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An Fe–S cluster in the conserved Cys-rich region in the catalytic subunit of FAD-dependent dehydrogenase complexes

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ABSTRACT

Several bacterial flavin adenine dinucleotide (FAD)-harboring dehydrogenase complexes comprise three distinct subunits: a catalytic subunit with FAD, a cytochrome *c* subunit containing three hemes, and a small subunit. Owing to the cytochrome *c* subunit, these dehydrogenase complexes have the potential to transfer electrons directly to an electrode. Despite various electrochemical applications and engineering studies of FAD-dependent dehydrogenase complexes, the intra/inter-molecular electron transfer pathway has not yet been revealed. In this study, we focused on the conserved Cys-rich region in the catalytic subunits using the catalytic subunit of FAD dependent glucose dehydrogenase complex (FADGDH) as a model, and site-directed mutagenesis and electron paramagnetic resonance (EPR) were performed. By co-expressing a hitch-hiker protein (γ-subunit) and a catalytic subunit (α-subunit), FADGDH γα complexes were prepared, and the properties of the catalytic subunit of both wild type and mutant FADGDHs were investigated. Substitution of the conserved Cys residues with Ser resulted in the loss of dye-mediated glucose dehydrogenase activity. ICP-AEM and EPR analyses of the wild-type FADGDH catalytic subunit revealed the presence of a 3Fe–4S-type iron–sulfur cluster, whereas none of the Ser-substituted mutants showed the EPR spectrum characteristic for this cluster.
The results suggested that three Cys residues in the Cys-rich region constitute an iron–sulfur cluster that may play an important role in the electron transfer from FAD (intra-molecular) to the multi-heme cytochrome *c* subunit (inter-molecular) electron transfer pathway. These features appear to be conserved in the other three-subunit dehydrogenases having an FAD cofactor.

1. Introduction

Several bacterial flavin adenine dinucleotide (FAD)-harboring dehydrogenase complexes have been reported, including FADGDH, fructose dehydrogenase (FDH), 2-keto-D-gluconate dehydrogenase (KDGH) and sorbitol dehydrogenase (SDH) complexes have been reported (Table 1) [1–9]. These FAD-dependent dehydrogenase complexes are composed of three distinct subunits: a catalytic subunit with FAD, a cytochrome *c* subunit containing three hemes, and a small subunit. These dehydrogenase complexes have the potential to transfer electrons directly to an electrode because of the presence of the cytochrome *c* subunit. FADGDH and FDH have both been reported to facilitate direct electron transfer to an electrode [10–14], enzyme fuel cells [15–20] and also novel principle of biosensor, BioCapacitor [21–24].

Both isolation and characterization of FADGDH from *Burkholderia cepacia* SM4 strain have been previously reported [1,2]. As is the case for other FAD-dependent dehydrogenase complexes, FADGDH is comprised the following three distinct subunits: the catalytic subunit (α subunit) that has an FAD cofactor in its redox center, shows catalytic activity, and oxidizes the first hydroxyl group of glucose; the small subunit (γ subunit), a hitch-hiker protein of the bacterial TAT secretion system, which is necessary for the proper folding and secretion of the α subunit [25]; and the cytochrome *c* subunit (β subunit) that is in responsible for the transfer of electrons between the active-site cofactor and external electron acceptors. The β subunit also confers the ability to transfer electrons directly to an electrode, making FADGDH ideal for the latest generation of glucose sensors, which rely on direct-electron transfer to avoid the use of artificial electron acceptors or mediators, and as well as prevent interference from oxygen. By co-expressing γ-subunit and α-subunit, FADGDH without β subunit, that is FADGDH γα complex, and the complex showed dye mediated glucose dehydrogenase activity and also characteristic FAD spectrum [3]. We also reported on the mediated type glucose sensor employing recombinantly prepared FADGDH γα complex and applied

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Table 1
FAD-dependent dehydrogenase complexes.

Dehydrogenase complex	Species	Catalytic subunit	Cyt *c* subunit	Small subunit	Ref.
		Mw(g/mol)			
Glucose dehydrogenase (FADGDH)	*Burkholderia cepacia*	59,832.04	45,577.83	18,033.55	[1–4]
Fructose dehydrogenase (FDH)	*Gluconobacter japonicus*	59,731.49	52,194.85	20,085.79	[5,6]
2-keto-D-gluconate dehydrogenase (KGDH)	*Gluconobacter oxydans*	60,095.74	51,322.03	23,381.97	[7,8]
Sorbitol dehydrogenase (SDH)	*Gluconobacter frateurii*	59,954.44	51,057.86	22,245.36	[9]

to the screen printed carbon electrode based disposable glucose sensor [26]. In that study, the electron transfer between enzyme and electrode was assumed to be occurred between reduced FAD and electrode mediated by the artificial electron acceptor. Similarly, Kawai et al. [6] also reported on the electrochemical studies of FDH complex without electron transfer subunit, and the electron transfer was also observed in the presence of artificial electron acceptors, suggesting that the electron transfer from reduced FAD to electrode via artificial electron acceptors. Protein engineering studies of the active site of the α subunit, by preparing and characterizing mutant FADGDH γα complexes, have also demonstrated that the enzyme is essentially specific for glucose [27], eliminating risks of broad substrate specificity in sensor application.

Despite various electrochemical applications and engineering studies of FAD-dependent dehydrogenase complexes, the intra/intermolecular electron transfer pathway has not yet been revealed. Based on amino acid sequence alignment of catalytic subunits of bacterial dehydrogenase complexes, we focused on sequential Cys residues (Cys-rich region). Using site-directed mutagenesis and electron paramagnetic resonance (EPR), we identified a 3Fe–4S-type iron–sulfur cluster in the FADGDH γα complex. The results of our study suggest that three Cys residues in the Cys-rich region constitute an iron–sulfur cluster that may have an important function in the electron transfer from FAD (intra-molecular) to the multi-heme cytochrome *c* subunit (inter-molecular) electron transfer pathway. These features seem to be conserved in other three-subunit dehydrogenases that have an FAD cofactor.

2. Experimental

2.1. Materials

Phenazine methosulfate (PMS), D-glucose, and 2,6-dichlorophenolindophenol (DCIP) were purchased from Kanto Chemical Co., Ltd. (Japan). All other chemicals were of reagent grade.

2.2. DNA manipulations

In order to prepare recombinant FADGDH γα complex, pTrc-γα(C-his) vector was constructed, which encodes γ and α subunits. By using pTrc-γαβ, which encodes structural genes of 3 subunits of FADGDH [4] as a template, a poly histidine tag (his-tag) in the C-terminus of α subunit was introduced by PCR amplification. The DNA region containing γα(C-his)β was obtained by PCR amplification using the primers 5′-ACCACC ACTGATAAGGAGGTCTGACCGTGCGGAAATCTAC-3′ and 5′-AGCCTGTGCG ACTTCTTCCTTCAGCGATCGGTGGTGGTGG-3′. The region containing γα(C-his) was obtained by PCR amplification using the γα(C-his)β vector, using the primers 5′-GGGCCCGGGCCCCGAGGAAGATCTGTA-3′ and 5′-GGCGGAAGCTTCAGTGGTGGTGGTGGTGGTGGCTA-3′. The PCR product was inserted into the *Eco*R1 and *Hin*dIII sites of the pTrc-γα vector to create pTrc-γα(C-his) vector and the nucleotide sequence was confirmed. All site-directed mutations were performed by PCR amplification using primers (Supplemental Table 1) and their sequences were confirmed.

2.3. Recombinant FADGDH γα complex expression and purification

Various pTrc-γα(C-his) constructs (wild type and mutants) were introduced into *Escherichia coli* BL21 (DE3). Transformed cells were aerobically cultivated at 25 °C for 24 h in ZYP-5052 medium (0.5% glycerol, 0.05% glucose, 0.2% lactose, 50 mM $(NH_4)PO_4$, 50 mM Na_2HPO_4, and 1 mM $MgSO_4$ [28]) containing 100 μg/ml of ampicillin. To prepare crude extracts for enzyme assays and purification, harvested cells were washed twice with 0.85% NaCl and were disrupted by French Press in buffer A (20 mM imidazole, 500 mM NaCl, and 20 mM sodium phosphate, pH 7.0). The homogenate was centrifuged at 10,000 ×*g* for 20 min to remove cell debris and inclusion bodies, while the supernatant was ultracentrifuged at 104,000 ×*g* for 60 min. The ultracentrifuge supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 7.0).

Recombinant FADGDH γα complex was purified by nickel affinity chromatography at room temperature. The supernatant after ultracentrifugation was applied to a 1-mL HisTrap HP column that was equilibrated with 20 column volumes of buffer A. After washing with 20 column volumes of 9.5% buffer B (500 mM imidazole, 500 mM NaCl, and 20 mM sodium phosphate, pH 7.0), purified protein was eluted with 20 column volumes of 20% buffer B and dialyzed against 10 mM potassium phosphate buffer (pH 7.0). Protein concentrations were determined using the DC Protein Assay Kit (BioRad, California, USA).

2.4. Enzyme assay

Activity of the purified enzyme or crude extracts of recombinant FADGDH γα complex was assayed according to previous studies [1,2,3] with slight modifications. The enzyme sample was incubated in 10 mM potassium phosphate buffer (pH 7.0) containing 6 mM PMS, 0.06 mM DCIP, and various concentrations of glucose at room temperature. The activity was determined by monitoring the decrease in the absorbance from DCIP at 600 nm and using the molar absorption coefficient of DCIP (16.3 mM/cm at pH 7.0) to calculate enzyme activity. One unit of enzyme activity is defined as the amount that oxidizes 1 μmol of glucose per min.

2.5. Electron paramagnetic resonance

EPR spectra were recorded using a JES-FA200 spectrometer. For the instrument, the following conditions were employed: microwave power of 1 mW, micro-frequency of about 9 GHz, and modulation frequency of 100 kHz. The sample cavity was cooled to 6 K. For the preparation of oxidized samples, purified FADGDH γα complex (final concentration of 0.4 mM) was incubated with PMS (final concentration of 2 mM) at room temperature for 1 h and was dialyzed against 10 mM potassium phosphate buffer (pH 7.0). The oxidized samples were incubated with 13.3 mM glucose at room temperature for 1 h followed by incubation at 4 °C for 24 h.

3. Results

3.1. Amino acid sequence alignment of catalytic subunits in FAD dependent dehydrogenase complexes

FADGDH is a member of the glucose/methanol/choline (GMC) oxidoreductase family. This large and diverse family shares a homologous structural backbone with a conserved FAD-binding motif, which includes an ADP-binding βαβ-fold close to the amino terminus, and