〈Brief Note〉

Easy preparation of a stable membrane-bound lactate dehydrogenase for application in lactate biosensor

Yuya Shimozawa[1], Saki Yoshida[2], Kazuya Ikeda[2], Yuri Kato[2], Fuka Toyama[2] and Yoshiaki Nishiya[1, 2,*]

Summary Flavin mononucleotide (FMN)-dependent lactate oxidase, which reduces the electric signals by transferring electrons to oxygen molecules, is now utilized in lactate biosensors. Although FMN-dependent lactate dehydrogenase (FMN-Ldh) could react anaerobically, being a membrane-bound protein, its time-consuming purification and instability are well known. We succeeded in overexpressing and briefly purifying the FMN-Ldh from *Escherichia coli* as a His-tagged protein. The enzyme was soluble without any detergents and stable at 4°C for at least 6 months. FMN-Ldh was unstable at a low concentration, and could be stabilized using the DnaK substrate-binding domain. The *E. coli* FMN-Ldh purified in this study will be useful for improving lactate biosensors.

Key words: Flavin, Lactate dehydrogenase, His-tag, *Escherichia coli*, Biosensor

1. Introduction

Flavin mononucleotide (FMN)-dependent lactate dehydrogenase [L-lactate dehydrogenase (cytochrome), EC 1.1.2.3, abbreviated FMN-Ldh] is identical to a flavohemoprotein cytochrome b2[1]. Ferricytochrome c is a natural electron mediator for this membrane-bound enzyme[1]. FMN-Ldh, which has been well studied in yeast and other eukaryotes, is also produced by some prokaryotes, such as *Escherichia coli* and *Pseudomonas* species[2-6]. Bacterial membrane-bound FMN-Ldh is not a hemoprotein; however, its primary structure is homologous to lactate oxidase (L-lactate: oxygen oxidoreductase, EC 1.1.3.2, abbreviated as Lox)[4]. Lox is also an FMN-dependent enzyme[7-9]. However, it is not membrane-bound, and is highly soluble.

Commercially available Lox, derived from the genera *Aerococcus* and *Enterococcus*, are already

[1]Division of Life Science, Graduate School of Science and Engineering, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.
[2]Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.

[*]Corresponding author: Yoshiaki Nishiya, Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.
Tel: +81-72-800-1151
Fax: +81-72-838-6599
E-mail: nishiya@lif.setsunan.ac.jp

Received for publication: June 9, 2020
Accepted for publication: July 29, 2020

used in clinical tests and lactate biosensors[7]. As for the application in biosensors, Lox and other flavin-containing oxidases have a disadvantage for accurate measurements because it reduces the electric signals by transferring electrons to oxygen molecules (Fig. 1). FMN-Ldh and other flavin-containing dehydrogenases that can react anaerobically are expected to overcome this disadvantage (Fig. 1). However, because of the hydrophobicity and low solubility, time-consuming purifications and instabilities of membrane-bound proteins, including FMN-Ldh, are well-known[3,6].

This report describes the overexpression and brief purification of FMN-Ldh as a His-tagged protein from *E. coli*. The purified enzyme was soluble without any detergents and stable at 4°C for long term (more than half a year). The FMN-Ldh purified in this study will be applied for improving biosensors.

2. Materials and Methods

Chemicals, bacterial strain, plasmid, and culture conditions

Compounds and reagents were purchased either from Nacalai Tesque (Kyoto, Japan) or from Wako Pure Chemical Industries (Osaka, Japan). The blocking peptide fragment, consisting of the DnaK substrate-binding domain, used as a stabilizer, was obtained from Toyobo (Osaka, Japan). *E. coli* BL21(DE3) (BioDynamics Laboratory Inc., Tokyo, Japan) and the plasmid pET29a (Km^r) (Novagen, Madison, WI, USA) were used for recombinant strain preparation and plasmid construction, respectively. Bacteria were grown in Luria-Bertani (LB) broth or on LB agar (LB broth plus 1.5% agar) at 30°C. The antibiotic used was kanamycin (30 μg/mL).

DNA manipulation and recombinant plasmid construction

Plasmid isolation, cleavage of DNA with restriction enzymes, ligation of DNA with T4 DNA ligase, and DNA sequencing were performed as described previously[7-9]. *E. coli* cells were transformed according to the chemical method[7-9]. Polymerase chain reaction (PCR) was performed according to the manufacturer's instructions (Toyobo).

The FMN-Ldh gene (DDBJ/EMBL/GenBank

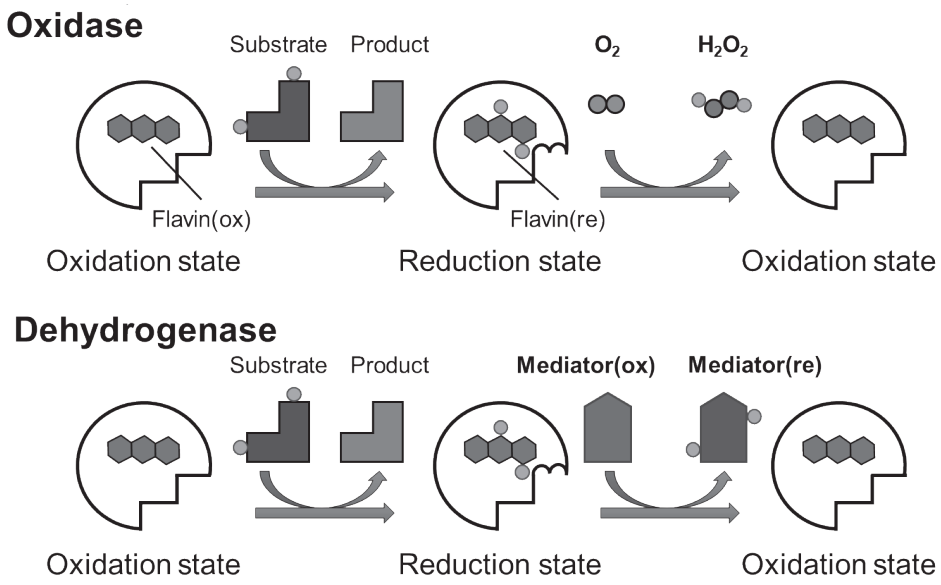

Fig. 1 Comparison of reactions between flavin-containing oxidase and dehydrogenase. Hydrogen and oxygen are represented by small and large gray colored balls, respectively.

accession number: CP011938) was obtained through colony PCR of *E. coli* BL21(DE3) using KOD-Plus-Neo DNA polymerase (Toyobo) using the following primers: 5'-TTTTTCATATGATTAT TTCCGCAGCCAGCGATTAT-3' (corresponding to the sequence that encodes the N-terminal peptide and contains an *Nde*I site marked by underlined nucleotides) and 5'-TTTTTGTCGACTGCCGCATT CCCTTTCGCCAT-3' (corresponding to the sequence that encodes C-terminal peptide and contains an *Sal*I site marked by underlined nucleotides). The amplified DNA was digested with *Nde*I and *Sal*I and ligated at the corresponding site in pET29a. The recombinant construct encoding FMN-Ldh and an additional 6×His-tag with spacer peptide (VDKLAAALEHHHHHH) introduced at the C-terminus was designated as pET-LdhHT. The DNA sequence of the construct was verified by sequencing. The pET-LdhHT carriers were induced to synthesize the gene product by the addition of isopropyl-L-D-thiogalactopyranoside (IPTG) to the culture medium.

Enzyme purification and assays

E. coli BL21(DE3)/pET-LdhHT was grown in LB broth (100 mL) containing kanamycin (30 μg/mL) at 30°C for 2.5 h with rotation at 150 rpm. Then, the culture was supplemented with IPTG (0.1 mmol/L) and the cells were cultured for an additional 7 h at 30°C to reach the stationary phase. The cells were harvested by centrifugation and resuspended in 20 mmol/L potassium phosphate buffer (pH 7.5). A crude extract was prepared by sonication of the cells followed by centrifugation. The supernatant was then loaded onto a His GraviTrap immobilized metal affinity chromatography column (GE Healthcare, Uppsala, Sweden). FMN-Ldh was eluted using 20 mmol/L potassium phosphate (pH 7.5) containing 500 mmol/L imidazole and 500 mmol/L NaCl. Pooled fractions exhibiting Ldh activity were further purified by dialysis with a Slide A-Lyzer (Thermo Fisher Scientific, Tokyo, Japan) and 20 mmol/L potassium phosphate (pH 7.5). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins based on the relative molecular mass.

Purification of the *Enterococcus* Lox (LoxL) was performed as described previously[7]. The lactate oxidase assay was also performed as described previously[7-9].

The lactate dehydrogenase assay was based on measuring the reduction of 2,6-dichloroindophenol coupled with substrate oxidation. The final assay mixture contained 50 mmol/L L-lactate, 0.2 μmol/L dichloroindophenol, 0.2 μmol/L phenazine methosulfate, and 50 mmol/L potassium Tris-HCl buffer (pH 8.0). The enzyme solution (50 μL) was incubated with the assay mixture (1,000 μL) at 37°C, and the amount of redox dye decreased by the dehydrogenase reaction was measured spectrophotometrically at 600 nm against a blank sample. One unit of enzyme activity was defined as the reduction of 1 μmol of dichloroindophenol per min at 37°C and pH 8.0.

Homology modeling

Molecular Operating Environment (MOE) software ver. 2019.01 (Chemical Computing Group Inc., Montreal, Canada) was used to align the primary structures, and to build a three-dimensional protein model of FMN-Ldh using the tertiary structure of the C203A mutant of lactate monooxygenase from *Mycobacterium smegmatis* (PDB ID: 6DVHa)[10] as a template. The sequence identity between FMN-Ldh from *E. coli* (396 amino acid residues) and the C203A mutant (394 amino acid residues) was approximately 33%. Energy minimization was applied to the model for further structure refining. The previously built[7] structural model of LoxL was refined in this study using MOE software. MOE and Pymol (https://pymol.org/) were used for molecular visualization.

3. Results and Discussion

Gene expression and enzyme purification

A lactate dehydrogenase activity of 18 U/mL was detected in the crude extract of cultured recombinant *E. coli* BL21(DE3)/pET-LdhHT. Subsequently, FMN-Ldh was easily purified in 3

steps as described in the materials and methods section (Fig. 2A). The purification yield was 11% with the increase in the specific activity by approximately 34-fold over the crude extract. The purified FMN-Ldh was soluble without any detergents. In the previous studies on FMN-Ldh, it needed to be purified using detergents, such as Tween 80 and Triton X-100. Moreover, the numbers of purification steps and the total yields were 6 and 2.6-3.2%, respectively[3,6]. The purified preparation gave almost a single protein band on the SDS-PAGE (Fig. 2B) and exhibited an absorption spectrum characteristic of a flavoprotein. The molecular weight was estimated at 42.8 kDa through the SDS-PAGE, which nearly agreed with that of the monomer His-tagged FMN-Ldh calculated from the deduced amino acid sequence and FMN (44.9 kDa). We succeeded in overexpressing and briefly purifying the FMN-Ldh from *E. coli* as a His-tagged protein.

Enzyme activity and stability

The lactate oxidoreductase activity of purified FMN-Ldh with and without oxygen was measured and compared with those of LoxL. The dehydrogenase and oxidase activities of FMN-Ldh were estimated to be 65 and 0.027 U/mg, respectively, whereas those of LoxL were 13 and 77 U/mg, respectively (Fig. 3). As expected, FMN-Ldh can hardly react with oxygen molecules. FMN-Ldh lacking in transferring electrons to oxygen must be suitable for application in the biosensors.

Investigation of long-term storage stability demonstrated that the purified FMN-Ldh (0.70 mg/mL) was stable at 4°C for at least 6 months (Fig. 4). In contrast, a low concentration of FMN-Ldh (0.0070 mg/L) diluted with 20 mmol/L potassium phosphate buffer (pH 7.5) was unstable. For example, its residual activity was 11% at 4°C for 3 days. However, it could be markedly stabilized to 90% of residual activity using the blocking peptide fragment[11] consisting of the DnaK substrate-binding domain (final concentration of 0.007%), which has a surfactant-like structure and functions as a stabilizer.

In this way, a stable FMN-Ldh could be easily prepared in this study, which will be useful for improving lactate biosensors.

Structural discussion

A three-dimensional structural model of *E. coli* FMN-Ldh was constructed by homology modeling

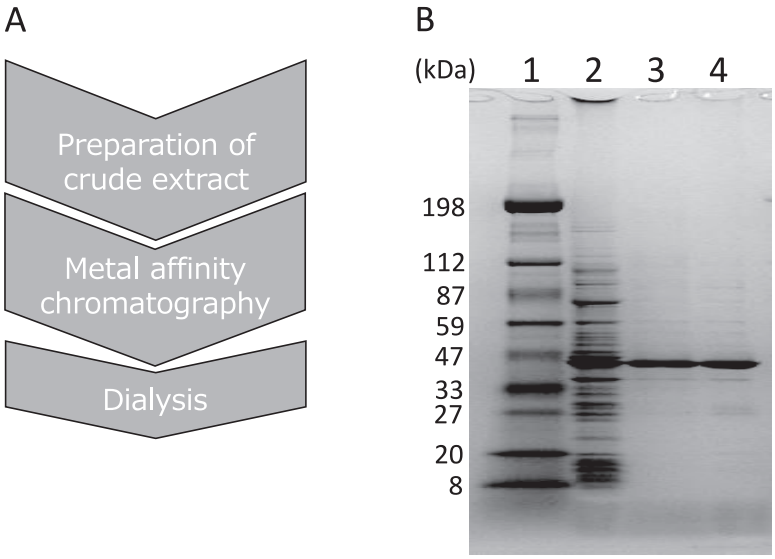

Fig. 2 *E. coli* FMN-Ldh purification flow and SDS-PAGE analysis at each purification step. (A) Purification flow. (B) SDS-PAGE analysis. 1: Molecular weight marker, 2: crude extract, 3: pooled fractions of metal affinity chromatography, and 4: final preparation after dialysis. Arrow indicates the band corresponding to the recombinant *E. coli* FMN-Ldh.

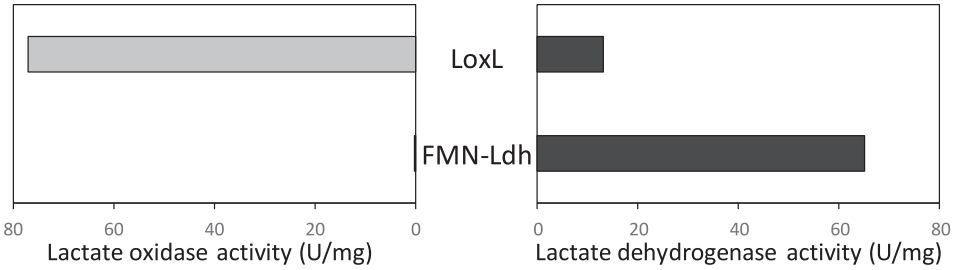

Fig. 3 Comparison of specific activities between *E. coli* FMN-Ldh and *Enterococcus* Lox. The lactate oxidase and dehydrogenase assays were measured as described in Materials and Methods section. Data are expressed as mean for triplicate determinations.

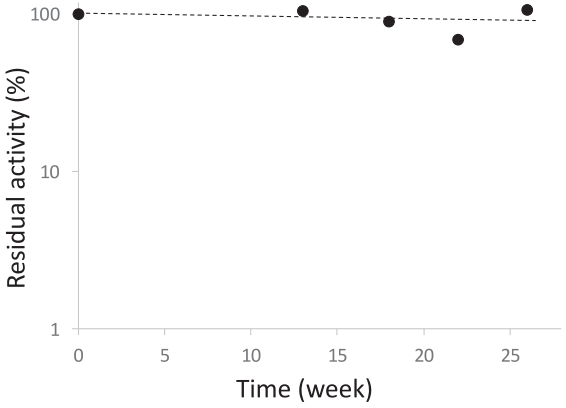

Fig. 4 Long-term storage stability of *E. coli* FMN-Ldh at 4°C. The residual activity was evaluated based on the lactate dehydrogenase activity of *E. coli* FMN-Ldh without the stabilizer (view the Materials and methods section). Data are expressed as mean for triplicate determinations.

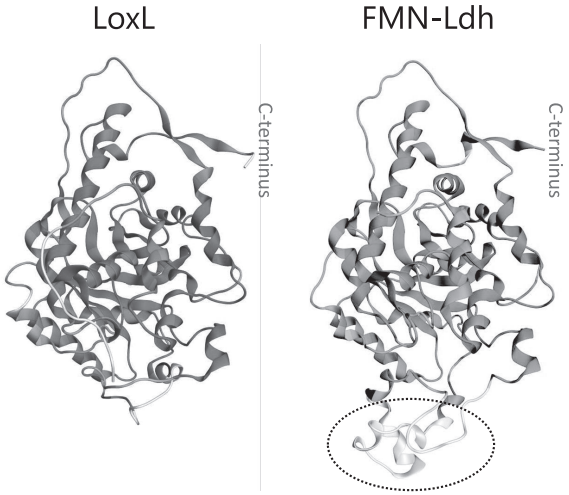

Fig. 5 Comparison of *E. coli* FMN-Ldh and *Enterococcus* Lox homology model structures. Secondary structures are shown in the ribbon representations. An extra part of *E. coli* FMN-Ldh is highlighted by a dotted circle.

using a template structure with the highest sequence identity (Fig. 5). The sequence identity between *E. coli* FMN-Ldh (396 amino acid residues) (DDBJ/EMBL/GenBank accession number: CP011938) and LoxL (367 amino acid residues) (DDBJ/EMBL/GenBank accession number: LC377909) was only 29%. On the other hand, the overall structures of FMN-Ldh and LoxL superimpose well, with a root mean square deviation (RMSD) for atomic Cα positions of 1.9 Å. As expected, both the structures are quite similar to each other. However, only FMN-Ldh retained an extra part that was expected to be a membrane-binding site (Fig. 5). It was positioned far from the C-terminus. In the prepared FMN-Ldh, the His-tag and spacer peptide consisting of 15 amino acid residues was added to the C-terminus. The effect of 6 consecutive His residues on the extra part is unknown.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgements

This work was supported by JSPS KAKENHI; Grant Number 18K06616. We would like to thank Editage (www.editage.jp) for English language

editing.

References

1. Dubois J, Chapman SK, Mathews FS, Reid GA, and Lederer F: Substitution of Tyr254 with Phe at the active site of flavocytochrome b2: consequences on catalysis of lactate dehydrogenation. Biochemistry, 29:6393-6400, 1990.
2. Garvie EI: Bacterial lactate dehydrogenases. Microbiol Rev, 44:106–139, 1980.
3. Futai M and Kimura H: Inducible membrane-bound L-lactate dehydrogenase from *Escherichia coli*. J Biol Chem, 252:5820-5827, 1977.
4. Dong JM, Taylor JS, Latour DJ, Iuchi S, and Lin EC: Three overlapping *lct* genes involved in L-lactate utilization by *Escherichia coli*. J Bacteriol, 175:6671-6678, 1993.
5. Ma C, Gao C, Qiu J, Hao J, Liu W, Wang A, Zhang Y, Wang M, and Xu P: Membrane-bound L- and D-lactate dehydrogenase activities of a newly isolated *Pseudomonas stutzeri* strain. Appl Microbiol Biotechnol, 77:91–98, 2007.
6. Gao C, Jiang T, Dou P, Ma C, Li L, Kong J, and Xu P: NAD-independent L-lactate dehydrogenase is required for L-lactate utilization in *Pseudomonas stutzeri* SDM. PLoS One, 7:e36519, 2012.
7. Hiruta M and Nishiya Y: Structural prediction and interpretation of the substrate specificity of a lactate oxidase from *Enterococcus* sp. NBRC3427. Int J Anal Bio-Sci, 5:30-36, 2017.
8. Hiruta M and Nishiya Y: Creation of an L-mandelate oxidase via structure-guided design of engineered lactate oxidase. Int J Anal Bio-Sci, 6:25-29, 2018.
9. Hiruta M, Yoshida S, Shimozawa Y, and Nishiya Y: Altered substrate specificities of mandelate oxidases generated by site-directed mutagenesis of L-lactate oxidase. Int J Anal Bio-Sci, 7:35-39, 2019.
10. Kean KM and Karplus PA: Structure and role for active site lid of lactate monooxygenase from *Mycobacterium smegmatis*. Protein Sci, 28:135-149, 2019.
11. Matsuno H, Date T, Kubo Y, Yoshino Y, Tanaka N, Sogabe A, Kuroita T, and Serizawa T: Enhanced adsorption of protein fused with polymeric material-binding peptide. Chem Lett, 38:834-835, 2009.