Investigation of the Key Heme in Cytochrome c_3 to the Electron Pool Effect by Highly Sensitive EQCM Technique

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ABSTRACT

Cytochrome c_3 , which has four bis-histidinyl coordinated hemes per molecule, is a redox protein, and stores electrons temporarily until recognizing a redox partner. This mechanism is called “electron pool effect”. In this study, highly sensitive EQCM measurement clarified which heme in cytochrome c_3 caused the electron pool effect. To investigate the key heme to the electron pool effect, cytochrome c_3 mutants in which the sixth axial ligand of one heme was changed were prepared, and the intermolecular electron transfer was measured by viologen-immobilized electrode. The kinetics of the electron transfer complex formation between cytochrome c_3 and immobilized viologen indicated that redox of the heme II in cytochrome c_3 caused the electron pool effect.

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1. Introduction

Intermolecular electron transfer between redox proteins is a key step in biological processes such as photosynthesis and electron transport systems in mitochondria. It has been suggested that redox proteins store electrons temporarily until donating electrons and select specific redox partners. The electron transfer chain is maintained by this mechanism, which is called “electron pool effect”.^{1–3}

In previous studies, the electron transfer rates between a redox protein and its partner were investigated.^{4–6} These experiments showed that the complex formation and dissociation processes were the rate-determining steps. The processes, however, have not been monitored directly and have not been revealed in experiment. To monitor the processes directly, we have improved electrochemical quartz crystal microbalance (EQCM) technique to give high sensitivity in solution.^{7–9} This technique makes it possible to monitor the access of molecules to the electrode or repulsion from the electrode. The complex formation and dissociation processes were investigated by highly sensitive EQCM. In this study, the electron pool effect of cytochrome c_3 was investigated by highly sensitive EQCM.

Cytochrome c_3 from *Desulfovibrio vulgaris* (Miyazaki) is a small water-soluble protein (14.1 kDa) containing four c -type hemes.^{10–14} The four hemes have two histidine as an axial ligand, so that redox potential of the hemes is low compared with Met-Fe-His heme, such as cytochrome c .¹⁵ Thus, cytochrome c_3 has multiple redox states caused by four hemes redox, and the electron pool effect works during the four hemes redox.

To reveal the electron pool effect in experiments, viologen-immobilized electrode was used, and viologen-cytochrome c_3 electrode was prepared. Redox potential of viologen is -420 mV (vs. SHE) close to the value of the hemes in cytochrome c_3 .¹⁶ Therefore, the electron transfer between cytochrome c_3 and immobilized viologen occurs. In viologen-cytochrome c_3 electrode, cytochrome c_3 is bound electrostatically onto immobilized viologen. Thus an electrostatic complex is formed. The process of electrostatic complex formation was monitored by QCM. Next, cyclic voltam-

metry was carried out on the viologen-cytochrome c_3 electrode, and the intermolecular electron transfer between cytochrome c_3 and viologen was investigated. Viologen is not a redox partner of cytochrome c_3 in vivo, so that the electron pool effect of cytochrome c_3 works when electron transfer from cytochrome c_3 to the viologen occurs. In other words, cytochrome c_3 hardly donates electrons to viologen. Reduced cytochrome c_3 seems to store electrons and the electron transfer complex is formed slowly. In this study, the process of interconversion between electrostatic and electron transfer complex was measured by highly sensitive EQCM.¹⁷

To clarify the key heme in cytochrome c_3 to the electron pool effect, the four types of cytochrome c_3 mutants, H23M, H26M, H36M and H71M, were prepared.^{18–20} Histidine is the sixth axial ligand to iron in the heme and each histidine was replaced by a methionine by site-directed mutagenesis. Changing ligand into methionine makes redox potential of the heme higher than that of a bis-histidinyl coordinated heme. Thus, the methionine ligand heme is out of the electron transfer pathway in cytochrome c_3 . Electron pool effect probably does not work where the key heme is changed.

2. Experimental

Cytochrome c_3 was purified from *Desulfovibrio vulgaris* (Miyazaki) according to the literature.^{21,22} Cytochrome c_3 mutants were obtained by site-directed mutagenesis.^{18,19,22} Four plasmids (H23M, H26M, H36M and H71M) were constructed by QuickChange Mutagenesis kit (Stratagene) using pKF3FPB as a template. The constructed plasmids were transfected into *Shewanella oneidensis* TSP-C cells to express cytochrome c_3 . The expressed cytochrome c_3 mutants were purified by the same method as wild type cytochrome c_3 . In the case of H23M cytochrome c_3 , the potential of heme I becomes 100–150 mV higher. In the H23M cytochrome c_3 , histidine of heme I was changed into methionine, so H23M cytochrome c_3 describes heme 1A cyt. c_3 . Likewise H36M cytochrome c_3 , H26M cytochrome c_3 and H71M cytochrome c_3 describe heme 2A cyt. c_3 , heme 3A cyt. c_3 , and heme 4A cyt. c_3 , respectively.

Figure 1. The schematic illustration of viologen-immobilized electrode.

EQCM measurements were performed with an ALS/CHI model 440 Electrochemical Quartz Crystal Microbalances. All electrochemical measurements were carried out in 100 mM Tris-HCl buffer (pH 7.4) with an Au-evaporated quartz crystal electrode as a working electrode, a saturated calomel electrode as a reference electrode and a Pt electrode as a counter electrode. All measurements were performed in a glove box (O_2 concentration was less than 0.1 ppm). All solutions were prepared with ultra pure water (Milli-Q Millipore 18.2 M Ω cm) and were filtered with 0.2 μ m just before use.

Figure 1 shows the schematic illustration of viologen-immobilized electrode. The viologen-immobilized quartz crystal Au electrode was prepared as follows. In the first step, a cleaned Au electrode immersed in 5 mM DL- α -lipoic acid ethanol solution for 3 h. In the second step, 1-(2-aminoethyl)-1'-methyl-4,4'-bipyridinium ($MV^{2+}C_2H_4NH_2Br^-I^-$) bound covalently to the carboxyl group of lipoic acid on the electrode by DCC coupling in DMF for 18 h. *N,N'*-diisopropylcarbodiimide (DIC) and 1-Hydroxy-benzotriazole-anhydrous (HOBt) were used as condensing agents. For counter-anion exchange from Br^- and I^- for Cl^- , cyclic voltammetry was carried out under 100 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl at a slow scan rate. The scan rate was 20 mV s $^{-1}$ from -0.7 to -0.3 V.

Cytochrome c_3 was bound electrostatically onto the viologen. The viologen immobilized electrode was set to a measurement cell and 4.4 μ M of cytochrome c_3 solution was injected. The electrostatic complex formation was monitored by QCM and cyclic voltammetry was carried out.

3. Results and Discussion

Figure 2(a) shows cyclic voltammograms of WT cytochrome c_3 bound onto the viologen-immobilized electrode (solid curve) and the viologen-immobilized electrode (dashed curve). The number of immobilized viologen was 1.1×10^{-10} mol cm $^{-2}$ calculated from the anode peak area of dashed curve. Cyclic voltammetry was carried out at scan rate of 10, 20, 50, 100, 300, 500 mV s $^{-1}$. The linear relationship between peak current and scan rate was observed, so that the immobilized viologen was prepared as a monolayer. Peak position of solid curve is the same as dashed curve though peak current becomes larger, indicating that cytochrome c_3 redox occurred via the viologen redox (V^{2+}/V^{+}). This result shows that intermolecular electron transfer between cytochrome c_3 and viologen occurs on electrode surface. The number of cytochrome c_3 was determined by subtracting the peak area of viologen redox from that of solid curve. The number of WT cytochrome c_3 was 1.0×10^{-12} mol cm $^{-2}$, corresponding to 8% of the number of immobilized viologen. These result shows that cytochrome c_3 is bound electrostatically onto the viologen immobilized electrode as a monolayer.

Figures 2(b), 2(c), 2(d) show cyclic voltammograms of heme 1A cyt. c_3 , heme 2A cyt. c_3 , and heme 3A cyt. c_3 bound onto the viologen-immobilized electrode, respectively. The results of these electrodes shows that the intermolecular electron transfer occurs in the case of heme 1A cyt. c_3 heme 2A cyt. c_3 , heme 3A cyt. c_3 ,

Figure 2. Cyclic voltammograms of (a) WT cyt. c_3 (b) heme 1A cyt. c_3 (c) heme 2A cyt. c_3 (d) heme 3A cyt. c_3 bound onto the viologen-immobilized electrode (solid line) and the viologen-immobilized electrode (dashed line). Scan rate is 50 mV s $^{-1}$.

Figure 3. EQCM responses of (a) WT cyt. c_3 (b) heme 1A cyt. c_3 (c) heme 2A cyt. c_3 (d) heme 3A cyt. c_3 bound onto the viologen-immobilized electrode.

respectively. In the case of cyclic voltammogram of heme 4A cyt. c_3 , peak area becomes smaller, indicating that heme 4A cyt. c_3 is not able to accept and donate electrons via immobilized viologen. Heme 4A cyt. c_3 does not maintain the native structure by replacing the 71 Histidine, so the electron transfer complex was not formed.

Figure 3(a) shows a potential step EQCM of WT cytochrome c_3 bound to the viologen-immobilized electrode. The potential was stepped between -0.64 and -0.36 V, and the stepped potential was kept at for 10 s. At -0.36 V before 0 s, electrostatic complex of cytochrome c_3 and viologen is formed. When the potential was

Table 1. The rate constants of electron transfer complex formation between cytochrome c_3 and viologen.

	Complex formation rate between oxidized cyto- chrome c_3 and $V^{+•}/s^{-1}$	Complex formation rate between reduced cyto- chrome c_3 and V^{2+}/s^{-1}
Wild Type	1.0	0.4
Heme 1A	2.0	0.5
Heme 2A	2.2	2.1
Heme 3A	1.9	0.3

stepped from -0.36 to -0.64 V, a quick frequency increase was observed. From the calculation of this curve according to fitting analysis, the rate constant was 1.0 s^{-1} . In this region from 0 s to 10 s, the electron transfer complex between oxidized cytochrome c_3 and reduced viologen ($V^{+•}$) is formed and electron transfer complex dissociates and returns quickly electrostatic complex after reduction of cytochrome c_3 .

On the other hand, a slow frequency decrease was observed when the potential stepped from -0.64 to -0.36 V at 10 s. The rate constant was 0.4 s^{-1} . During this potential step the electron transfer from reduced cytochrome c_3 to V^{2+} occurred. The slow frequency decrease shows that the electron transfer complex formation is slow. This result indicates that the electron pool effect works, and that cytochrome c_3 stores electrons temporarily.

Figures 3(b), 3(c), and 3(d) show potential steps of heme 1A cyt. c_3 , heme 2A cyt. c_3 and heme 3A cyt. c_3 , respectively. Each rate constant of complex formation was calculated from fitting analyses of each curves, and the calculated data were listed in Table 1. Heme 1A cyt. c_3 , heme 3A cyt. c_3 , and WT cyt. c_3 are similar in rate constant. Therefore, the electron pool effect works in heme 1A cyt. c_3 and heme 3A cyt. c_3 . These results indicate that heme I and heme III have nothing to do with the electron pool effect and do not cause the structural change leading to the electron pool effect.

In the case of heme 2A cyt. c_3 , the rate of complex formation between oxidized cytochrome c_3 and $V^{+•}$ (2.2 s^{-1}) is nearly equal to that between reduced cytochrome c_3 and V^{2+} (2.1 s^{-1}). This result shows that the electron pool effect does not work when heme II is out of the electron transfer in cytochrome c_3 , and that redox of heme II is due to the electron pool effect, which is probably caused by structural change of cytochrome c_3 . This result suggests that heme II redox, causing axial ligand state changes, allows cytochrome c_3 to dynamic conformational change.

4. Conclusion

The intermolecular electron transfer mechanism of cytochrome c_3 was investigated by using highly sensitive EQCM technique. In this study, it is clarified that the electron pool effect of cytochrome c_3 works by the redox of heme II. These results show that heme II is a key heme to the electron pool and that heme II causes dynamic conformational change of cytochrome c_3 in order to select a specific partner during period the conformational change.

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