried out using KOD DASH DNA polymerase (Toyobo, Osaka, Japan) on a thermal cycler (Bio-Rad Laboratories, Hercules, CA). The PCR fragments were gel-purified by with GFX PCR DNA and a gel band purification kit (Amersham Biosciences, Buckinghamshire, UK), ligated into pCR2.1, and introduced into *E. coli* DH5α by a standard transformation protocol. After the integrity of the PCR-amplified fragment was confirmed by DNA sequencing, the insert DNA was recovered from the recombinant plasmid by digestion with *Bam*HI and *Sal*I and ligated into pET-28a(+), yielding pET-bfmBC. The produced recombinant enzyme (termed rBfmBC) contained a 6xHis-tag at the N-terminus.

Purification of rBfmBC. E. coli BL21 (DE3) harboring pET-bfmBC was grown overnight at 37 °C in 500 ml of LB medium containing 40 μg/ml of kanamycin. IPTG was added to a final concentration of 1 mM and incubated for a further 5 h at 37 °C. The cells were harvested by centrifugation at 4,000 × *g* for 20 min, and resuspended in 30 ml of lysis buffer (pH 7.4) containing 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole. The cells were disrupted by sonication, and the soluble fraction of the recombinant protein was obtained by centrifugation at 4,500 × *g* for 25 min. The supernatant was used as the crude enzyme solution and was purified by HiTrap Chelating HP (Amersham Biosciences) column chromatography according to the supplier's protocol. Active fractions were collected and desalted by dialysis using 50 mM sodium phosphate buffer (pH 7.4). The presence of about 50-kDa recombinant protein rBfmBC was confirmed by SDS–PAGE (12.5%) analysis. Protein was determined by the Bradford method[12] or by UV absorption.

Molecular weight determination by gel filtration. The molecular weight of native rBfmBC was determined by gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences). The column was eluted with 50 mM morpholineethanesulfonic acid buffer (pH 7.5) containing 0.15 M NaCl at a flow rate of 30 ml/h. Horse spleen apoferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) were used as molecular weight standards in this experiment.

Enzyme assays. DLD activity was assayed at 30 °C in 50 mM potassium phosphate-2 mM EDTA (pH 7.0) containing 0.4 mM DLA and 1 mM NAD^+. The reaction mixture (final volume, 1 ml) was started with the addition of the enzyme, and its progress was monitored by the increase in A_{340}.[13] The extinction coefficient for NADH ($6.22 \times 10^3\ M^{-1}\ cm^{-1}$) was used in the calculation of enzyme activity. One unit of enzyme activity was defined as the amount of enzyme required for the production of 1 μmol of NADH in 1 min at 25 °C.

Diaphorase activity against DCPIP (extinction coefficient, $2.1 \times 10^4\ M^{-1}\ cm^{-1}$) was determined by measuring the decrease in absorbance at 600 nm.[8] Diaphor-

ase activity against 0.5 mM NBT was assayed by measuring the reduction in absorbance at 585 nm with 9 mM NADH in 0.3 ml of 50 mM Tris–HCl (pH 8.0) at 37 °C.[14] Unless otherwise stated, enzyme activity was measured with a Shimadzu spectrophotometer BioSpec-1600 (Kyoto, Japan) at 30 °C. One unit of enzyme was defined as the amount of enzyme that reduces 1 μmol of 2,6-DCPIP per min at 25 °C at pH 8.5.

The effect of pH on DLD and diaphorase activities was investigated using Britton and Robinson's universal buffer (50 mM phosphoric acid, 50 mM boric acid, 50 mM acetic acid; the pH was adjusted to 3–11 using 1 M NaOH). To determine pH stability, the enzymes were pre-incubated overnight at 4 °C in Britton and Robinson's universal buffer solution at different pHs. The residual activities were then measured under standard assay conditions.

The effect of temperature on DLD and diaphorase activities was measured using a Shimadzu spectrophotometer UV-1200 with a temperature control system. Thermostability was measured by pre-incubating the enzyme at various temperatures (30 to 70 °C) for 30 min, followed by measurement of the residual enzyme activities under standard assay conditions.

The apparent K_m values of rBfmBC for LA, DLA, NADH, and NAD^+ were determined in 50 mM potassium phosphate-2 mM EDTA (pH 7.0). The concentrations of LA and DLA were varied between 0.2 and 1.0 mM, with fixed concentrations of NADH (0.1 mM) and NAD^+ (1 mM) respectively. The apparent K_m values for NADH and NAD^+ were measured with varying concentrations of NADH (0.02–0.1 mM) and NAD^+ (0.1–1.0 mM) at constant concentrations of LA (1 mM) and DLA (1 mM) respectively.

Cofactor determination. Identification of flavin cofactor present in recombinant enzyme was carried out as described previously.[15] rBfmBC was heated at 100 °C for 20 min and then centrifuged at 14,000 × *g* to remove white precipitated protein. An appropriate amount of the supernatant was applied to a Kieselgel 60 TLC plate (merck) along with FAD and FMN as standards, and developed with a solvent system of butanol:acetic acid:water (4:1:1). The molar content of cofactor FAD present in the recombinant enzyme was determined using extinction coefficient $10.3 \times 10^3\ M^{-1}\ cm^{-1}$.

UV-Visible and fluorescence spectral analyses of rBfmBC. UV-Visible absorption spectra of rBfmBC were obtained with Shimadzu Spectrophotometer BioSpec-1600. Measurement was carried out between 250 and 800 nm at room temperature in 50 mM phosphate buffer (pH 7.4).

Fluorescent spectra were recorded using an RF-spectrophotometer (Shimadzu). The protein solution (1 μM) in 50 mM phosphate buffer (pH 7.4) was excited at 260 nm and 296 nm. Following 260-nm and 296-nm excitation, the emission pattern was recorded from 300 to 600 nm and 300 to 560 nm respectively at room temperature. The influence of NAD^+ on the fluorescence spectrum was monitored by the addition of NAD^+ to a final concentration of 0.05 mM. The effect of DLA on the fluorescence spectrum was monitored after the addition of the substrate to give a final concentration of 0.25 mM at 0 min and 5 min.

Results and Discussion

Expression and purification of rBfmBC

rBfmBC was highly expressed by *E. coli* BL21(DE3) harboring pET-rbfmBC, and purified using a HiTrap HP Chelating column in one step, with a recovery of about 70%. The expression level of rBfmBC was calculated to be about 10 mg per liter of the culture. The purified fractions showed a strong yellow color, suggesting that rBfmBC is a flavoprotein. Expression was found to be better in LB Broth EZMix (Sigma-Aldrich) than in normal LB or Super broth (3.5% Bacto Tryptone, 2% yeast extract, and 0.5% NaCl) media. As shown in Fig. 1, the purified protein showed a single band on SDS–PAGE. The molecular size of the purified protein (about 50 kDa) was in good agreement with the calculated molecular weight of rBfmBC, including a 6xHis-tag (52,983).

The apparent molecular weight of native rBfmBC was determined by gel filtration on a Superdex 200 HR 10/30 column as described in "Materials and Methods." After repeated experiments, we concluded that the molecular size of rBfmBC in the native form was about 110 kDa. A comparison of this value with the theoretical molecular size (53 kDa) suggested that rBfmBC might exist as a dimer in the native state. This was consistent with previous observations in which native forms of many DLDs were reported to exist as dimers consisting of two identical subunits (Table 1), which contained 1 mol of FAD and 2 redox-active Cys residues per subunit.[16,17] Although several dihydrolipoyl dehydrogenases with a typical molecular mass or nicotinamide-

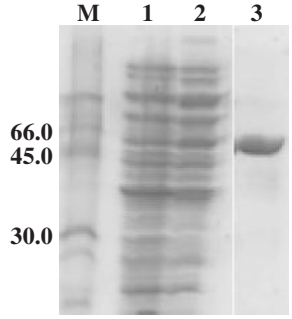

Fig. 1. SDS–PAGE of rBfmBC.

Protein samples were analyzed on a 12.5% polyacrylamide gel. Lane 1, cell-free extracts from uninduced cells; lane 2, cell-free extracts from cells induced with 1 mM IPTG; lane 3, purified rBfmBC; M, molecular size marker.

Table 1. Comparison of Structural and Functional Properties of Dihydrolipoamide Dehydrogenase from Different Sources

Source	Molecular mass (kDa)		Coenzyme		Metabolic role	References
	Native	Subunit[a]	NADH	NADPH		
Mammalian, yeast	100–115	50–55	+	−	Mitochondrial 2-oxo acid oxidation	2, 3, 26, 27
Trypanosoma cruzi	100	55	+	−	Probably 2-oxo acid oxidation	28
Aerobic eubacteria	100–110	50–55	+	−	2-oxo acid oxidation, but *cf.* (29) for a third DLD of *P. putida* without known function	2, 3, 29
Archaebacteria	115–122	58–61	+	−	Function not known	30–32
Anabaena sp.	104	53	+	−	Probably photorespiratory glycine oxidation	16
Clostridium cylindrosporum	105	52	+	+	Uric acid and glycine utilization	17
Clostridium sporogenes	100	52	−	+	Function not known	17
Eubacterium acidaminophilum	68	34.5	(+)[b]	+	Glycine utilization	17
Clostridium kluyveri	110[c]	50	+	−	Function not known	This study

[a]Values obtained by SDS–PAGE.
[b]NADPH was the preferred cofactor; this enzyme is actually thioredoxin reductase, like flavoprotein, exhibiting some DLD activity.
[c]This value was determined for the recombinant protein that contained a 6xHis-tag and some additional amino acid residues.

nucleotide specificity are known in anaerobic and glycine-utilizing bacteria,[17] they were reported to form dimers in the native form.

BfmBC is expected to be a component of the glycine cleavage system, since some genes downstream of *bfmBC* have high similarity to those responsible for glycine cleavage, but the molecular size and nicotinamide-nucleotide specificity of rBfmBC were similar to the usual DLDs, but not to clostridial enzymes involved in glycine cleavage (Table 1). A comparison of the BfmBC sequence with DLD sequences registered in the protein sequence database on Pfam website (http://pfam.sanger.ac.uk/) revealed that BfmBC consisted of a Pyr_redox_2 domain and a Pyr_redox_dim domain, and that the former domain included a flavin-binding domain (the 6th to the 27th amino acids), and highly conserved redox-active sites (the 39th to the 53rd amino acids), including two conserved Cys residues.

TLC analysis clearly revealed the presence of FAD but not that of FMN in the recombinant enzyme as a cofactor (Fig. 2), as expected from the presence of a FAD-binding site conserved in many DLDs. The molar ratio of FAD to rBfmBC monomer was calculated to be 0.97.

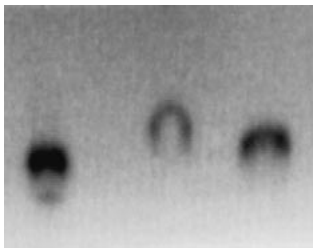

Fig. 2. Identification of Flavin Cofactor Present in rBfmBC.
The flavin cofactor of rBfmBC was released by boiling and analyzed by TLC with FMN and FAD as standards. S, sample.

Enzyme properties

When the DLD and diaphorase activities of rBfmBC were assayed at various temperatures, rBfmBC was optimally active at 40 °C. rBfmBC activities gradually decreased at temperatures higher than 40 °C, and rBfmBC showed about 20% of the highest activity at 70 °C. The enzyme was stable to incubation at 40 °C for 30 min in the absence of the substrates, and it kept its activity after 3 months of storage at 4 °C. It showed slight activity even after incubation in the absence of the substrate at 60 °C for 30 min. The small diaphorase, which is a major component of the commercial diaphorase of *C. kluyveri*, showed negligible activity at 60 °C.[9] *C. cylindrosporum* and *C. sporogenes* DLDs lost about 60% and 10% respectively of their specific activities upon storage at −20 °C for 6 months.[18] Based on these observations, rBfmBC appears to be comparatively thermostable, although some microorganisms have been found to produce thermostable DLDs, *e.g.*, DLD of thermophilic bacterium *Bacillus stearothermocphilus* was stable at 65 °C,[19] and incubation of DLDs from halophilic archaebacteria at 95 °C for 15 min in the presence of 4 M NaCl resulted in no detectable loss of their enzyme activities.[20] Although high stability of enzymes is generally an important factor for usage, the requirement of extreme reaction conditions is not desirable.

The optimum pH of rBfmBC was found to be about 7.0, and it showed high activity in a pH range of 6.0 to 8.0 in both DLD and diaphorase assays. Overnight incubation of the enzyme at 4 °C in Britton and Robinson's buffer solution at various pH, the levels

Table 2. Kinetic Constants of *C. kluyveri* rBfmBC

Type of activity	Substrate	K_m (mM)	Specific Activity (U/mg)
DLD	DLA	0.3	139
	NAD	0.5	131
	LA	0.42	112
	NADH	0.038	88
Diaphorase	DCPIP	0.12	675
	NADH	0.25	1200

indicated that the recombinant enzymes were stable at pH 6.0, and retained about half of the initial activity at pH 6.0 and 9.0.

The influence of metal ions on DLD and diaphorase activities were examined by assaying enzyme activity in the presence of each metal ion at 1 mM of metal ions such as Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Pb^{2+}, and Hg^{2+}, no ion enhanced the enzyme activity of rBfmBC. Pb^{2+} and Hg^{2+} ions showed strong inhibitory effects on rBfmBC activity (more than 80% inhibition). About 30% inhibition of rBfmBC activity was observed with 5 mM Zn^{2+} ions.

Kinetic parameters

From graphs showing reaction rates plotted against respective substrate concentrations, the apparent K_m values for DLA and NAD^+ were estimated to be 0.3 mM and 0.5 mM (Table 2) respectively under the assay conditions described above under "Materials and Methods." In the reverse reaction, the apparent K_m values for LA and NADH were determined to be 0.42 mM and 0.038 mM respectively. Although the K_m values for DLA and LA were similar to each other, the K_m value for NADH was smaller than that for NAD^+. This enzyme did not use NADPH as the electron donor. The similar trend was observed in many DLDs, as shown in Table 1, but DLDs from some anaerobic *Clostridium* spp. and glycine-utilizing bacteria differ from other DLDs in their coenzyme specificity, *i.e.*, the latter is highly specific to NADH.[21] It is interesting that *C. kluyveri*, being strictly anaerobic bacteria, has a preference for NADH as a coenzyme, in contrast to some other DLDs from anaerobic bacteria (Table 1).

One of the characteristics of DLDs is that they can function with benzyl viologen, DCPIP, ferricyanide, menadione, methylene blue, and NBT in addition to LA and lipoic acid in a wide range of artificial electron acceptors, and with NADH or NADPH as electron donor.[22] In diaphorase activity, the apparent K_m values of rBfmBC for DCPIP and NADH were 0.12 mM and 0.25 mM respectively. No diaphorase activity was detected when NADH was replaced by NADPH, as observed in the DLD of halophilic archaebacteria[20] and the hyperthermophilic archaeon *Thermococcus kodakaraensis*.[23] At high concentrations of DCPIP, the reaction mixture was too dark in color to detect a change in absorbance, leading to the limited assay conditions used in this study, as described under "Materials and Methods." rBfmBC showed strong diaphorase activity with NBT as an electron acceptor, although quantitative analysis was not carried out because of insoluble precipitate (formazan) formation due to the enzyme reaction.

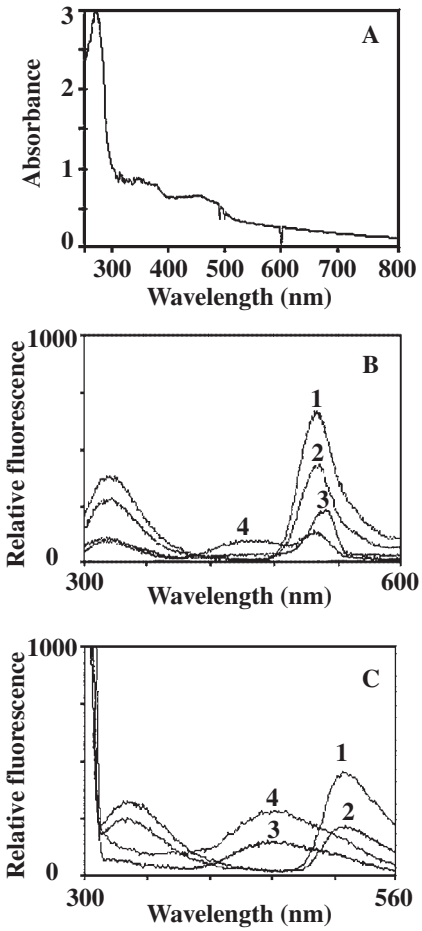

Fig. 3. UV-Visible Absorption Spectrum (A) and Fluorescent Spectra (B and C) of rBfmBC.

A, Measurement was carried out between 250 and 800 nm at room temperature in 50 mM phosphate buffer (pH 7.4). B, Fluorescent spectra were recorded between 300 and 600 nm by excitation at 260 nm in 50 mM phosphate buffer (pH 7.4). Treatments were as follows: *trace* 1, as-isolated rBfmBC; *trace* 2, rBfmBC plus 50 μM NAD^+ after 5 min; *trace* 3, rBfmBC plus 50 μM NAD^+ and 250 μM DLA; *trace* 4, rBfmBC plus 50 μM NAD^+ and 250 μM DLA after 5 min. C, Fluorescent spectra were recorded between 300 and 560 nm by excitation at 296 nm in 50 mM phosphate buffer (pH 7.4). Treatments were as follows: *trace* 1, as-isolated rBfmBC; *trace* 2, rBfmBC plus 50 μM NAD^+ after 5 min; *trace* 3, rBfmBC plus 50 μM NAD^+ and 250 μM DLA; *trace* 4, rBfmBC plus 50 μM NAD^+ and 250 μM DLA after 5 min.

UV-Visible and fluorescence spectral analyses of rBfmBC

UV-Visible spectral analysis of the recombinant protein showed two absorption peaks, at 270 nm due to protein and at 453 nm due to the FAD cofactor

(Fig. 3A). The ratio of absorption at 280 nm to that of 450 nm was 4.98. This factor was conveniently used as a criterion to determine the purity of DLD. The calculated value was comparable to those of enzymes previously reported,[18,24] confirming that a subunit molecule contained an FAD molecule.

Since rBfmBC contains no Trp residue but does contain 14 Tyr and 10 Phe residues, we attempted to measure the intrinsic fluorescence spectrum due to Tyr or Phe residues at an excitation wavelength of 260 nm.[23] When the intrinsic fluorescence spectrum of as-isolated rBfmBC was recorded, emission peaks were observed around 330 and 510 nm (Fig. 3B). Excitation at 260 nm excited amino acid residues, probably tyrosine residues in rBfmBC, which emitted the absorbed energy at 330 nm. This emitted energy was transferred to the FAD cofactor, which in turn emitted energy at 510 nm. When NAD^+ was added to the enzyme mixture, the peaks at 330 and 510 nm decreased greatly. The decrease in these peaks upon the addition of NAD^+ suggests a change in the local environment of FAD and the aromatic amino acid residues due to binding of NAD^+ to the protein. When the enzyme was reduced with DLA in the presence of NAD^+, both emission peaks drastically decreased at 330 nm and 510 nm, since the FAD molecule was reduced. Concomitantly, a 460 nm emission appeared. It represented the formation of NADH as result of the oxidation of DLA by rBfmBC. This peak increased after 5 min of incubation, indicating the ongoing reaction and formation of NADH, but this 460 nm peak disappeared over time (data not shown). This might have been due to dissociation of NADH from rBfmBC. These observations were similar to those for recombinant human DLD.[25]

In many intrinsic fluorescence spectral studies, a wave length of about 295 nm has been used to excite Trp residues and to avoid excitation of Tyr and Phe residue(s). Although rBfmBC contains no Trp residue and does contain 14 Tyr and 10 Phe residues in the molecule, emission peaks were obtained at 330 and 510 nm by excitation at 296 nm. The fluorescence spectrum due to 296-nm excitation was similar to that due to 260 nm excitation (Fig. 3C).

DLDs have been studied in eukaryotes and a wide range of aerobic bacteria, but less studied in strictly anaerobic clostridia such as *C. kluyveri*. In this study, we expressed and purified a *C. kluyveri* DLD (BfmBC) that was different from the major diaphorase of this bacterium, and found that the recombinant protein showed enzyme properties similar to those of DLDs isolated from other origins (Table 1). However, the high stability of rBfmBC may make it useful for practical use.

Acknowledgments

We thank Dr. Hideaki Yukawa of RITE for providing unpublished sequence data for *C. kluyveri*.

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