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Mutation of heme *c* axial ligands in D-fructose dehydrogenase for investigation of electron transfer pathways and reduction of overpotential in direct electron transfer-type bioelectrocatalysis

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ABSTRACT

D-Fructose dehydrogenase (FDH), a flavoprotein-cytochrome *c* complex, exhibits high activity in direct electron transfer (DET)-type bioelectrocatalysis. One of the three types of heme *c* in FDH is the electron-donating site to the electrodes, and another heme *c* is presumed to not be involved in the catalytic cycle. In order to confirm the electron transfer pathway, we constructed three mutants in which the sixth axial methionine ligand (M301, M450, or M578) of one of the hemes was replaced with glutamine, which was selected with the expectation that it would shift the formal potential of the hemes in the negative direction. An M450Q mutant successfully reduced the overpotential by approximately 0.2 V, giving a limiting current close to that of the native FDH. In contrast, an M301Q mutant remained almost unchanged and an M578Q mutant drastically decreased DET-type catalytic activity. The results indicate that the electron transfer in the native FDH occurs in sequence from the flavin, through the heme *c* with M578, to the heme *c* with M450 (as the electron-donating site to the electrodes), without going through the heme *c* with M301. The M450Q mutant will be useful for biofuel cells because of the decreased overpotential.

1. Introduction

Bioelectrocatalysis is an electrode reaction coupled with a redox enzymatic reaction, which is useful for the construction of bioelectrochemical devices such as biofuel cells and biosensors, and for fundamental research into the electron transfer reactions of enzymes. The electrode reactions are classified into two types: direct electron transfer (DET)-type bioelectrocatalysis and mediated electron transfer (MET)-type bioelectrocatalysis [1–7]. In particular, DET-type bioelectrocatalysis is valuable for use in constructing simple bioelectrochemical devices and in minimizing thermodynamic energy losses in bioelectrocatalysis [1,2].

D-Fructose dehydrogenase (FDH) from *Gluconobacter japonicus* NBRC 3260 is a membrane-bound heterotrimeric enzyme comprising subunits I (67 kDa), II (50 kDa), and III (20 kDa) [8]. Subunit I has a flavin adenine dinucleotide (FAD) while subunit II has three heme *c* moieties [9]. FDH exhibits high activity in DET-type bioelectrocatalytic oxidation of fructose, and is useful for the construction of biosensors and biofuel cells [10,11]. In our previous research, it was pointed out that the FAD oxidizes D-fructose into 5-keto-D-fructose, one of the three heme *c* moieties is the electron-donating site to the electrode, and another heme *c* is not involved in the catalytic cycle [12].

Although the crystal structure of FDH remains unknown, we attempted to identify the heme *c* acting as the electron-donating site as well as the non-catalytic heme *c* by utilizing a protein engineering technique. Glutamine (Gln) exhibits stronger electron-donating character than methionine (Met) as the axial ligand of the native heme *c*. Therefore, the replacement of the natural axial Met ligand with Gln can be expected to cause a shift in the formal potential ($E^{\circ\prime}$) of the corresponding heme *c* in the negative direction [13], as demonstrated in the mutation of the axial ligand of type I copper in a multicopper oxidase, bilirubin oxidase [14]. Our hypothesis in this work is that a Gln mutation of the axial Met ligand of the electron-donating heme *c* site will cause a negative shift in the half-wave potential ($E_{1/2}$) of the DET-type sigmoidal catalytic wave; however, practically no change is expected for a Gln mutation of the axial Met ligand of the non-catalytic heme *c*. A negative shift in $E_{1/2}$ of the DET-type catalytic wave indicates a decrease in the overpotential of FDH-catalyzed fructose oxidation, which could be used to improve the performance of fructose/O_2 biofuel cells.

In this work, we constructed three mutants in which the sixth axial Met ligand (M301, M450, or M578) of one of the three types of heme *c* was replaced with Gln. The effects of the mutation on the catalytic waves were examined with cyclic voltammetry. In this work, the three types of heme *c* coordinated by M301, M450, and M578 as the axial ligands are called heme 1*c*, heme 2*c*, and heme 3*c*, respectively. The three mutants exhibited significant differences in voltammograms.

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In particular, an M450Q mutation on heme 2*c* significantly reduced the overpotential of the DET-type bioelectrocatalytic wave by approximately 0.2 V. On the basis of the changes in the catalytic waves caused by the mutations, we propose the most likely electron transfer pathway in the native FDH.

2. Experimental

2.1. Materials

We performed site-directed mutagenesis by inverse polymerase chain reactions to prepare M301Q, M450Q, and M578Q mutants. The plasmid pSHO13 [9] harboring the complete FDH_{ATG} gene was used for expressing the mutants. The site-directed mutation was introduced to the plasmid by replacing one of the ATG codons corresponding to Met301, Met450, or Met578 by the CAG codon (corresponding to Gln). FDH from *G. japonicus* and the mutants were expressed in *Gluconobactor oxydans* NBRC12528 Δ*adhA*::Km^r and purified, as described in a previous paper [9]. The concentration of FDH and the mutants were spectrophotometrically determined using an adsorption coefficient of heme *c* at 550 nm (ε_{550} = 23.000 M^{-1} cm^{-1}; M = mol dm^{-3} [15]).

Potassium ferricyanide was purchased from Nakalai Tesque (Japan). Other chemicals were acquired from Wako Pure Chemical Industries (Japan).

2.2. Electrochemical measurements

Cyclic voltammetry was performed in a McIlvain buffer (pH 4.5) at 25 °C on an ALS 611s voltammetric analyzer under anaerobic conditions. The working electrode was an Au electrode. The Au electrode was polished to a mirror-like finish with Al_2O_3 powder (0.05-μm particle size), rinsed with distilled water, and sonicated in distilled water. The reference and counter electrodes were a handmade Ag|AgCl|sat.KCl electrode and a Pt wire, respectively. Here, all potentials are referred to the reference electrode. All electrochemical measurements were performed in 1.0 mL of McIlvain buffer (pH 4.5) containing 0.1 M D-fructose under anaerobic conditions at a scan rate of 10 mV s^{-1}. In measurements of bioelectrocatalytic currents, 3 μL each of the enzyme stock solutions (native FDH: 28 μM, M301Q: 17 μM, M450Q: 15 μM, M578Q: 16 μM) was added to the buffer solution.

2.3. Other analytical methods

FDH activity was measured photometrically with potassium ferricyanide (as an electron acceptor) and the ferric dupanol reagent, as described in Ref. [8]. One FDH unit was defined as the amount of enzyme oxidizing 1 μmol of D-fructose per min at pH 4.5. The protein concentration was determined with a DC protein assay kit (Bio-Rad, CA) using bovine serum albumin as a standard.

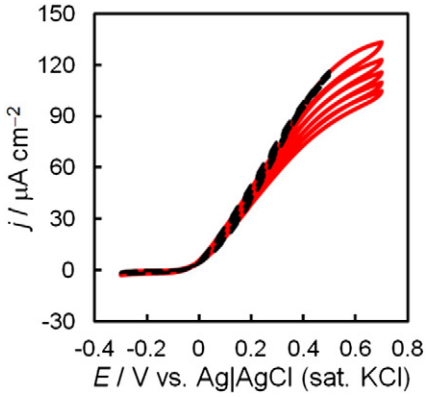

Fig. 2. Multi-scan cyclic voltammograms of D-fructose (0.1 M) at an FDH-adsorbed Au electrode in a McIlvain buffer (pH 4.5) containing 0.1 M D-fructose under anaerobic conditions at a scan rate of 10 mV s^{-1}. The broken line indicates a multi-can cyclic voltammogram in the potential range from −0.3 V to 0.5 V.

3. Result and discussion

Fig. 1(A) shows cyclic voltammograms of native FDH- and M450Q mutant-adsorbed Au electrodes in the presence of D-fructose. Each of the enzymes was physically adsorbed on a bare Au electrode from an electrolysis solution containing the corresponding enzyme. Clear DET-type catalytic waves corresponding to fructose oxidation were observed at both of the enzyme-adsorbed electrodes. The current–potential curves were not affected by stirring. Therefore, the catalytic current is controlled by the enzyme kinetics and the interfacial electron transfer kinetics alone [15–20]. The current approached a limiting value at 0.5 V at the M450Q mutant-adsorbed electrode. The limiting catalytic current (I_{lim}; to be observed at more positive potentials) is completely controlled by the enzyme kinetics [15–20]:

$$I_{lim} = n_E F A k_{cat} \Gamma, \quad (1)$$

where n_E is the number of electrons in one catalytic turnover of enzyme, *F* is the Faraday constant, *A* is the electrode surface area, k_{cat} is the catalytic constant, and Γ is the surface concentration of enzyme. It can be assumed that the surface concentrations of the enzymes are almost identical with each other under our experimental condition, as evidenced by quartz crystal microbalance measurements for other FDH mutants [16]. In order to discuss k_{cat} in Eq. (1), it is important to record I_{lim} at sufficiently positive potentials. However, measurements at potentials more positive than 0.5 V cause a decrease in the catalytic current because of the formation of an Au oxide layer [16]. In reality, when the potential was scanned up to 0.7 V, the current decreased gradually during the multiple scans, as shown in Fig. 2. Such a decrease in the

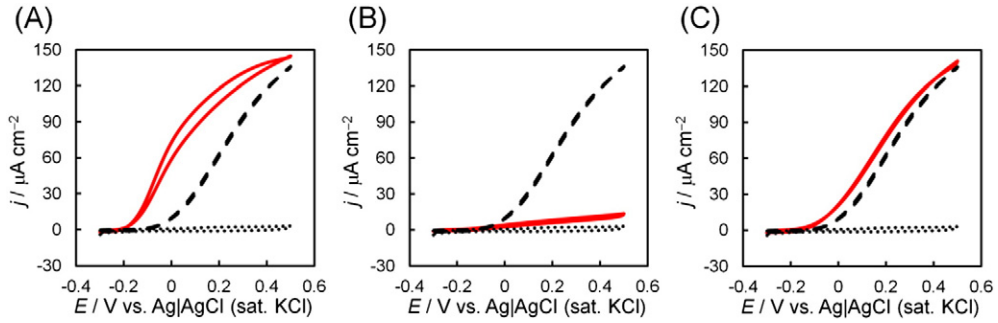

Fig. 1. Cyclic voltammograms of D-fructose oxidation at (A–C: broken line) a native FDH-adsorbed Au electrode and (solid lines) (A) M450Q, (B) M578Q, and (C) M301Q mutant adsorbed Au electrodes in a McIlvain buffer (pH 4.5) containing 0.1 M D-fructose under anaerobic conditions at a scan rate of 10 mV s^{-1}. The dotted lines indicate a background voltammogram at a bare Au electrode.