Discussion on Direct Electron Transfer-Type Bioelectrocatalysis of Downsized and Axial-Ligand Exchanged Variants of α-Fructose Dehydrogenase

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ABSTRACT

α-Fructose dehydrogenase (FDH) gives a clear direct electron transfer (DET)-type bioelectrocatalytic wave even at planar gold (Au) electrodes. The recombinant (native) FDH (r_FDH) has three hemes c in subunit II (1c, 2c, and 3c from N-terminus). With a view to downsize the enzyme and shorten the distance between an electrode-active site and an electrode, we constructed a variant that lacked 143 amino acid residues involving the heme 1c moiety (Δ1cFDH) and a variant that lacked 199 amino acid residues involving the heme 1c and 2c moieties (Δ1c2cFDH). In order to shift the redox potential of heme 2c of Δ1cFDH to the negative direction, the M450 residue as the axial ligand of heme 2c was also replaced with glutamine (M450Δ1cFDH). The DET-type catalytic properties of r_FDH and the three variants at planar Au electrodes were compared with each other, and the steady-state waves were analyzed on a random orientation model. The orientation of the enzymes on the electrode was also discussed. In addition, in order to examine the electron transfer pathway in the DET-type reaction of Δ1c2cFDH, ESR measurements and inhibition of DET-type reaction by cyanide ion were performed.

Keywords : Fructose Dehydrogenase, Downsizing, Redox Potential Shift, Orientation

1. Introduction

Bioelectrocatalysis, in which the electrode reaction and the catalytic reaction of a redox enzyme are coupled with each other, have attracted increasing attention from viewpoints of environment, energy, and health thanks to enzymatic properties of highly selective and active catalysts under the mild conditions. There are two types of bioelectrocatalysis: direct electron transfer (DET) and mediated electron transfer (MET) types. Especially, DET-type bioelectrocatalysis, in which the electrode and enzymatic reactions are directly coupled, plays an significant role in the construction of mediator-free and simple bioelectrochemical-devices for biosensing (biosensors) and biochemical electricity production (biofuel cells) with minimum overpotential in theory.1–16

In order to improve or investigate the DET-type bioelectrocatalysis, several protein-engineering methods have been examined: the point mutation around the active site to change its catalytic characteristics,15–20 the deglycosylation to shorten the distance between an electrode and the redox center buried in the enzyme and improve the interfacial electron transfer kinetics,18,21,22 and the insertion of a tag sequence or cysteine residue(s) to control the characteristics,17

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recombinant (native) FDH (r.FDH) and the three variants: Δ1cFDH, Δ1c2cFDH, and M450QΔ1cFDH. Planar Au electrodes were intentionally used as scaffolds of the enzymes in order to eliminate (or minimize) the effects of the roughness factors of the electrode surface. Data obtained by electrochemical measurements at each enzyme-adsorbed Au electrode were analyzed based on a random orientation model and the obtained parameters were compared with each other. The orientation of the variants on the electrode was also discussed. In addition, in order to examine the pathway of electrons in the DET-type reaction of Δ1c2cFDH, electron spin resonance (ESR) measurements and inhibition of DET-type reaction by cyanide ion were performed.

2. Experimental

2.1 Materials

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

2.2 Preparation of the mutants and FDH

According to the literature,27,30–32 r.FDH, Δ1cFDH, M450QΔ1cFDH, and Δ1c2cFDH were purified with some little modifications as described below. Concentration gradient of a Mcllvaine buffer solution (MeB, pH 6.0) from 20-fold to 40-fold diluted McBs containing 1 mM 2-mercaptoethanol and 0.1% (w/v) Triton X-100 (M = mol dm⁻³) was employed to elution of the Δ1c2cFDH mutant from DEAE-Sepharose. In case of Δ1cFDH and M450QΔ1cFDH, elution was performed from 20-fold to 5-fold diluted McBs.

2.3 Electrochemical measurements

The working electrodes were planar Au disk electrodes (3-mm diameter, BAS Inc., Japan), which were polished with Al₂O₃ powder (0.05-μm particle size) to a mirror-like finish, rinsed with distilled water, and sonicated in distilled water. The reference electrode was handmade Ag | AgCl | sat.KCl and the working electrode was Pt wire. A 3 μL aliquot of each enzyme solution was added to a McB solution (pH 4.5) in an electrochemical cell for measurements of bioelectrocatalytic currents (L = cm³). As a result, the final concentration of Triton X-100 and 2-mercaptoethanol was 3 ppm (w/v) and 6 μM, respectively. In this paper, all the potentials are referred to the reference electrode and all electrochemical measurements were performed under anaerobic conditions. Cyclic voltammograms were recorded by using an ALS1000 electrochemical analyzer at 25°C. In order to record the rotating disk voltammetry, a rotating disk electrode (RDE-1, BAS Inc., Japan) was employed.

2.4 Electron spin resonance spectroscopy

The Δ1c2cFDH solution was concentrated to 20 μM for the measurements. The sample solution was placed into a glass capillary cell with an inner diameter of 0.5 mm. ESR spectra were recorded on a JES-FA100 spectrometer (JEOL, Japan). The microwave power was set to 2 mW.

2.5 Other analytical methods

The enzyme activity was spectrophotometrically measured using potassium ferricyanide (as an electron acceptor) and the ferric duponol reagent, as described in the literature.36

3. Results and Discussion

Each variant was successfully constructed and purified. The enzyme concentrations were spectrophotometrically determined using a molar extinction coefficient of the reduced heme c at 550 nm (ε₅₅₀nm = 23000 M⁻¹ cm⁻¹), regarding that r.FDH, Δ1cFDH, M450QΔ1cFDH, and Δ1c2cFDH have three, two, two, and one heme c moieties (moeity), respectively. Before electrochemical measurements, each enzyme solution was diluted to 7.7 μM with a 50 mM phosphate buffer solution (pH 6.0) containing 1 mM 2-mercaptoethanol and 0.1% (w/v) Triton X-100. The catalytic activities of r.FDH, Δ1cFDH, M450QΔ1cFDH, and Δ1c2cFDH in their solutions (kcat(sol)) were evaluated to be 2.0 × 10¹⁰ U mol⁻¹, 1.2 × 10¹⁰ U mol⁻¹, 1.3 × 10¹⁰ U mol⁻¹, and 3.0 × 10¹⁰ U mol⁻¹, respectively.

Usually, mesoporous electrodes are very useful (or sometime essential) to observe DET-type bioelectrocatalytic waves in order to increase the possibility of the productive orientation of redox enzymes by curvature effects.34 However, as described in our previous papers,36–32 clear steady-state waves of α-fructose oxidation were observed at planar Au electrodes on which r.FDH, Δ1cFDH, M450QΔ1cFDH, or Δ1c2cFDH was adsorbed (Fig. S3(A)). Since the scan rate (from 1 to 50 mV s⁻¹) and the rotating speed (from 0 to 4000 rpm) did not affect the catalytic waves, the catalytic currents were independent of the mass transfer of the substrate, and were controlled by the interfacial electron transfer kinetics or the enzyme kinetics.33–39 The steady-state catalytic current density (jcat) under such conditions for enzymes with single orientation is given by:40

\[
 j_{\text{cat}} = \frac{j_{\text{lim}}}{1 + k_b/k_f},
\]

where \( k_b \) is the catalytic rate constant of the enzyme reaction, but is not necessarily proportional to \( k_{cat(sol)} \). The parameters \( k_f \) and \( k_b \) correspond to the rate constants of the forward (anodic) and the reverse (cathodic) electrode reactions, respectively (Eqs. (S2) and (S3)). \( j_{\text{lim}} \) is the limiting steady-state current density (Eqs. (S3)). Detailed description on Eq. (1) is given in Appendix S1. Equation (1) predicts sigmoidal waves with a limiting current region giving \( j_{\text{lim}} \). Such ideal sigmoidal waves were observed at the M450QΔ1cFDH- and Δ1c2cFDH-adsorbed Au electrodes (Fig. S3(A)). The exponentially increasing part on the waves was clearly observed, indicating fast electron transfer between each enzyme and the electrode. It can be considered that M450QΔ1cFDH and Δ1c2cFDH adsorbed in rather ordered orientations on the planar Au electrode and that the orientation is suitable for the DET-type bioelectrocatalysis. The Δ1c2c-downsizing may induce an attractive interaction between the electrode and the electrode-active site (heme c as described later). Since the orientation of M450QΔ1cFDH is more suitable to DET than Δ1cFDH, the M450Q mutation might cause some change in the enzyme surface conditions near the heme c moiety.

In contrast, the r.FDH- and Δ1cFDH-adsorbed electrodes gave a linearly increasing region on the catalytic wave (Fig. S3(A)). This is called residual slope35,36 and is ascribed to the random orientation of enzymes with an eccentrically located electrode-active site (Appendix S1). \( j_{\text{lim}} \)

values were not clearly defined in these cases within the potential window of the measurements. At potentials more positive than 0.5 V, the DET-type reaction is inhibited by gold oxide formed at such high potentials.37,39 It can be considered that r.FDH and Δ1cFDH adsorbed randomly on the planar Au electrode.31,32 Unfortunately, we could not find clear explanation on a question why the M450Q mutation could improve the orientation. However, similar improvement in the catalytic wave was observed by the M450Q mutation in r.FDH.37

The observed CVs were normalized against the current density at 0.5 V (jₓₓₓₓ). The normalized waves at the r.FDH- and Δ1cFDH-adsorbed electrodes were almost identical with each other (Fig. S3(B)). This is the evidence that the electrode-active site of
The half-wave potential of the normalized CVs at the M450QΔ1cFDH-adsorbed electrode was approximately 0.08 V and were 0.19 V more negative than that of the wave of the Δ1cFDH-adsorbed electrode. This negative shift in the half-wave potential of the catalytic wave at the M450QΔ1cFDH-adsorbed electrode is ascribed to the negative shift in the formal potential of the electrode-active redox center (heme 2c) in the enzyme (\(E_{1/2}^o\)) by replacing methionine as the native 6th axial ligand of heme 2c at the 450th amino acid residue with glutamine with an electro-donating property. The result also supports that heme 2c is the electrode-active site of M450QΔ1cFDH.

In order to clarify the interfacial electron transfer pathway of Δ1c2cFDH on electrodes, ESR measurements were carried out under \(\alpha\)-fructose-reducing conditions. The FAD in the variant is 2-electron reduced by the hydride transfer from \(\alpha\)-fructose. The electron-reduced FAD is ESR-silent, but the substrate-reduced Δ1c2cFDH yielded a strong isotropic ESR signal at \(g \approx 2\) (Fig. 1). This signal is assigned to one of the two electrons in the reduced FAD is transferred to heme 3c to yield the semiquinone radical in the enzyme, obviously indicating that one of the two electrons in the reduced FAD is ESR-silent, but the substrate-reduced Δ1c2cFDH yielded a strong isotropic ESR signal at \(g \approx 2\) (Fig. 1). This signal is assigned to its FAD semiquinone radical in (Fig. 1). This signal is assigned to its FAD semiquinone radical in (Fig. 1). This signal is assigned to its FAD semiquinone radical in (Fig. 1). This signal is assigned to its FAD semiquinone radical in the enzyme, obviously indicating that one of the two electrons in the reduced FAD is transferred to heme 3c to yield the semiquinone radical in the enzyme. Such a coordination of CN⁻ is eliminated in the Δ2c-downsizing caused a decrease in the surface concentration of the enzyme \((I_E)\) by assuming that \(k_r\) remained almost unchanged by the mutations. Comparing the values of \(J_{0.5V}\) of M450QΔ1cFDH and Δ1cFDH-adsorbed electrode to those of other mutants adsorbed electrode, the Δ1c-downsizing is effective to increase \(I_E\). As described before, M450QΔ1cFDH adsorbed rather orderly, while Δ1cFDH adsorbed randomly on the electrode. Therefore, the Δ1c-downsizing effect is more evident for M450QΔ1cFDH compared with that of Δ1cFDH.

Contrary to our expectation, the \(J_{0.5V}\) value at the Δ1c2cFDH-adsorbed electrode was smaller than that of Δ1cFDH (M450QΔ1cFDH) and almost same as that at the rΔ1cFDH-adsorbed electrode (Fig. S3(A)). The Δ1c2c-downsizing caused a decrease in the surface concentration of the enzyme \((I_E)\). This may probably due to a decrease in the hydrophobic property of subunit II by the Δ1c2c-downsizing. Such change in the hydrophobic property seems to be very significant in FDH enzymes, since Triton X-100 is essential to solubilize the enzymes. Triton X-100 may competitively prevent the adsorption of Δ1c2cFDH to some extent. The hydrophobicity of enzyme seems to play an important role in the adsorption of such membrane-bound enzymes.

All explanations described above are qualitative. In order to discuss quantitatively, the recorded voltammograms were analyzed on the basis of the random orientation model for the DET-type bioelectrocatalysis. A steady-state catalytic current without the concentration polarization of the substrate was used, and Eq. (7) was fitted to the steady-state waves of the forward scan at the Δ1c2cFDH- and M450QΔ1cFDH-adsorbed electrodes using nonlinear regression analysis by Gnuplot with \(k_{max}/k_r, \beta, \Delta d, \) and \(E_{1/2}^o\) as adjustable parameters by setting \(J_{lim} = J_{0.5V}\) and the transfer coefficient \((\alpha) = 0.5\) (Fig. S4(C, D)), where \(k_{max}\) is the standard rate constant at the closest approach when the enzyme is most suitable orientation for the electron transfer reaction, \(\Delta d\) is the difference of the distance of electrode-active center from the electrode surface between the closest and farthest approach of the electrode.
enzyme, and $\beta$ is the coefficient in the long range electron transfer
the transfer coefficient. The physical meanings of the other
parameters are given in Appendix S1. The catalytic current of the
forward scan at the r.FDH- and Δ1cFDH-adsorbed electrodes were
also analyzed in the same manner (Fig. S4(A, B)), but $k_c'\Gamma$ was also
employed as an adjustable parameter, because clear $j_{\text{lim}}$ could not be
determined by the voltammogram.

The evaluated values of the fitting parameters for each enzyme-
adsorbed electrode are summarized in Table 1. The $E^{\text{on}}_c$ values for
M450QΔ1cFDH was evaluated to be 18 ± 1 mV, which is more
negative than those of the r.FDH (54 ± 1 mV)$^{31}$ and Δ1cFDH (34 ± 1 mV).$^{31}$ These data indicate that M450QΔ1cFDH can
transfer the electrons at a more negative potential than r.FDH due
to its negatively shifted electron donating site, heme 2c, by the
M450Q mutation. The $E^{\text{on}}_c$ values evaluated for Δ1c2cFDH (20 ± 1 mV)$^{31}$
was also more negative than those of the r.FDH and Δ1cFDH and is in good agreement with the potential of the non-
catalytic wave measured at the r.FDH-immobilized glassy-carbon
electrode on which anthracene-modified single-walled carbon
nanotubes were deposited.$^{45}$ This result supports that heme 3c is the
electrode-active site in Δ1c2cFDH, while heme 2c in r.FDH and
Δ1cFDH variants.$^{31}$ The $E^{\text{on}}_c$ value for r.FDH was almost the same
as the value for heme 2c of r.FDH determined by a mediated
spectroelectrochemical titration (60 ± 5 mV) at pH 5.0,$^{42}$ while
those values of Δ1c2cFDH and Δ1cFDH were slightly more
positive than that of heme 3c spectroelectrochemically determined
(−10 ± 4 mV). In these variants, some change in the environment
(especially in water accessibility) around the heme c moieties may
occur by the mutation.

The evaluated values of $k_c'\Gamma$ support the above discussion; the
Δ1c-downsizing is effective to increase $\Gamma'$, while Δ1c2c-downsizing
causes a decrease in $\Gamma'$. The $k_{\text{max}},k_c$ values of the M450QΔ1cFDH
and Δ1c2cFDH-adsorbed electrodes were notably larger than those
of the r.FDH- and Δ1cFDH-adsorbed electrodes. This situation is
related to our discussions that M450QΔ1cFDH and Δ1c2cFDH
adsorbed on the planar Au electrode rather homogeneously in
orientations suitable for the DET-type reaction. The distance
between the electrode and the electrode-active site in
M450QΔ1cFDH (heme 2c) and Δ1c2cFDH (heme 3c) was
shortened by the Δ1c- and Δ1c2c-downsizings, respectively, and thus $k_{\text{max}}'$ increased.

Figure 3 shows predicted orientations suitable for the DET-type
reaction of r.FDH, Δ1cFDH, and Δ1c2cFDH, in which the
orientations were set so as to minimize the distance between the
electrode-active site and the electrode. The heme 3c moiety in
Δ1c2cFDH is located relatively near the surface of the enzyme.$^{31}$
Therefore, the orientation of the variant in Fig. 3(C) may be the
most possible one to realize the fast heterogeneous electron transfer.
The occupied area of the variant on the surface is small in this
orientation. However, the adsorption affinity of the variant (to compete Triton X-100) does not seem to be enough in this
orientation. This may responsible for a decrease in $\Gamma'$ for
Δ1c2cFDH.

r.FDH and Δ1cFDH adsorb in random orientation. Therefore, Fig. 3(A) and (B) are one of the possible orientations for r.FDH and
Δ1cFDH. However, M450QΔ1cFDH adsorbs rather ordered
orientation suitable for the DET-type reaction, as described above.
The orientation in Fig. 3(B) may be a possible one to minimize the
distance between heme 2c and the electrode. However, the distance
does not seem to be sufficiently short to realize the fast heterogeneous electron transfer. Some other suitable orientation or
some conformational change by the M450Q mutation may occur.

FDH can do MET-type bioelectrocatalytic reaction in the
presence of a suitable mediator. In the MET-type reaction, the
mediator can shuttle electrons between the enzyme and an electrode.
The movement of the mediator near the electrode surface may
affected by the orientation of the adsorbed enzyme. Therefore, we
measured the MET-type bioelectrocatalytic wave under the given
conditions of the DET-type reaction of the enzymes. On the addition
of ferrocene dimethanol as a mediator, the MET-type catalytic
currents were added on the DET-type catalytic currents, as shown in
Fig. S5. The concentrations of the mediator were set to 2 μM and
4 μM to maintain a linear response of the MET-type catalytic current
to the concentration of the mediator. Unfortunately, the MET-type

Table 1. Evaluated parameters by fitting.

| Enzyme                  | $E/mV$ (vs. Ag | AgCl | sat.KCl) | $k_c'\Gamma$ | $k_{\text{max}}$ | $\Delta d/\AA^1$ |
|------------------------|----------------|------|----------|--------------|-----------------|----------------|
| Recombinant FDH$^{31}$ | 54 ± 1         |      |          | 0.76 ± 0.01  | 0.41 ± 0.01     | 5.1 ± 0.1      |
| Δ1cFDH$^{31}$          | 34 ± 1         |      |          | 1.15 ± 0.01  | 0.79 ± 0.01     | 5.9 ± 0.1      |
| M450QΔ1cFDH            | 18 ± 1         |      |          | 1.38 ± 0.01  | 20 ± 3          | 5.7 ± 0.1      |
| Δ1c2cFDH$^{31}$        | 20 ± 1         |      |          | 0.65 ± 0.05  | 11 ± 6          | 4.5 ± 0.1      |

a) The $\Delta d$ values were evaluated by assuming that $\beta = 14 \text{ Å}^{-1}$.44

Figure 3. Schematic of predicted orientations suitable for DET-type reaction of (A) r.FDH, (B) Δ1cFDH, and (C) Δ1c2cFDH. The
structures of subunit I (green) and subunit II (cyan) were prepared by FAD-glucose dehydrogenase from Aspergillus flavus (PDB 4YNT) and
thiosulfate dehydrogenase from Marichromatium purpuratum (PDB 5LO9) as templates, respectively, in the homology modeling.$^{46}$ Since the
structural information of similar proteins is not obtained, the subunit III was not shown in the structures.
catalytic current was almost the same for all of the four FDH variants. This was contrary to our expectation that the MET-type catalytic current for Δ1c2cFDH and M450QΔ1c1FDH would be smaller than those of the other FDH variants due to the suitable orientation at a decreased distance between the electrode-active site and the electrode. Change in Å-orders of the distance by the fitting might not affect the actual movement of the mediator.

The stability of each FDH variant was also investigated by measuring $j_{0.5V}$ values every hour, and the values were normalized against $j_{0.5V}$ at the start of the measurement ($t = 0$ h) in the presence of α-fructose (Fig. S6). Unfortunately, the Δ1c- and Δ1c2c-downsizings caused a decrease in the stabilization. However, the M450Q mutation seems to increase the stability as judged from the comparison between M450QΔ1cFDH and Δ1cFDH. The reason was not clear.

4. Conclusions

We re-examined the properties of DET-type bioelectrocatalysis of r.FDH and the effects of the Δ1c- and Δ1c2c-downsizings and the M450Q mutation by comparison of the DET-type bioelectrocatalytic waves and their wave analyses. Heme 3c plays as the electrode-active site in the DET-type bioelectrocatalysis of the Δ1c2cFDH variant, while heme 2c is the electrode-active site in r.FDH, Δ1cFDH, and M450QΔ1cFDH. The electron transfer pathway of Δ1c2cFDH was verified by the ESR measurements and the KCN inhibition experiment. As a result, the Δ1c2c-downsizing successfully decreased the overpotential in the bioelectrocatalytic oxidation of fructose. The Δ1c2c-downsizing was also useful to improve the orientations suitable for the DET reaction and then to improve the heterogeneous electron transfer kinetics. The positive effects were comparable with those observed in the M450Q mutation in Δ1cFDH. On the other hand, the Δ1c-downsizing was effective to increase the surface concentration of the enzyme, while Δ1c2c-downsizing caused a decrease in the surface concentration. The decrease seems to be ascribed to a decrease in the hydrophobicity of the membrane-bound enzyme in the presence of Triton X-100. Most of qualitative interpretations of the catalytic waves were supported by solid analysis of the waves. Additional mutations might be required in subunit II to increase the hydrophobicity around heme 3c in Δ1c2cFDH.

Supporting Information

The Supporting Information is available on the website at DOI: https://doi.org/10.5796/electrochemistry.20-00029.

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