Electrochimica Acta 210 (2016) 689–694

Contents lists available at ScienceDirect

Electrochimica Acta

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

Bioelectrochemical characterization of the reconstruction of heterotrimeric fructose dehydrogenase from its subunits

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ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 28 April 2016
Accepted 28 May 2016
Available online 31 May 2016

Keywords:
fructose dehydrogenase
bioelectrocatalysis
direct electron transfer
heme C subunit
electron transfer site

ABSTRACT

D-Fructose dehydrogenase (FDH) is a heterotrimeric-membrane-bound enzyme capable of direct electron transfer (DET)-type bioelectrocatalysis. Subunit II contains three heme C moieties and is presumed to play a key role in the DET reaction because the subunit I/III subcomplex (without subunit II) lacks DET-type activity. We constructed an expression system for subunit II. A non-turnover signal from subunit II was not observed on voltammograms, and a subunit II-adsorbed electrode did not exhibit DET-type activity in the presence of the subunit I/III subcomplex and fructose. Gel filtration column chromatography indicated that subunit II formed multimeric complexes with four or more subunits. The aggregation of subunit II seemed to interfere with its direct communication with the electrodes. In contrast, when subunit II was mixed with the subunit I/III complex in a solution, they formed a 1:1 full complex, and the complex recovered DET-type bioelectrocatalytic activity. These results strongly suggest that the heme C in subunit II is the electron transfer site for the DET-type bioelectrocatalytic activity of FDH.

1. Introduction

D-Fructose dehydrogenase (FDH; EC 1.1.99.11) was isolated from *Gluconobacter japonicas* (former name: *Gluconobacter frateurii*) NBRC3260 as a heterotrimeric-membrane-bound enzyme [1]. FDH catalyzes the oxidation of D-fructose to produce 5-keto-D-fructose. Subunits I (67 kDa) and II (51 kDa) comprise flavin adenine dinucleotide (FAD) and three heme C moieties, respectively, as redox prosthetic groups [1,2]. Subunit III (20 kDa) has no cofactor and is presumed to be related to the stability of FDH. FDH works as the primary dehydrogenase in the respiratory chain; FDH oxidizes fructose and transfers the electrons to ubiquinone embedded in the membrane. FDH displays strict substrate specificity to fructose as an electron donor but can transfer the electron to several artificial electron acceptors [2]. FDH-catalyzed reactions are coupled with electrode reactions via these artificial electron acceptors. This reaction is called mediated electron transfer (MET)-type bioelectrocatalysis. Some studies have reported the determination of fructose in food and diagnostic analyses based on the MET-type reaction of FDH [3,4].

There is another method for coupling enzymatic reactions with electrode reactions wherein redox enzymes directly react with the electrodes. This is called direct electron transfer (DET)-type bioelectrocatalysis. Only a limited number of redox enzymes clearly exhibit DET-type bioelectrocatalytic activity [5–9]; FDH is one such enzyme [10]. The DET-type catalytic activity of FDH is quite high in comparison with that of alcohol dehydrogenase, which is structurally similar to FDH [11,12]. The DET-type bioelectrocatalysis of FDH on the anode of a biofuel cell has achieved high power density (2.6 mW cm^{-2}) [13]. The critical factors that govern DET-type bioelectrocatalysis remain unknown. Previously, we suggested that the heme C subunit (subunit II) in FDH functions as an electron transfer site in direct electrochemical communication with the electrodes. This hypothesis was based on the observation that a subunit I/III subcomplex (without subunit II) lacked DET-type activity [14].

The direct electrochemistry of redox proteins has long been studied to elucidate the electron transfer mechanisms for application in bioelectrochemical devices, including biosensors and biofuel cells [15–19]. The size of proteins seems to correlate with non-catalytic DET-signals. Horse heart cytochrome *c*, a small protein with a molecular mass of *ca.* 12 kDa [20] and a spherical shape ($2.6 \times 3.2 \times 3.0\,nm^3$) [21], exhibits a clear non-turnover signal on voltammograms. Small-sized proteins increase the surface concentration of redox-active sites on the electrodes and

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http://dx.doi.org/10.1016/j.electacta.2016.05.193
0013-4686/

decrease the length between electron transfer sites and the electrode surface. Although the crystal structure of FDH has not been solved, the size of FDH was reported to be *ca*. 7 nm from atomic force micrographs [22]. The size of the protein seems to be too large to yield clear non-catalytic DET signals on a voltammogram; however, very small, non-catalytic faradaic current density could potentially be amplified by the catalytic turnover of FDH. This would result in a large faradaic current density, which is associated with DET-type bioelectrocatalysis.

This work aimed to elucidate the key factors that govern direct communication between the heme C moiety and the electrodes by isolating subunit II of FDH. We constructed an expression system for subunit II of FDH and investigated the electrochemical properties of subunit II. We also attempted to reconstruct FDH from a subunit I/III subcomplex and subunit II on an electrode surface and in solution.

2. Experimental

2.1. Bacterial strains, plasmids, and growth conditions

Gluconobacter spp. were grown on a ΔP medium (potato dextrose medium without potato) consisting of 5 g of glucose, 20 g of glycerol, 10 g of hipolypeptone, and 10 g of yeast extract per liter at 30 °C. pSHO8 [2] was used as an expression vector; it comprises the broad-host-range vector pBBR1MCS-4, into which a fragment of a putative promoter region of the *adhAB* gene of *G. oxydans* 621 H was inserted. *Escherichia coli* DH5α was used for plasmid construction [23]. *E. coli* HB101 harboring pRK2013 [24] was used as a helper strain for conjugative plasmid transfer. The *E. coli* cells were grown in Luria broth at 37 °C.

2.2. Chemicals

Restriction endonucleases were purchased from Takara Shuzo (Japan). Yeast extract was purchased from Oriental Yeast (Japan). 2,3-Dimethoxy-5-farnesyl-1,4-benzoquinone (Q_1) was obtained from Sigma-Aldrich Co. (USA). 2-Mercaptoethanol was purchased from Nacalai Tesque (Japan). All other chemicals were from Wako Pure Chemical Industries (Japan).

2.3. Expression of subunit I/III complex (FdhSL) and subunit II (FdhC)

The expression of the subunit I/III complex (FdhSL, where S and L indicate small and large subunits, respectively) was carried out as described in a previous paper [2]. Subunit II (*FdhC*, where C indicates a heme C–containing subunit) comprised a gene that was amplified by polymerase chain reaction (PCR) with Herculase II Fusion DNA Polymerase (STRATAGENE) using two phosphorylated primers (5′-gaattcATGCCGGCATTTTAAG-3′ and 5′-ggatCCTTGAATCTTATTAAGC-3′; the residues printed in capital letters are complementary to the template sequence) from the genome DNA of *G. japonicus* NBRC3260. The PCR product was inserted into pSHO8 [2] treated with *Eco*RI and *Bam*HI to yield pSHO21. The nucleotide sequence was confirmed by Fasmac sequencing service (Kanagawa, Japan). *G. oxydans* NBRC12528 ΔadhA:Km^r was transformed with pSHO21 as described in a previous paper [2].

2.4. Purification of the subunit I/III complex and subunit II

Gluconobacter harboring pSHO16 and pSHO21 were used for purification of the subunit I/III complex and subunit II, respectively. The *Gluconobacter* cells were collected, suspended, and then disrupted with a French pressure cell (Otake Works, Japan) as described in a previous paper [2]. Ultracentrifugation at 100,000*g* was used to separate the resulting lysate into a supernatant fraction and a membrane fraction. The supernatant and membrane fractions were used for isolation of the subunit I/III complex and subunit II, respectively, because the subunit I/III complex is soluble and subunit II has a membrane-anchoring region [2].

The supernatant was injected into a DEAE-Sepharose Fast Flow column equilibrated with 20-fold–diluted MacIlvaine buffer (pH 6.0). The subunit I/III complex was eluted under a linear gradient ranging from the diluted MacIlvaine buffer to the original buffer; the fractions containing the subunit I/III complex were collected. Ammonium sulfate (50% saturation) was added to the fractions, which were stirred at 4 °C for 1 h then centrifuged at 10,000*g* and 4 °C for 30 min. The supernatant was collected and injected into a Buthyl Toyopearl 650 M column equilibrated with a 50 mM sodium phosphate buffer (NaPB; pH 6.0; M = mol dm^{-3}). The elution of the subunit I/III complex was carried out under a linear gradient from 50% saturated $(NH_4)_2SO_4$ to 0% $(NH_4)_2SO_4$. The sample was concentrated and dialyzed overnight against 50 mM NaPB (pH 6.0) containing 150 mM NaCl. After dialysis, the sample was further purified by size exclusion chromatography on a Superdex-200 column equilibrated with 50 mM NaPB containing 150 mM NaCl.

After ultracentrifugation of the lysate, the membrane fraction was suspended in 20-fold–diluted MacIlvaine buffer (pH 6.0) containing 1% TritonX-100, 1 mM 2-mercaptoethanol, and 1 M KCl; it was stirred at 4 °C for 1 h. The solubilized supernatant was collected by ultracentrifugation at 100,000*g* and precipitated with 30% $(NH_4)_2SO_4$. The precipitate was dissolved in 20-fold–diluted MacIlvaine buffer (pH 5.0) containing 0.1% TritomX-100 and 1 mM 2-mercaptoethanol; it was dialyzed overnight against the same buffer. All of the solutions used for the purification of subunit II contained 0.1% TritonX-100 and 1 mM 2-mercaptoethanol. After dialysis, the sample was injected into a Toyopearl CM-650 column equilibrated with 20-fold–diluted MacIlvaine buffer (pH 5.0). Subunit II was eluted under a linear gradient from the diluted MacIlvaine buffer to the original buffer. The fractions were collected, concentrated, and dialyzed against 50 mM NaPB (pH 6.0).

The purities of the subunit I/III complex and subunit II were measured on an SDS-PAGE using Coomassie brilliant blue R-250 staining. The concentrations of the subunit I/III complex and subunit II were spectrophotometrically determined using the extinction coefficient of free FAD (11,300 M^{-1} cm^{-1} [25]) and heme C in FDH (23,000 M^{-1} cm^{-1} [26]).

2.5. Electrochemical measurements

Cyclic voltammetry and linear sweep voltammetry were carried out in McIlvaine buffers (pH 5.0 or 6.0) at 25 °C on a BAS CV–50 W electrochemical analyzer under anaerobic conditions. The working electrodes were made of glassy carbon (GC) or Au. The reference and counter electrodes were a handmade Ag|AgCl|sat.KCl electrode and a Pt-wire, respectively. All potentials in this paper are given with respect to the reference electrode.

3. Results and discussion

3.1. Construction and expression of the subunit I/III complex and subunit II

We have previously reported the expression and partial purification of the subunit I/III complex [2]. Its purification was carried out using a modified version of the method in Ref. [14]; this successfully isolated the complex at a higher purity than previous work. The specific activity was improved from 40 ± 3 U mg^{-1} to 75 ± 10 U mg^{-1} at an optimum pH value of 6.0 [14]. The purified subunit I/III complex showed a clear FAD signal on UV–vis spectroscopic measurements and two major bands on SDS-PAGE