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Ultimate downsizing of \( \text{D}-\text{fructose dehydrogenase} \) for improving the performance of direct electron transfer-type bioelectrocatalysis

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ABSTRACT

\( \text{D}-\text{Fructose dehydrogenase (FDH), a membrane-bound heterotrimeric enzyme, shows strong activity in direct electron transfer (DET)-type bioelectrocatalysis. An FDH variant (Δ1c2cFDH) which lacks 199 amino acid residues including two heme c moieties from N-terminus was constructed, and its DET-type bioelectrocatalytic performance was evaluated with cyclic voltammetry at Au planar electrodes. A DET-type catalytic current of D-fructose oxidation was clearly observed on Δ1c2cFDH-adsorbed Au electrodes. Detailed analysis of the steady-state catalytic current indicated that Δ1c2cFDH transports the electrons to the electrode via heme 3c at a more negative potential and at more improved kinetics than the recombinant (native) FDH.} \)

1. Introduction

Bioelectrocatalysis, which couples the electrode reaction and the catalytic function of the redox enzyme, attracts attention from the view point of environment, energy, and health [1–10]. Especially, direct electron transfer (DET)-type bioelectrocatalysis that directly couples the two reactions plays an important role in constructing mediator-free biofuel cells and biosensors with simplicity and minimum thermo-dynamic energy loss.

\( \text{D}-\text{Fructose dehydrogenase (FDH) from } \text{Gluconobacter japonicus} \) NBRC3260 is a heterotrimeric membrane protein consisting of subunit I (67 kDa), II (50 kDa), and III (20 kDa). Subunit I contains a flavin adenine dinucleotide (FAD) as the catalytic center, while subunit II contains three heme c moieties that we call heme 1c, 2c, and 3c from N-terminus of subunit II [11,12]. FDH catalyzes the oxidation of \( \text{D}-\text{fructose} \) and shows high DET-type bioelectrocatalytic activity [13]. In previous researches in our group, it was pointed out that the electron was transferred from the substrate-reduced FAD, to heme 3c, heme 2c, and an electrode, and that heme 1c was not involved in the catalytic electron transfer [14]. In addition, an FDH variant in which the region containing the heme 1c binding site was largely deleted (Δ1cFDH) showed an increase in the catalytic current density [15]. This was presumably due to the downsizing of the enzyme, which resulted in an increase of the surface concentration of Δ1cFDH.

In this study, we constructed an FDH variant which lacks 199 amino acid residues including heme 1c and 2c moieties (Δ1c2cFDH, Fig. 1) with the expectations of (1) an increase in the surface concentrations due to the downsizing and (2) the electron transfer from heme 3c directly to electrodes. The two expectations may lead to an increase in the limiting current density and the reduction of the over potential for the catalytic oxidation of \( \text{D}-\text{fructose} \), respectively.

2. Experimental

2.1. Materials

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

2.2. Preparation of the mutants and FDH

In the preparation of the Δ1c2cFDH mutant, in-frame deletion was introduced into plasmid pYUF3 [15]. pYUF3 is a vector pT7Blue (Novagen, Merck, USA) into which a 3.5 kbp DNA fragment corresponding to most of subunit I and all of subunit II and III are inserted. Except for the sequence containing heme 1c and heme 2c, pYUF3 was amplified by inverse polymerase chain reaction using herculase II fusion DNA polymerase. The primers used were fdhC_NSTLKTBD(+) (5′-AATAG TACTCTGACAAAAACAACCGAT-3′) and fdhC_SignalTerminal(−) (5′-TTGCGCCCGTACGTTCGTCCCTGCGAG-3′), and the PCR product was self-ligated by Ligation-High to form pYKF1. Subsequently, PCR was carried out using pYKF1 as a template and primers UF (5′-
N-terminal  
MRYFRPLSATAMTTVVLLAGNTNVRAQPTEPTPASSHRPSISRGHLYIAADCAACHT
NGRDQGFLAGGAYISSPMGNYNSTIPSKTHGIGNYTLQFSKALKRHGIRADGACL
YPAMPYDAYNRLTDENVKLYAYIMTEVKVDPAPSPTQPLPFSSNASGLWKIAAR
IEGKPYVFDHHTNDDWNWGRYVLVDELAHCGECHTPRNFLAPNQASAYLAGADIGS
WRAPNTNAFQGGGWSQDLFQYKLTGTKAHTAHAAAGMPAEIHSLQYLPADAI
SAIVTLRBSVPAKASEGQTWANFHEAGRPSYSVANANSRXSNSSLTCTDGALEYA
VCASCHQSDGKGSDKGYYPSLGVGNTTQQLNPNDLIALYGVDRTBDNEILMPAF
GPDQLVQLPDTLEQIALIATIDYVLSSFNGAQTAVSADAKVQVRAGKGQVKLAPSGV
MLLLCTGGILGAILVAGLWPLLSSRRKRKSAC

C-terminal  

Fig. 1. The amino acid sequence of FDH subunit II. The underlined regions were deleted. Three marked sequences (CXCH) are the heme c-binding sites.

3. Results and discussion

We constructed and purified Δ1c2FDH. The SDS-PAGE results showed that Δ1c2FDH was satisfactorily purified, and subunit II was reduced in size from 51 kDa to 20 kDa (data not shown), which is consistent with that predicted by the protein engineering. The heme-based enzyme concentrations were determined by spectrophotometric measurements using the molar extinction coefficient of the reduced heme c at 550 nm (\(\varepsilon_{550\text{ nm}} = 23000\text{ M}^{-1}\text{ cm}^{-1}\)) [17], considering that the recombinant (native) FDH, Δ1cFDH, and Δ1c2FDH have three, two, and one heme c moiety (moieties), respectively. The activities of the recombinant (native) FDH, Δ1cFDH, and Δ1c2FDH were evaluated to be 2.0 \(\times 10^{10}\text{ U mol}^{-1}\), 1.2 \(\times 10^{10}\text{ U mol}^{-1}\), and 3.0 \(\times 10^{9}\text{ U mol}^{-1}\), respectively.

Fig. 2(A) shows cyclic voltammograms (CVs) measured in the presence of n-fructose with the Au electrodes on which the recombinant (native) FDH, Δ1cFDH, and Δ1c2FDH were adsorbed. A clear DET-type catalytic wave attributed to the n-fructose oxidation was observed at all of the enzyme-adsorbed electrodes. These waves were independent of the scan rate (from 1 to 50 mV s\(^{-1}\)) and the rotating speed (from 0 to 4000 rpm), indicating that the catalytic currents were independent of the mass transfer of the substrate due to a high concentration of the substrate and were controlled by the interfacial electron transfer kinetics and the enzyme kinetics [18–22].

The CVs were normalized against the current density at 0.5 V (\(j_{0.5}\)), as shown in Fig. 2(B). The half-wave potential of the steady-state catalytic wave at the Δ1c2FDH-adsorbed electrode was slightly smaller than that at the recombinant (native) FDH-adsorbed electrode. This may be ascribed to a decrease in the surface concentration of Δ1c2FDH probably due to a decrease in the hydrophobic property by the downsizing of the hydrophobic subunit II. Triton X-100 may in part competitively block the adsorption of Δ1c2FDH.

Next, ESR measurements were done for Δ1c2FDH that was reduced by n-fructose. The two-electron reduced variant yielded a strong isotropic ESR signal of an organic radical at \(g = 2\) (data not shown). This signal is assigned to the FAD semiquinone radical. The data clearly indicate that one of the two electrons in the fully reduced FAD remains on the FAD to generate the semiquinone radical and that the other electron is transferred to heme c from the fully reduced FAD.

In addition, in order to clarify the electron transfer pathway in the DET reaction by Δ1c2FDH, potassium cyanide (KCN) was added to the reaction buffer at a final concentration of 1 mM. The catalytic current density greatly decreased in the presence of KCN, (Fig. 2(C)). This should be due to the coordination of cyanide ion to the axial ligand of...
the heme iron, which may cause a redox potential shift of approximately 0.4 V to the negative potential direction [23]. Therefore, the electron transfer from the reduced flavin to the CN−-coordinated heme is electrochemically determined for heme 3c becomes thermodynamically difficult. The very small catalytic wave was observed even in the presence of KCN. The wave showed a residual current density (j) without sigmoidal characteristics, indicating a slow electron transfer kinetics from the CN−-treated Δ1cFDH to the electrode [21,22]. The possibility could not be ruled out that the electron might be transferred directly from the reduced FAD to the electrode.

Now, the steady-state catalytic waves were analyzed by considering the random orientation of the enzymes. A steady state model without the concentration polarization of the substrate was used. The following equation is given to this model [19,21]:

\[ j_{\text{lim}} = n_{E} F k_{c} \Gamma \]

where \( n_{E} \) is the number of electrons in the rate determining step of the interfacial electron transfer process (\( =1 \) in this case since the number of the electron for the heme 3c in Δ1c2FDH); \( F \), Faraday constant; \( R \), the gas constant; \( T \), the absolute temperature; \( k'_{\text{max}} \), the standard rate constant at the closest approach in the best orientation of the enzyme; \( \Delta d \), the distance between the closest and farthest approach of the enzyme; \( \alpha \), the transfer coefficient; \( \beta \), the coefficient in the long range electron transfer; \( E'_{\text{F}} \), the formal potential of the redox center of the enzyme for electrochemical communication with the electrode.

\[ j = \frac{1}{k'_{\text{max}}} \left( 1 + \exp \left( \frac{\Delta d}{R} (E - E'_{\text{F}}) \right) \right) + k_{c} \exp \left( \frac{\Delta d}{R} (E - E'_{\text{F}}) \right) \]

\[ \times \ln \left( k'_{\text{max}} \exp(-\beta d) \left[ 1 + \exp \left( \frac{\Delta d}{R} (E - E'_{\text{F}}) \right) \right] + k_{c} \exp \left( \frac{\Delta d}{R} (E - E'_{\text{F}}) \right) \right) \]

\[ \Gamma = \frac{n_{E} F k_{c} \Gamma}{j_{\text{lim}}} \]

where \( j_{\text{lim}} \) is the catalytic current density (\( j_{\text{lim}} \)) value completely controlled by the enzyme kinetics is expressed by the following equation [18–22]:

\[ j_{\text{lim}} = n_{E} F k_{c} \Gamma \]

Fig. 2. (A) Original CVs and (B) normalized CVs of α-fructose oxidation at the recombinant (native) FDH-, Δ1cFDH-, and Δ1c2FDH-adsorbed electrodes in McB (pH 4.5) in the presence of 100 mM α-fructose under anaerobic conditions at \( v = 10 \) mV s\(^{-1}\). The broken line in panel (A) indicates the background current at the bare Au electrode. In panel (B), the background current was subtracted. In panel (C), the solid line is the CV at the Δ1c2FDH-adsorbed electrode in McB (pH 4.5) in the presence of 100 mM α-fructose under anaerobic conditions; the broken line is background, and the dotted line is a CV after the addition of KCN (1 mM in final concentration) at \( v = 10 \) mV s\(^{-1}\). Panels (D) to (F) show fitted curves obtained by non-linear least square method. The circles are the waves of the forward scan measured with (D) the Δ1c2FDH-, (E) recombinant (native) FDH-, and (F) Δ1cFDH-adsorbed electrodes. The insets indicate the evaluated parameters. The \( \Delta d \) values were obtained by assuming \( \beta = 14 \) nm\(^{-1}\) [25]. The error bars were evaluated by the Student t-distribution at a 90% confidence level.
were slightly more positive than the spectroelectrochemically determined ones of heme 3c (−10 ± 4 mV) and heme 2c for the recombinant (native) FDH. The mutation seems to cause change in the environment around the heme moiety in the variant. The relative values of $k_c'\Gamma$ were 1: 1.15: 1.74 for Δ1c2cFDH-, recombinant (native) FDH, and Δ1cFDH. The value of $\Gamma$ could not be separately evaluated, but was indirectly compared by measuring mediated electron transfer (MET)-type bioelectrocatalytic wave. MET-type-bioelectrocatalytic wave was superimposed on the DET-type wave at potentials more positive than 0.2 V on the addition of K₄[Fe(CN)₆] as a mediator (Fig. 4). The relative heights of the limiting MET-type waves (the remaining limiting currents after subtraction of the DET-type currents) were 1: 1.1: 1.8 for Δ1c2cFDH-, recombinant (native) FDH, and Δ1cFDH. The ratio is close to that of $k_c'\Gamma$. Therefore, the ratio of $k_c'\Gamma$ seems to reflect the ratio of $\Gamma$ at almost constant $k_c$. On the other hand, the mutation did not lead to a big difference in $\Delta\theta$, suggesting that the extent of the random orientation seems to be similar to each other. All these quantitative considerations support the proposed schematic of the productive orientation of the enzymes (Fig. 3). The increase of $k_{\text{max}}$ in the Δ1c2cFDH mutant seems to be attributed to a decrease in the distance between the electrochemically communicating heme and the electrode.

4. Conclusions

We successfully constructed the Δ1c2cFDH variant that lacks 199 amino acid residues including heme 1c and 2c. The Δ1c2cFDH variant showed bioelectrocatalytic wave for D-fructose oxidation at the planar gold electrode. In the DET reaction of Δ1c2cFDH, the electrons were transferred from D-fructose to the electrode via FAD and heme 3c in this order. The Δ1c2cFDH variant transferred the electrons to the electrode at a more negative potential than the recombinant (native) FDH (Fig. 3). Therefore, the energy loss in the DET-type bioelectrocatalysis of FDH successfully decreased. The interfacial electron transfer kinetics were also improved probably by shortening the distance between heme c and the electrode. However, the maximum current density decreased, which is opposite to what we expected from the downsizing mutation. This seems to be due to a decrease in the hydrophobicity of the variant.

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