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Construction of a protein-engineered variant of D-fructose dehydrogenase for direct electron transfer-type bioelectrocatalysis

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ABSTRACT

D-Fructose dehydrogenase (FDH), a heterotrimeric membrane-bound enzyme, exhibits strong activity in direct electron transfer- (DET-) type bioelectrocatalysis. We constructed a variant (Δ1*c*FDH) that lacks 143 amino acid residues involving one heme *c* moiety (called heme 1*c*) on the N-terminus of subunit II, and characterized the bioelectrocatalytic properties of Δ1*c*FDH using cyclic voltammetry. A clear DET-type catalytic oxidation wave of D-fructose was observed at the Δ1*c*FDH-adsorbed Au electrodes. The result clearly indicates that the electrons accepted at the flavin adenine dinucleotide catalytic center in subunit I are transferred to electrodes via two of the three heme *c* moieties in subunit II without going through heme 1*c*. In addition, the limiting current density of Δ1*c*FDH was one and a half times larger than that of the native FDH in DET-type bioelectrocatalysis. The downsizing protein engineering causes an increase in the surface concentration of the electrochemically effective enzymes and an improvement in the heterogeneous electron transfer kinetics.

1. Introduction

Direct electron transfer- (DET-) type bioelectrocatalysis involves coupling of redox enzymatic reactions and electrode reactions without mediators [1–9]. DET-type bioelectrocatalysis is attracting significant attention for the construction of mediator-free biosensors and biofuel cells and for the fundamental analysis of redox properties of enzymes [1,2,10,11]. In DET-type bioelectrocatalysis, it is important that the redox active site of an enzyme is located near the surface of the enzyme and that the enzyme is adsorbed in a proper orientation that is suitable for direct electrochemical communication. However, in most redox enzymes, the redox active site is embedded in insulating peptides. Therefore, only a limited number of redox enzymes exhibit clear DET-type bioelectrocatalytic activity [7,8,12–14].

We have focused on D-fructose dehydrogenase (FDH) from *Gluconobacter japonicus* NCBR 3260 as a model enzyme with DET-type bioelectrocatalytic activity [15,16]. FDH that is adsorbed on electrodes produces a catalytic wave of D-fructose oxidation at large current densities [17,18]. FDH shows strict substrate specificity to D-fructose and is used in diagnosis and food analysis [19,20]. A biofuel cell with the DET-type bioelectrocatalysis of FDH on the anode exhibited a high power density (2.6 mW cm^{-2}) [21]. FDH is a membrane-bound enzyme with a molecular mass of ca. 140 kDa. It is a heterotrimeric enzyme complex that consists of subunits I (67 kDa), II (51 kDa), and III (20 kDa) [22]. Subunit I has one flavin adenine dinucleotide (FAD), and subunit II has three heme *c* moieties [23]. In this work, the three heme *c* moieties are called heme 1*c*, 2*c*, and 3*c* from the N- to the C-terminus. FAD in FDH oxidizes D-fructose to 5-keto-D-fructose. It is presumed that the electron is transferred from the reduced FAD to heme 3*c*, heme 2*c*, and an electrode and that heme 1*c* is not involved in the DET-type catalytic cycle [24,25]. Therefore, it is important to construct a variant that lacks heme 1*c* to verify our assumption and to demonstrate a strategy of protein engineering to improve the DET-type bioelectrocatalytic activity.

In this study, we constructed a variant FDH that lacks 143 amino acid residues and heme 1*c* (Δ1*c*FDH) on the N-terminus of subunit II (Fig. 1). Our hypothesis is as follows. Because the rate constant of the heterogeneous electron transfer (k°) increases exponentially with a decrease in the distance between the active sites of the enzyme and the electrode surface [26], heme 2*c* in FDH (as an electron-donating site to an electrode) may come close to the electrode surface via the proposed protein engineering, resulting in an increase in k°. In addition, the protein engineering makes FDH compact and leads to an increase in the surface density of the enzyme monolayer on the electrode.

In cyclic voltammetry, the Δ1*c*FDH-adsorbed electrodes exhibited a large steady-state catalytic wave of D-fructose oxidation compared with native FDH-adsorbed electrodes. To date, several downsizing protein engineering approaches have been attempted, for example, for Cu efflux oxidase to delete a helical region [27] and for horseradish peroxidase [28], glucose oxidase [29] and cellobiose dehydrogenase [30] for deglycosylation. However, this study is the first trial of the deletion of such a long region that includes one of the prosthetic groups to enhance the DET-type bioelectrocatalytic activity.

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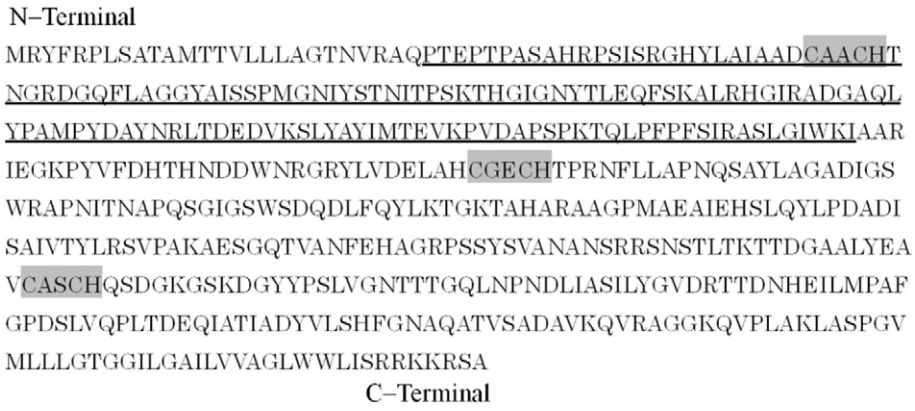

Fig. 1. The amino acid sequences of subunit II. The underlined amino acid sequences were deleted. Three hatched amino acid sequences (CXXCH) are motifs for the heme *c* covalently bound sites. The N-terminal sequences from RYFRP to NVRAQ are the signal peptide.

2. Experimental

2.1. Materials

Herculase II fusion DNA polymerase and restriction endonucleases were purchased from Agilent Technologies (Santa Clara, CA) and Takara Shuzo (Japan), respectively. DNA ligase was obtained from Toyobo (Japan). Potassium ferricyanide was obtained from Nacalai Tesque (Japan). Other chemicals were obtained from Wako Pure Chemical Industries (Japan).

2.2. Preparation of the mutants and FDH

To prepare the Δ1*c*FDH variant, an in-frame deletion in the *fdh* gene was introduced to the plasmid pSHO13 that was used for the expression of FDH [23]. pSHO13 is a broad-host-range vector pBBR1-MCS4 [31] inserted with a fragment of the putative promoter region of the *adhAB* gene of *G. oxydans* 621H and a fragment of the complete fdh_{ATG} gene. The N-terminal amino acid sequences from RYFRP to NVRAQ (Fig. 1) are believed to be a signal peptide that relates to the expression of subunit II; hence, it was not deleted. The signal peptide was predicted by SignalP4.1 [32]. pSHO13 was treated with *Hin*dIII and *Bam*HI, and a 3.5 kbp DNA fragment corresponding to most of subunit I and all of subunits II and III was inserted into pT7Blue (Novagen, Merck, USA), which was treated with *Hin*dIII and *Bam*HI to yield pYUF3 as a template of the following inverse polymerase chain reaction. The sequences of pYUF3, except for a part corresponding to heme 1*c*, were amplified by Herculase II fusion DNA polymerase using primers fdhC_AARIEGK(+) (5′-GCGGCAAGAATCGAAGGCAAACCC-3′) and fdhC_SignalTerminal(−) (5′-TTGCGCCCGTACGTTCGTCCCTGCGAG-3′), and the polymerase chain reaction (PCR) products were self-ligated to yield pYUF26. pYUF26 was treated with *Hin*dIII and *Bam*HI, and a 3.1 kbp DNA fragment was inserted into pSHO13 that was treated with *Hin*dIII and *Bam*HI to yield pYUF27. An in-frame deletion was introduced to pSHO13 to form pYUF27. All nucleotide sequences of the fdh_{ATG} gene of PCR products were confirmed by Fasmac sequencing service (Japan).

G. oxydans NBRC12528 Δ*adhA*::Km^r was transformed with pYUF27 via a triparental mating method using the HB101 strain that includes pRK2013 [33]. *Gluconobacter* cells were cultivated, and then FDH and the mutant were purified as described in a previous paper [23] with a little modification as described below. The elution of Δ1*c*FDH from a DEAE-Sepharose column was performed using a concentration gradient of McIlvain buffers (McB) at pH 6.0 from 20-fold-diluted McB to 4-fold-diluted McB that contained 1 mM 2-mercaptoethanol and 0.1% (w/v) Triton X-100 ($M = mol\ dm^{-3}$).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12.5% acrylamide gel at 100 V at room temperature, and proteins were stained with Coomassie Brilliant Blue.

2.3. Electrochemical measurements

Cyclic voltammetry and chronoamperometry were performed on an ALS 611 s voltammetric analyzer under anaerobic conditions. The working electrode was an Au electrode, whereas the reference and counter electrodes included a handmade Ag|AgCl|sat.KCl electrode and a Pt wire, respectively. All potentials are referred to the reference electrode in this paper. Cyclic voltammograms (CAs) were recorded at 25 °C and a scan rate (v) of 10 mV s^{-1} in 1.0 mL of a McB (pH 4.5) that contained 0.1 M D-fructose ($L = dm^3$). Chronoamperograms (CAs) were recorded under the same conditions. For measurements of the DET-type catalytic waves, a 3 μL aliquot of the corresponding enzyme stock solution was added to the buffer solution. We fixed the enzyme activity per volume (activity-based enzyme concentration, c_A) at 4×10^2 U mL^{-1} and the concentration of Triton X-100 at 0.1% (w/v) for the enzyme stock solutions used in this work. Here, one unit (U) of the FDH activity is defined as the amount of enzyme oxidizing 1 μmol of D-fructose per minute at pH 4.5.

2.4. Other analytical methods

The FDH activity was spectrophotometrically measured with potassium ferricyanide (as an electron acceptor) and the ferric dupanol reagent, as described in the literature [22]. The total protein concentration (c_t) was determined using a DC protein assay kit (Bio-Rad, CA) that used bovine serum albumin as the standard. Oxygen consumption rate (v_{O_2}) of whole cells was measured as described in a previous paper [23].

3. Result and discussion

We constructed and purified Δ1*c*FDH. The SDS-PAGE results indicated that Δ1*c*FDH was acceptably purified, and subunit II was downsized from 51 kDa to 36 kDa (data not shown). The evaluated molecular mass of subunit II is close to that expected from the protein engineering procedure. The heme-based enzyme concentration (c_E) of the native FDH was spectrophotometrically determined using the adsorption coefficient of the reduced heme *c* at 550 nm ($\varepsilon_{550\ nm} = 23{,}000\ M^{-1}\ cm^{-1}$ [34]). c_E of Δ1*c*FDH was evaluated by assuming that the adsorption coefficient of Δ1*c*FDH is two-thirds that of the native FDH, as there are two heme *c* moieties in Δ1*c*FDH and three in the native FDH. c_E determined spectrophotometrically and c_t determined using the DC protein assay kit were 15 μM and 1.7 g L^{-1}, respectively, for the native FDH solution; the values were 28 μM and 10 g L^{-1}, respectively, for the Δ1*c*FDH solution. The results indicate that the purity of the Δ1*c*FDH solution is lower than that of the native FDH solution; c_E/c_t was 2.8 μmol g^{-1} and 8.8 μmol g^{-1} for Δ1*c*FDH and the native FDH, respectively. Because c_A was set at 4×10^5 U L^{-1} for both of the enzyme solutions, the data