Electrochemistry Communications 38 (2014) 28–31

Contents lists available at ScienceDirect

Electrochemistry Communications

journal homepage: www.elsevier.com/locate/elecom

Short communication

The electron transfer pathway in direct electrochemical communication of fructose dehydrogenase with electrodes

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ARTICLE INFO

Article history:
Received 21 August 2013
Received in revised form 18 October 2013
Accepted 18 October 2013
Available online 25 October 2013

Keywords:
Fructose dehydrogenase
Direct electron transfer
Bioelectrocatalysis
Heme C
Electron transfer pathway

ABSTRACT

A heterotrimeric membrane-bound fructose dehydrogenase (FDH) complex from *Gluconobacter japonicus* NBRC3260 catalyzes oxidation of D-fructose into 2-keto-D-fructose and is one of typical enzymes allowing a direct electron transfer (DET)-type bioelectrocatalysis. Subunits I and II have a covalently bound flavin adenine dinucleotide and three heme C moieties, respectively. We have constructed subunit I/III subcomplex (ΔcFDH) lacking of the heme C subunit. ΔcFDH catalyzes the oxidation of D-fructose with several artificial electron acceptors, but loses the DET ability. The formal potentials ($E^{\circ\prime}$) of the three heme C moieties of FDH have been determined to be -10 ± 4, 60 ± 8 and 150 ± 4 mV (vs. Ag|AgCl|sat. KCl) at pH 5.0, while the onset potential of FDH-catalyzed DET-type bioelectrocatalytic wave is −100 mV. Judging from these results, we conclude that FDH communicates electrochemically with electrodes via the heme C, and discuss the pathway of the electron transfer in the catalytic process.

1. Introduction

Direct electron transfer (DET)-type bioelectrocatalysis is an intriguing phenomenon. DET reactions have the benefit to minimalize the loss of thermodynamic efficiency in bioelectrocatalytic reactions and may be utilized in several bioelectrochemical devices such as biofuel cells and biosensors [1,2]. Although DET bioelectrocatalysis is observed only for some limited redox enzymes, the interfacial electron transfer (ET) between electrodes and redox enzymes is one of the most important and interesting subjects in this field. There must be some factors of critical importance to govern DET reactions. However, the factors remain to be elucidated.

D-Fructose dehydrogenase (FDH; EC 1.1.99.11) from *Gluconobacter japonicus* NCBR 3260 is a heterotrimeric membrane-bound enzyme complex with a molecular mass of ca. 140 kDa, consisting of subunits I (67 kDa), II (51 kDa), and III (20 kDa). Subunits I and II have a covalently bound flavin adenine dinucleotide (FAD) and three heme C moieties, respectively. FDH shows strict substrate-specificity to D-fructose, and is utilized in diagnostic and food analyses [3,4]. On the other hand, FDH provides large catalytic current density in DET bioelectrocatalysis [5] as well as some hydrogenases [6,7]. It is thought that FDH reacts with electrodes at the heme C subunit [5], but no direct evidence has been reported to support the idea. In this study, we have constructed subunit I/III subcomplex (ΔcFDH) that lacks subunit II containing the three heme C moieties, and compared the electrochemical features with those of FDH in order to examine the ET pathway from FDH to electrodes. Based on bioelectrocatalytic kinetics, thermodynamics and protein engineering, we will propose an idea that two of the three heme C moieties are involved in the catalytic cycle and the DET reaction, while the other heme with the highest formal potential remains reduced.

2. Experimental

2.1. Chemicals

$(NH_4)_2OsCl_6$, $Ru(NH_3)_6Cl_3$, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0), and 2,3-dimethoxy-5-farnesyl-1,4-benzoquinone (Q_1) were obtained from Sigma–Aldrich Co. (USA). Na[Fe(edta)] (edta; ethylenediamine tetraacetate) was purchased from Dojindo Laboratory (Japan). Other chemicals were from Wako Pure Chemical Industries (Japan).

2.2. Purification of FDH and ΔcFDH

The expression and purification of FDH and ΔcFDH were carried out as described in a previous paper [8]. However, some modifications were done for the purification of the soluble ΔcFDH. In the literature [6], all of the solutions used for the purification contain TritonX-100 and mercaptoethanol, as in the case of the FDH purification. However, TritonX-100 may adsorb on electrodes and mercaptoethanol may form self-assembled layers on Au electrodes. Since these situations might inhibit DET reactions of redox proteins, the two reagents were removed from all of the solutions used for the purification of ΔcFDH. In brief, the freshly harvested cells were suspended with 20-fold-diluted McIlvaine buffer (pH 6.0) and then disrupted with a French pressure cell press (Thermo Fisher Scientific, Waltham, MA). The resulting lysate

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http://dx.doi.org/10.1016/j.elecom.2013.10.024

was centrifuged at 100,000 × *g* for 1 h. The supernatant was applied to a DEAE-Sepharose column equilibrated with 20-fold-diluted McIlvaine buffer (pH 6.0). The purity of ΔcFDH was checked on SDS-PAGE by Coomassie brilliant blue R-250 staining.

2.3. Electrochemical measurements

Cyclic voltammetry was carried out in McIlvaine buffers (pH 5.0 and 6.0) at 25 °C on a BAS CV-50 W electrochemical analyzer under anaerobic conditions. The working electrode was an Au electrode. The reference and counter electrodes were a handmade Ag|AgCl|sat.KCl electrode and a Pt wire, respectively. All the potentials are referred to the reference electrode in this paper.

2.4. Spectroelectrochemical determination of protein redox potentials

The formal potentials of the hemes C in FDH were determined by a mediated spectroelectrochemical titration method according to the literature [9]. Mediated bulk electrolysis was carried out at 25 °C on a BAS CV-50 W electrochemical analyzer under stirring and anaerobic conditions in McIlvaine buffer (pH 5.0) containing 0.3% (w/w) Antifoam PE-L (Wako Pure Chem., Japan) as an antifoaming reagent. Spectral change of the electrolysis solutions was monitored on a MultiSpec-1500 photodiode array (Shimadzu, Japan). The redox mediators used were 200 μM $Ru(NH_3)_6Cl_3$ ($E_m = -172$ mV; E_m being the midpoint potential determined with cyclic voltammetry at pH 5.0), 200 μM Na[Fe(edta)], ($E_m = -91$ mV); 200 μM $(NH_4)_2OsCl_6$ ($E_m = 35$ mV), and 100 μM $K_3[Fe(CN)_6]$ ($E_m = 225$ mV).

3. Results and discussion

We constructed a strain to produce ΔcFDH and purified ΔcFDH in the absence of TritonX-100 and mercaptoethanol. The concentration of the purified ΔcFDH was spectrophotometrically determined using an extinction coefficient of free FAD (11,300 M^{-1} cm^{-1} [10]). D-Fructose oxidation activity of ΔcFDH was measured with $K_3[Fe(CN)_6]$ as an electron acceptor as described in the literature [3]. ΔcFDH shows a high activity to oxidize D-fructose of 40 ± 3 U mg^{-1} at the optimum pH (6.0), which is compared with that of FDH (260 ± 20 U mg^{-1} at the optimum pH (5.0)).

However, in contrast to FDH, any DET catalytic current was not observed with ΔcFDH (even at ca. 3 μM) at a bare Au electrode (in the presence of 0.2 M D-fructose, Fig. 1A). FDH loses the DET activity by removing the heme C subunit. When *p*-benzoquinone (*p*BQ) was added in the electrolysis solution containing ΔcFDH and D-fructose, a clear catalytic current of D-fructose oxidation was observed (Fig. 1A), in which *p*BQ works as an ET mediator.

The bi-molecular reaction rate constant (k) between ΔcFDH and the quinones was assessed from the dependence of the limiting catalytic current on the quinone concentration according to the literature [11] (Fig. 1A, inset). The result is depicted in Fig. 2A. The log(k) values increase linearly with E_m of the quinone with a slope of 30 V^{-1}. This property is called linear free energy relationships (LFER) observed for non-specific interactions of a series of related compounds with the identical main structure. Since the slope is given by $\beta nF/(2.303RT)$ (n: the number of electron in the rate determining step, β: proportional constant in LFER ($0 < \beta < 1$); other ones have usual meanings), β is evaluated to be 0.5 by assuming $n = 1$. Thus we can conclude that the *p*BQs work as non-specific electron acceptors of ΔcFDH.

The k values between FDH and the *p*BQs were also evaluated in a similar manner based on MET reaction, where TritonX-100 (1% as a final concentration) was added in the electrolyte solution to inhibit the DET reaction (Fig. 1B, inset). It is very interesting that the log(k) values of Q_0 and Q_1 are obviously larger than those expected from the LFER characteristics observed for ΔcFDH (Fig. 2B, where the broken line represents a tentative one based on the characteristics of ΔcFDH). The heme C subunit is essential to bind FDH to the membrane and for *in vivo* fructose oxidizing respiratory activity [3]. Then we can conclude that the electron-donating site of FDH locates on the heme C subunit. In addition, some ubiquinone in the membrane is the most plausible natural electron acceptor of FDH. These considerations and the present results suggest that some specific attractive interaction occurs between the methoxy groups on the 2 and/or 3 positions of *p*BQ derivatives and the heme C moiety of FDH.

We determined the formal potential ($E^{\circ\prime}$) of the three heme C moieties in FDH at pH 5.0 using a spectroelectrochemical method based on mediated bulk electrolysis [9]. Fig. 3A shows the spectral change on bulk electrolysis of FDH at stepwise constant-potentials from −200 mV to 150 mV. Clear isosbestic points are observed in the wavelength region from 500 to 600 nm, where no absorption band appears for the mediators used. The spectral change is assigned to the redox reaction of the heme C moieties in FDH. In this work, the following sequential three-step one-ET model was used for the spectral analysis;

$$Ox + e^- \rightleftarrows Red_1 \left(E_1^{\circ\prime}\right) \text{ (the first step at heme } C_1) \quad (1a)$$

$$Red_1 + e^- \rightleftarrows Red_2 \left(E_2^{\circ\prime}\right) \text{(the second step at heme } C_2) \quad (1b)$$

$$Red_2 + e^- \rightleftarrows Red_3 \left(E_3^{\circ\prime}\right) \text{(the third step at heme } C_3) \quad (1c)$$

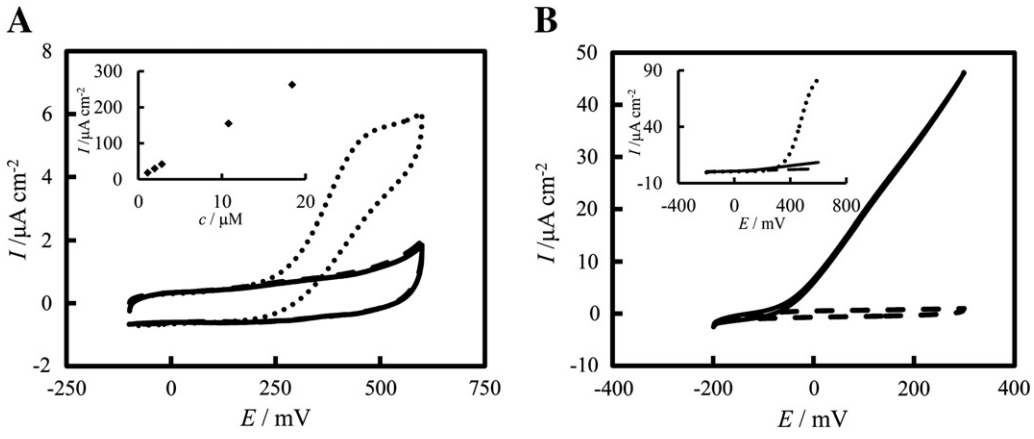

Fig. 1. (A): Cyclic voltammograms (CVs) of fructose oxidation (0.2 M) catalyzed by (A) ΔcFDH (pH 6.0) and (B) FDH (pH 5.0) at $v = 10$ mV s^{-1} and at an Au electrode. Dash line: fructose only, solid line: enzyme + fructose, dotted line: enzyme + *p*BQ + fructose. The inset (A) shows the linear dependence of the limiting MET current on the *p*BQ concentration, from the slope the rate constant can be evaluated. The inset (B) shows CVs of FDH-catalysis in the presence of 1% TritonX-100. The data indicate the inhibition of the DET ability and the appearance of MET activity of FDH by addition of TritonX-100.