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Construction and characterization of flavin adenine dinucleotide glucose dehydrogenase complex harboring a truncated electron transfer subunit

Junko Okuda-Shimazaki [a, **], Noya Loew [b, c, ***], Nana Hirose [c], Katsuhiro Kojima [a], Kazushige Mori [a], Wakako Tsugawa [b], Koji Sode [a, b, c, *]

[a] Ultizyme International Ltd., Japan, 1-13-16, Minami, Meguro, Tokyo, 1520013, Japan
[b] Department of Biotechnology and Life Science, Graduate School of Engineering, Tokyo University of Agriculture and Technology, Japan, 2-24-16, Naka-cho, Koganei, Tokyo, 1848588, Japan
[c] Joint Department of Biomedical Engineering, The University of North Carolina at Chapel Hill, North Carolina State University, Chapel Hill, NC, 27599, USA

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ABSTRACT

One of the most prominent glucose dehydrogenases (GDHs) capable of direct electron transfer with electrodes is the FADGDH complex derived from *Burkholderia cepacia*. This FADGDH complex comprises the following three distinct subunits: the catalytic subunit (α subunit) that has an FAD cofactor in its redox center, a hitch-hiker protein from the bacterial TAT secretion system (γ subunit), and the electron transfer subunit (β subunit). The electron transfer subunit (β subunit) of the FADGDH complex is a three-heme c containing cytochrome *c* like molecule (heme 1, heme 2 and heme 3 from the N-terminal). In this study, an FADGDH complex harboring a truncated electron transfer subunit composed of only heme 3 was constructed, and its enzymatic activity and electrochemical properties were investigated to elucidate the role of heme 3 and its region. A truncated electron transfer subunit, trβ subunit, was designed using the 3D structures of homologous cytochrome *c* proteins. The designed trβ subunit was expressed as soluble and functional heme c molecules forming complexes with γα catalytic complexes. Thus, the formed FADGDH complex has inter-molecular electron transfers from the FAD to the trβ subunit, and from the trβ subunit to the external electron acceptor, showing electron transfer subunit-mediated characteristic dye-mediated dehydrogenase activity with a Ru-complex. Therefore, heme 3 in the electron transfer subunit is responsible for accepting the electron from the γα catalytic complex. Moreover, the FADGDH complex harboring the trβ subunit showed DET activity toward the electrode. Spectroelectrochemical observations revealed that the trβ subunit possessed a lower formal potential than any of the 3 hemes in the whole electron transfer subunit. These unexpected electrochemical properties of the heme in the trβ subunit may potentially result in the construction of a DET principle-based glucose sensor, which can be operated at a much lower potential than those achieved using the FADGDH complex with a whole electron transfer subunit.

1. Introduction

Glucose monitoring is essential for proper metabolic control of diabetes mellitus. Self-monitoring of blood glucose (BG) is essential and the most common method for patients to determine proper treatments and learn how activities affect their blood glucose levels. To achieve tight glycemic control, a continuous glucose monitoring (CGM) system is widely employed as the current state-of-the-art technology. Recently, the U.S. Food and Drug Administration approved an artificial pancreas system consisting of a CGM system, a continuous subcutaneous insulin infusion pump, and control algorithms, increasing the demand for CGM sensors with more accuracy and precision.

Among the variety of enzymes/proteins utilized for glucose

* Corresponding author. Joint Department of Biomedical Engineering, The University of North Carolina at Chapel Hill, North Carolina State University, Chapel Hill, NC 27599, USA.
** Corresponding author.
*** Corresponding author. Joint Department of Biomedical Engineering, The University of North Carolina at Chapel Hill, North Carolina State University, Chapel Hill, NC 27599, USA.
E-mail address: ksode@email.unc.edu (K. Sode).

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monitoring, glucose oxidase (GOx) and glucose dehydrogenase (GDH) are the most widely studied and commercially utilized [1]. Three principles have been reported for electrochemical sensors to measure glucose using either GOx and/or GDH. The first-generation sensors are blood glucose monitors employing oxygen as the electron acceptor and determining the glucose concentration by following either the consumption of oxygen or the liberation of hydrogen peroxide. The second-generation sensors are based on the use of artificial electron acceptors (or electron mediators) instead of oxygen to avoid interferences from other redox species. The third-generation sensors employ the direct electron transfer (DET) principle. In this principle, no electron mediator or oxygen is used, eliminating toxic artificial electron mediators and avoiding errors due to variations in the concentration of oxygen in blood samples. However, enzymes capable of DET are limited. GOx and fungi-derived flavin adenine dinucleotide (FAD) FADGDHs, which are the most common enzymes in BG systems, are difficult to confirm their DET ability, since their redox cofactor, FAD, is buried deeply in the protein molecules. Considering the elegant method reported by Nöll and co-worker [2], which showed only free FAD of GOx showed voltammetry, whereas bound FAD did not, and also recent article claimed the suspicious of DET ability of GOx [3], achievement of DET using GOx or fungal FADGDH, the use of advanced nanotechnology-based modification of electrodes would be mandatory.

One of the most prominent GDHs capable of DET is the FADGDH complex derived from *Burkholderia cepacia* [4–8]. This bacterial FADGDH complex comprises the following three distinct subunits: the catalytic subunit (α subunit), which has an FAD cofactor in its redox center; the small subunit (γ subunit), which is necessary for the proper folding and secretion of the α subunit into the periplasmic space [6]; and the electron transfer subunit (β subunit). The γα catalytic complex also shows dye-mediated dehydrogenase activity but scarcely shows DET activity because of the absence of the β subunit. The FADGDH complex belongs to a family of bacterial FAD dependent dehydrogenase complexes, including fructose dehydrogenase (FDH), 2-keto-D-gluconate dehydrogenase (KDGH) and sorbitol dehydrogenase (SDH) complexes [4,5,7–13]. Recently, we have reported that the catalytic subunit harbors a 3Fe-4S cluster [14]. The presence of 3Fe-4S cluster serves as a new electron transfer pathway between the catalytic subunit and heme c containing electron transfer subunit of the FAD dependent dehydrogenase complex. The reductive half reaction at the catalytic site reduces the FAD, transfers the electron to the 3Fe–4S cluster in the catalytic subunit, consequently, the electron is transferred from the 3Fe–4S in the catalytic subunit to β. These features seem to be preserved in other three-subunit bacterial dehydrogenases that have an FAD cofactor.

The electron transfer subunit (β subunit) of the FADGDH complex is a three-heme c containing cytochrome *c* like molecule (heme 1, heme 2 and heme 3 from the N-terminal) responsible for the transfer of electrons between the active-site cofactor and external electron acceptors, which is proposed based on its primary structure. The Cys-Xaa-Xaa-Cys-His motif, which is a typical heme *c* binding motif, well conserved in the primary structures of the electron transfer subunit in the bacterial FAD dependent dehydrogenase complexes [15]. Bacterial heme *c* is covalently bound to the polypeptide by thioether bonds of the two Cys residues in the binding motif. The heme iron is six-coordinate, and in which four ligands are provided by the nitrogen atoms of the porphyrin ring and two residues from the polypeptide provide the axial coordinates. The fifth ligand is the His of the above mentioned heme binding motif, and the sixth residue is provided from the polypeptide chain. In the heme c harboring electron transfer subunit, they are all Met, which is fully conserved, found in the downstream of each of the three heme binding motifs. Therefore, the axial ligands of these hemes are all His-Met.

The elucidation of the mechanism and the role of each heme in the intra- and inter-molecular electron transfers is necessary to achieve further improvements in of the DET ability of the FADGDH complex. The detail analyses of the electron transfer pathway for fructose dehydrogenase (FDH), which is also a DET-type hetero-oligomeric FAD dependent dehydrogenase harboring heme c containing electron transfer subunit, has been reported recently [16–18]. Their mutagenesis studies and the preparation of an engineered heme c containing subunit by deleting heme 1 region, showed the strong suggestion that heme 2 is responsible for DET. They also suggested the possible contribution of heme 3 as to accept electron from FDH catalytic subunit. Recently, we reported the mutagenesis studies of the 6th heme axial ligands, Met109, Met263 and Met386, to elucidate the electron transfer pathway of FADGDH complex to the electron mediators and/or the electrode [15]. These mutagenesis studies of each 6th axial ligand revealed that heme 1 with Met109, is responsible for electron transfer with external electron acceptor, heme 2 with Met263 is responsible for DET, and heme 3 with Met386 seemed to be the electron acceptor from the catalytic subunit, since the mutation negatively affected not only the dye-mediated dehydrogenase activity but also the DET ability. From the mutagenesis studies, two electron transfer pathways were proposed depending on the electron acceptors. Electrons are transferred from the catalytic subunit to heme 3, then to heme 2, to heme 1 and finally to electron acceptors in solution. However, if the enzyme complex is immobilized on the electrode and is used as electron acceptors, electrons are passed to the electrode from heme 2 (Scheme 1). However, the direct approval to identify the role of heme 3 is remained to be done.

These results prompted us to construct an engineered electron transfer subunit composed of only heme 3 region, by deleting heme 1 and heme 2 region, in order to elucidate the role of heme 3 and its region.

In this study, an FADGDH complex harboring a truncated electron transfer subunit composed of only heme 3, i.e., the truncated β (trβ) subunit, was designed. After preparing the FADGDH complex with the trβ subunit, spectroscopic observations were carried out to determine the dye-mediated dehydrogenase activity and electrochemical analyses were carried out to investigate the DET ability. The trβ subunit formed a complex with the γα catalytic complex and exhibited dye mediated dehydrogenase activity, i.e., intermolecular electron transfer occurred from FAD to the trβ subunit to an external electron acceptor. Surprisingly, the FADGDH complex harboring the trβ subunit showed DET activity toward the electrode. Spectroelectrochemical observations revealed that the trβ subunit possessed a lower formal potential than any of the 3 hemes present in the whole electron transfer subunit.

2. Experimental

2.1. Materials

Hexaammineruthenium(III) chloride ($Ru(NH_3)_6Cl_3$), ammonium hexachloroosmate(IV) ($[(NH_4)_2Os]Cl_6$), and sodium dithionite were purchased from Sigma-Aldrich. Ethylenediamine tetraacetic acid iron(III) sodium salt (Na[Fe(III)-EDTA]) and Antifoam PE-L were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), phenazine methosulfate (PMS), 2,6-dichlorophenolindophenol (DCIP), and D(+)-glucose were purchased from Kanto Chemical co. Inc. (Tokyo, Japan). Dithiobis(succinimidyle hexanoate) (DSH) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Dojindo Molecular Technologies, Inc.