Improvement in Electron Transfer Efficiency Between Multicopper Oxidase and Electrode by Immobilization of Directly Oriented Enzyme Molecules

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Abstract: We investigated the effects of the distance between redox site of multicopper oxidase from Pyrobaculum aerophilum (McoP) and the electrode surface on efficient electron transfer to optimize biodevice performance. The O2 reduction peak current densities of the electrode on which McoP was directly immobilized using C-terminus His-tagged and Cys residue introduced McoP, and the electrode on which McoP was immobilized using C-terminus His-tagged McoP and self-assembled monolayer (SAM) were 203.6 µA/cm² and 167.4 µA/cm², respectively. The O2 reduction current per unit enzyme activity of McoP was 88.9 mA/units in the electrode on which McoP was directly immobilized using C-terminus His-tagged and Cys residue introduced McoP, and 68.4 mA/units in the electrode on which McoP was immobilized using C-terminus His-tagged McoP and SAM. McoP was directly immobilized onto Au electrode while controlling McoP orientation using cysteine residue on the C-terminus of McoP. As a result, O2 reduction current was increased. Improving the electron transfer efficiency between redox site of McoP and electrode surface was achieved by reducing the distance between them.

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1. Introduction

Biofuel cells (BFCs) have received a lot of attention as renewable source of energy because BFCs can use various organic resource as fuel [1,2]. In addition, BFCs have advantages such as operation at mild condition and ease of miniaturization. Therefore, in future, BFCs are expected as substitute for conventional fuel cells and implantable device [3]. However, low current density and short life due to low long-term stability of enzyme are limitations on practical applications of BFCs.

In order to improve BFC life, we have reported construction of electrode using enzymes from hyperthermophilic archaea [4]. Enzymes from hyperthermophilic archaea show high stability (e.g. long-term stability, thermal stability, pH stability) compared with enzymes from bacteria and fungi [5, 6]. The biocathode using multicopper oxidase from hyperthermophilic archaeon Pyrobaculum aerophilum (McoP) maintained current density of 70% after 14 days [4]. Therefore, in this study, McoP was used as biocatalyst for cathode.

McoP catalyzes the four-electron reduction from O2 to water as other multicopper oxidases (MCOs) such as laccase and bilirubin oxidase. McoP consists of
four copper atoms classified into type 1, 2, and 3 (T1, T2, and T3, respectively) as cofactors [7]. T1Cu receives an electron from an electron donor. Then, the electrons transfer from T1Cu to T2/T3Cu cluster and water is produced by reduction of dioxygen (O2) using four electrons at T2/T3Cu cluster. MCOs such as laccase have been also used for bleaching dye and effluent treatment in fiber processing [8, 9].

In biocatalytic electrode, electron transfer between electrode and enzyme is classified into two types, direct electron transfer (DET) and mediated electron transfer (MET). In DET type electrode, electron transfer occurs from the electrode to the enzyme. In MET type electrode, electron transfer between electrode and enzymes is mediated by redox mediator. Previously, in MCO modified electrodes, there were many research using MET because MET type electrodes showed higher current density than that of DET type electrodes [10]. However, most mediators showed low stability and are co-immobilized on the electrode with enzyme is necessary. On the other hand, recently, current density in DET type electrodes has been improved by development of nanomaterials such as carbon nanotubes (CNTs) and nanoparticles and development of enzyme immobilization method onto electrode [11, 12]. In DET type electrode, electron transfer efficiency is affected by the distance between the T1 site of MCO and the electrode surface [13]. Electron transfer efficiency increases with the decrease in the distance between T1 site of MCO and electrode surface. As a result, O2 reduction current increases and over potential for O2 reduction decreases. In most MCOs, T1 site is not located in the center of the MCO structure [14, 15]. Therefore, the orientation of MCO with respect to the electrode surface is also important for shortening the distance between T1 site and electrode surface [13, 16].

Previously, we reported about oriented immobilization of MCO obtained from Pyrobaculum aerophilum (McoP) onto multi-walled carbon nanotube (MWCNT) modified with a pyrene/nitrilotriacetic acid (NTA)/Ni linker using the affinity between His-tag and Ni coordinated to the NTA group on MWCNT (MWCNT-McoP) [17]. The biocathode using McoP showed long-term stability. The current density of MWCNT-McoP modified electrode was lower than that reported in previous studies on the biocathode [18, 19]. This is because the distance between the T1 site of McoP and the electrode surface was increased by using a linker. Therefore, in the present study, to improve the performance of the McoP-modified electrode, an oriented immobilization method without linker was investigated (Figure 1). McoP was immobilized on the electrode surface using an Au-S bond between the Au electrode and the thiol group of cysteine at the McoP terminus. C-terminal His-tagged McoP (McoP (C-His)) and C-terminal cysteine introduced McoP (C-His-Cys) were prepared. McoP (C-His) was immobilized onto an Au electrode because of the affinity between His-tag and Ni coordinated to the NTA group of 3,3'-dithiobis[N-(5-amino-5-carboxypentyl) propionamide]-N',N'-diacetic acid] dihydrochloride (C2-NTA) (Figure 1a). McoP (C-His-Cys) was immobilized onto the Au electrode using the thiol group of cysteine of McoP (C-His Cys) (Figure 1b). The length of C2-NTA (from S to COOH) was estimated to be 1.6 nm. Alkane thiol immobilized on the Au surface is oriented approximately 30 degrees from the Au surface [20]. Therefore, the distance between the electrode surface and Ni coordinated to NTA group was estimated to be 1.4 nm. When McoP (C-His Cys) was immobilized onto the electrode via Au-S bond, the distance between T1 site of McoP and electrode surface was approximately 1.4 nm shortened compared to that in an McoP (C-His)-modified electrode. In addition, the protein immobilized on the electrode via peptide tags fluctuate in solution [21]. Therefore, in McoP-modified electrode, electron transfer between T1 site and electrode occurred when T1 site approached the electrode surface or C2-NTA monolayer surface. McoP (C-His-Cys)-modified electrode is expected to show high current density and low over potential for O2 reduction compared to McoP (C-His) modified electrode by shortening the distance between T1 site of McoP and electrode surface. McoP (C-His-Cys)
modified electrode was prepared to improve the performance of the cathode for BFC.

2. Experimental

2.1 Chemicals and apparatus

PrimeSTAR GXL, In-Fusion HD Cloning Kit, and PrimeSTAR Mutagenesis Basal Kit were purchased from Takara Bio (Shiga, Japan). 3,3'-dithiobis[N-6-aminooxypropyl]-propion amide-N', N'-diacetic acid] dihydrochloride (C2-NTA) was obtained from Dojindo Laboratories (Kumamoto, Japan). Quartz crystal microbalance experiments were performed with an Affinix QN Pro (ULVAC, Inc, Kanagawa, Japan). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Sigma-Aldrich (Dorset, UK).

2.2 Preparation of McoP (C-His) and McoP (C-His-Cys)

The expression vector for the McoP (C-His) was constructed as follows: pae 1888 gene was amplified (without the region encoding the stop codon) by PCR using Primestar GXL DNA polymerase and primer sets (sense (S1): 5’-ATGACTGGTGAAGTCAAGAGG-CCTGAGACA-3’ and anti-sense (AS1): 5’-TTTAACTGCTATGGTTTATCATCATGCCCCC-3’). The stop codon deleted pae 1888 was used as template for PCR with following primer sets (sense (S 2): 5’-GAAGGAGATATACTATGACTGGAAGTCAAGAGG-CCTGAGACA-3’ and anti - sense ( AS 2 ) : GCTCGAATTCGGATCTTTAACTGCTATGTTTCA-3’). Ligation-independent cloning was achieved using an In Fusion HD Cloning kit, according to the manufacturer’s instructions. The amplified PCR product was cloned into linearized pET22 b (Novagen, Merck Millipore Japan, Tokyo, Japan) using In-Fusion HD Cloning Kit to generate pET22 b/PAE 1888 plasmid. Introduction of pae 1888 into pET22 b was verified by DNA sequencing.

For the construction of pET22 b/PAE 1888-C-Cys, PCR was carried out on the plasmid pET22 b/PAE 1888 using PrimeSTAR Mutagenesis Basal Kit and primer sets (sense (S 3): 5’-CACCAGTGTGAGATCGAGTCTGGTACAG-3’ and anti-sense (AS 3): GCCGGAATTCGGATTTAAGTCTGCTATGTTATTAC-3’). Ligation-independent cloning was achieved using an In Fusion HD Cloning kit, according to the manufacturer’s instructions. The amplified PCR product was cloned into linearized pET22 b (Novagen, Merck Millipore Japan, Tokyo, Japan) using In-Fusion HD Cloning Kit to generate pET22 b/PAE 1888 plasmid. Introduction of pae 1888 into pET22 b was verified by DNA sequencing.

McoP (C-His) and McoP (C-His-Cys) were expressed in Escherichia coli BL 21-CodonPlus (DE 3)-RILP (Agilent technology, California, USA), as previously described. In brief, McoP was obtained from a cell-free extract and purified by a three-step process involving heat treatment at 80°C for 10 min, anion-exchange chromatography, and Ni-NTA chromatography. The activities of McoP (C-His) and McoP (C-His-Cys) were measured using ABTS, as described previously. One unit activity was defined as the activity of McoP that oxidized 1 µmol of ABTS substrate per min at 50°C.

2.3 Enzyme immobilization onto Au electrode

To construct McoP (C-His)-modified electrode by physical adsorption, McoP (C-His) solution (2 mg/ml) was dropped on an Au electrode surface and kept at 4°C for 24 h.

To construct a McoP (C-His)-modified electrode, an Au electrode was immersed in 0.1 mM C2-NTA solution at 25°C for 3 h. Then the electrode was immersed in 50 mM NiSO4 at room temperature for 10 min. Finally, McoP (C-His) solution (2 mg/ml) was dropped on the electrode surface and kept at room temperature for 1 h.

To construct McoP (C-His-Cys)-modified electrode, McoP (C-His-Cys) solution (2 mg/ml) was dropped on an Au electrode surface and kept at 4°C for 24 h.

2.4 Electrochemical experiments

Linear sweep voltammetry (LSV) was conducted using a three-electrode cell on an ALS electrochemical analyzer (BAS Inc., Tokyo, Japan). The working electrode was an Au electrode (002014, geometrical area: 0.02 cm2, BAS Inc.), the counter electrode was a platinum wire, and the reference electrode was Ag/AgCl. All potential values were reported with respect to the Ag/AgCl reference electrode. The electrolyte solution was O2-saturated 100 mM acetate buffer, pH 5.0. The LSV measurements were recorded at potential scan rate of 0.01 V/s at 50°C. The working electrode was sonicated in 50 mM H2SO4, and the surface was then polished with a microcloth and alumina slurry suspension (φ = 1.0 and 0.05 µm, respectively). The electrode was scanned 50 times at a potential scan rate of 0.5 V/s, with potential ranging from -1.0 to 1.0 V. Finally, the electrode was sonicated in Milli-Q water.

2.5 QCM (quartz crystal microbalance) measurement

The QCM electrode (geometrical area: 4.909x 10⁻³ cm²) was washed using piranha solution (30% H2O2/H2SO4, (1:3, v/v)) and modified in the same way as the electrode for electrochemical measurements. The QCM measurements were performed in 50 mM...
HEPES buffer, pH 7.5 at 25 °C. The apparent mass alteration of immobilized enzyme was estimated from the frequency change using AFFINIX QN Pro Viewer (ULVAC Inc., Kanagawa, Japan). The number of enzyme molecules was calculated from the apparent mass alteration of immobilized enzyme.

3. Results and discussion

3.1 Characterization of McoP (C-His) and McoP (C-His-Cys)

To decrease the distance between the T1 site of McoP and the electrode surface (Figure 1), McoP (C-His) and McoP (C-His-Cys) were prepared by genetic engineering. The sequence analysis indicated that *pae* 1888 with 6×His tag at C-terminus was correctly inserted into pET22 b, resulting in 7.2 kb plasmids without gene mutation. Furthermore, introduction of cysteine into pET22 b/PAE 1888 was also confirmed. The specific activities of McoP (C-His) and McoP (C-His-Cys) were 2.82 ± 0.83 units/mg and 3.49 ± 0.52 units/mg, respectively.

3.2 Electrochemical reaction of McoP (C-His) or McoP (C-His-Cys)-modified electrode

The electrochemical O2 reduction of McoP (C-His)-adsorbed electrode, C2-NTA/Ni/McoP (C-His)-modified electrode and McoP (C-His-Cys)-modified electrode were compared using LSV measurement. Figure 2 shows linear sweep voltammograms of these electrodes. From the results of LSV of two electrodes, O2 reduction current at 0 V of McoP (C-His)-adsorbed electrode, C2-NTA/Ni/McoP (C-His)-modified electrode and McoP (C-His-Cys)-modified electrode were 141.4 ± 5.6 µA/cm², 167.4 ± 7.0 µA/cm² and 203.6 ± 9.2 µA/cm², respectively. Two types of oriented McoP immobilized electrodes showed higher O2 reduction current than that of McoP (C-His)-adsorbed electrode. This is because the distance between the electrode surface and the T1 site was shorter by oriented immobilization. Therefore, the number of electrochemically active enzyme on the electrode increased. In McoP (C-His-Cys)-modified electrode, the distance between T1 site and electrode surface was shorter than that of C2-NTA/Ni/McoP (C-His) as shown in Fig. 1. Therefore, the electron transfer between T1 site of McoP (C-His-Cys) and electrode was more efficient compared to that between T1 site of C2-NTA/Ni/McoP (C-His) and electrode. Consequently, the current density for O2 reduction of McoP (C-His-Cys)-modified electrode was increased.

3.3 Evaluation of the amount of immobilized C2-NTA/Ni/McoP (C-His) and McoP (C-His-Cys)

For further analysis, the amount of enzyme on the electrode was investigated by QCM measurement. The number of McoP molecules on the QCM electrode surface and the O2 reduction current per unit enzyme activity of McoP calculated from the QCM and LSV results are shown in Table 1. To investigate the effects of decreasing the distance between T1 site and electrode surface, the O2 reduction current per unit enzyme activity was calculated from the LSV and results from QCM because the two electrodes were different with respect to specific activity of enzyme and the amount of immobilized enzyme. In O2 reduction current per unit of McoP, McoP (C-His-Cys)-modified electrode showed high current compared to C2-NTA/Ni/McoP (C-His)-modified electrode. This result indicates that improving the electron transfer efficiency by shortening the distance between the T1 site of McoP and the electrode surface is mediated via increasing the catalytic current.

4. Conclusion

In the present study, the distance between the T1 site of McoP and the electrode surface decreased by removing NTA-Ni linker for increasing the catalytic current of biocathode for O2 reduction. O2 reduction current per unit of McoP (C-His-Cys)-modified electrode is 122% higher than that of C2-
NTA/Ni/McoP (C-His)-modified electrode. By decreasing the distance between the T1 site and the electrode surface, electron transfer efficiency was improved. Further research is needed to construct a biocathode using more precise orientation control of enzyme on electrode via site-directed mutagenesis (e.g. cysteine) on enzyme surface near T1 site to obtain high power and high voltage biofuel cells. These results also suggest that high performance McoP immobilization might be also useful for application of McoP for textile industry.

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