Int. J. Electrochem. Sci., 9 **(2014)** 1390 - 1398

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Bioelectrocatalytic Oxygen Reduction Reaction by Bilirubin Oxidase Adsorbed on Glassy Carbon and Edge-Plane Pyrolytic Graphite Electrodes: Effect of Redox Mediators

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Received: 14 October 2013 / *Accepted:* 2 November 2013 / *Published:* 5 January 2014

Direct electron transfer (DET) of bilirubin oxidase (BOx, from *Myrothecium verrucaria*) adsorbed on edge-plane pyrolytic graphite (EPPG) electrode as well as an electron transfer between the EPPG or glassy carbon (GC) electrode and the BOx (adsorbed on these electrodes) via a mediation by redox mediators ($[Fe(CN)_6]^{3-}$ and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS)), i.e., a mediator-assisted electron transfer, have been examined under anaerobic and aerobic conditions by cyclic voltammetry and differential pulse voltammetry. On differential pulse voltammograms on the EPPG electrode two redox processes with formal potentials of 0.50 and 0.29 V vs. Ag/AgCl/NaCl(3M) were clearly observed in 0.05 M phosphate buffer (pH 5.0), corresponding to the redox transformations of the T1 site and the T2/T3 cluster of the BOx, respectively. DET was not observed on the GC electrode. A mediator-assisted electron transfer between the electrode and the BOx adsorbed on it was realized in the absence and the presence of $[Fe(CN)_6]^{3-}$ and ABTS through the bioelectrocatalytic oxygen reduction reaction (ORR) by the BOx, and in addition its overall electron transfer mechanism is discussed briefly including the DET, mediator-assisted electron transfer and an intramolecular electron transfer (IET) from the T1 site to the T2/T3 cluster site, in which an uphill IET reaction during the catalytic ORR is suggested.

Keywords: Bilirubin oxidase, Direct electron transfer, Intramolecular electron transfer, Oxygen reduction reaction, Bioelectrocatalysis, Redox mediators

1. INTRODUCTION

Bilirubin oxidase (BOx) is one of multicopper oxidases (MCO), which contain three different copper centers (T1, T2 and T3) with coverall four copper ions, and catalyzes the oxidation of bilirubin

to biliverdin and thereby reduces molecular oxygen to water [1,2]. Generally, it has been accepted for MCO that the T1 site is the primary electron accepter from the substrate and electrons are transferred via an intramolecular electron transfer (IET) to the T2/T3 cluster site which converts molecular oxygen to water in a four-electron reaction [3-5]. Thus, a bioelectrocatalysis of BOx immobilized on electrodes to the oxygen reduction reaction (ORR), which is essential in its practical applications, for example, as the biocathode in biofuel cells [6-8], is considered to depend on an electron transfer between the BOx and the electrode as well as the IET from the T1 site to the T2/T3 cluster site. The former electron transfer can be realized actually via a direct electrochemistry [9-20], i.e., a so-called direct electron transfer (DET) or via a mediation by redox mediators [14, 21-23], i.e., a mediator-assisted electron transfer.

In this study, the bioelectrocatalysis of BOx from *Myrothecium verrucaria*, which is adsorbed on glassy carbon (GC) and edge-plane pyrolytic graphite (EPPG) electrodes, towards the ORR is examined in the absence and the presence of redox mediators with different redox potentials which are chosen by taking the formal potentials of the T1 and T2/T3 sites into account. In this case, the DET takes place on EPPG, but not on GC. Here, the preliminary results regarding the dependency of the bioelectrocatalysis of the BOx towards the ORR upon the DET as well as redox mediator-assisted electron transfer between the BOx and the electrode will be reported together with the overall electron transfer mechanism of the catalyzed ORR.

2. EXPERIMENTAL

2.1. Materials

Bilirubin oxidase from *Myrothecium verrucaria* (BOx, E.C. 1.3.3.5, 10 unit/mg solid) was purchased from Sigma-Aldrich and used without further purification. $K_3[Fe(CN)_6]$ was purchased from Kanto Chemicals Co., Inc. Japan and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) from Sigma-Aldrich. Phosphate buffer solution (PBS, 0.05 M, pH 5.0), prepared using sodium dihydrogenphosphate and disodium hydrogenphosphate (Kanto Chemicals Co., Inc.), was used as the supporting electrolyte for electrochemical measurements. All aqueous solutions were prepared with deionized water purified by a Milli-Q water system (Millipore, Japan). A glassy carbon (GC, 3 mm is diameter) and edge-plane pyrolytic graphite (EPPG, 3 mm is diameter) were purchased from BAS, Japan.

2.2. Electrochemical measurements

Cyclic voltammetric and differential pulse voltammetric measurements and open circuit potential measurements were performed using a computer-controlled electrochemical analyzer (CHI 760DS) (ALS) in a two-compartment and three-electrode cell. The bare and BOx-adorbed GC and EPPG electrodes were used as working electrodes, Ag/AgCl/NaCl(3 M) as a reference electrode and a platinum spiral wire as a counter electrode. Prior to use, GC and EPPG electrodes were polished with

aqueous slurries of fine alumina powder (particle size: 1.0 and 0.06 μm) with the help of a polishing microcloth. To remove the residual alumina particles the polished electrodes were ultrasonicated in Milli-Q water for 10 min. A volume of 8 μL of BOx solution (10 mg/ml H_2O) was placed on the thus pretreated electrode surface, allowed to adsorb, and after 2 h the BOx-adsorbed electrodes were used for the electrochemical measurements. For the experiments conducted under anaerobic (or aerobic) conditions, the electrolyte solution was bubbled with pure Ar (or O_2) gas for more than 30 min and Ar (or O_2) was flushed over the solution during the electrochemical measurements. All the electrochemical measurements were carried out at room temperature (25±1℃). All the current densities were calculated on the basis of the geometric surface area of the relevant working electrodes. All reported potentials are indicated with respect to Ag/AgCl/NaCl(3 M), unless otherwise noted.

3. RESULTS AND DISCUSSION

Figure 1 shows the typical differential pulse voltammograms obtained for the BOx adsorbed on the EPPG and GC electrodes in 0.05 M PBS (pH 5.0) under Ar atmosphere.

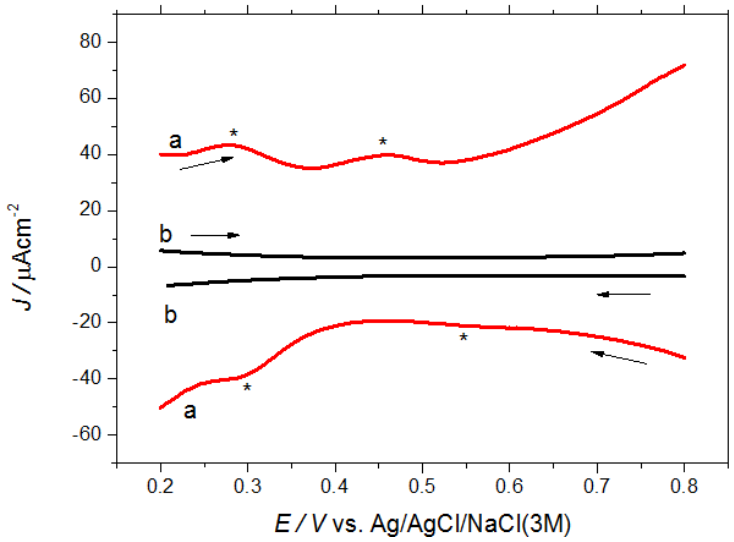
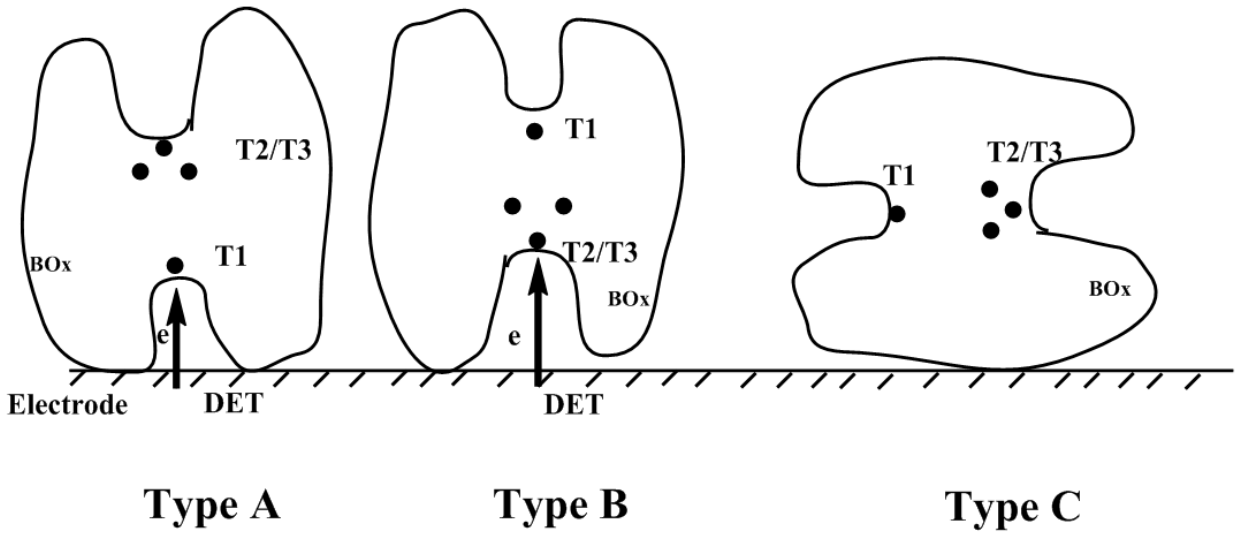


Figure 1. Differential pulse voltammograms obtained for the BOx adsorbed on (a) EPPG and (b) GC electrodes in 0.05 M PBS (pH 5.0) under Ar atmosphere. Differential pulse voltammetric conditions: amplitude 0.05 V, pulse width 0.05 s, pulse period 0.2 s. The arrows show the direction of potential pulse application.

Two redox processes were observed at the EPPG electrode: one had a formal potential ($E^{0\prime}$) of 0.29 V and another had an $E^{0\prime}$ of 0.50 V. These $E^{0\prime}$values were estimated as $(E_p^a + E_p^c)/2$, where E_p^a and E_p^c, indicated by $*$, are the anodic and cathodic peak potentials, respectively. Assuming a random orientation of the adsorption of BOx on the electrode surface as considered generally [24-26] and considering that BOx contains two redox active centers with different formal potentials, i.e., the T1 site

and T2/T3 cluster site [3-5, 27], this fact suggests at least two different types of adsorption orientation, i.e., the presence of some BOx molecules orientated by their T1 site and T2/T3 cluster site to the electrode is expected to lead to the observed two redox processes, as schematically shown in Scheme 1 (Type A and Type B). The redox reactions of these types are called as a so-called direct electron transfer (DET) between the active site of the enzyme and the electrode [28-40].



Scheme 1. Probable orientations of the adsorption of BOx at EPPG and GC electrodes. (Tpye A): the T1 site is in DET contact with the electrode surface, (Type B): the T2/T3 cluster site is in DET contact with the electrode surface and (Type C): BOx is not in DET contact with the electrode surface.

On the other hand, no redox response was observed at the GC electrode, indicating that there is no orientation of the adsorption of BOx allowing its DET, for example, as shown as Type C. Of course, in this case also BOx is surely adsorbed on the GC electrode as mentioned below. Here it should be also noted that the large difference in the background (residual) currents obtained on the EPPG and GC electrodes is due to the difference in the double layer capacitances at both electrodes.

Figure 2 shows the cyclic voltammograms (CVs) obtained at the BOx-adsorbed EPPG electrode in 0.05 M PBS (pH 5.0) in the absence and the presence of redox mediators ($[Fe(CN)_6]^{3-}$ and ABTS) under O_2 or Ar atmosphere. As can be seen from Figs. 2A(c) and 2B(c), in the absence of redox mediators and the presence of the BOx substrate (O_2), a reduction current begins to flow at around 0.5 V. In this case, if a potential at the current density of 0.1 μAcm^{-2} is taken as the onset potential (E_{onset}) of the oxygen reduction reaction (ORR), E_{onset} is estimated as 0.56 V. The open circuit potential (E_{ocp}) was estimated as 0.56 V under the same condition. This E_{ocp} value (0.76 V vs. NHE) is only 80 mV lower than the thermodynamic reversible potential for the O_2/H_2O couple at the same pH (0.84 V vs. NHE). The fact that the ORR current flows in the absence of redox mediators indicates that electrons are directly transferred from the electrode to the BOx adsorbed on the electrode surface, i.e., DET and the BOx possesses its essential enzymatic activity. Based on these results along with the fact

that the enzymatic four-electron ORR by BOx takes place via an intramolecular electron transfer (IET) from the T1 site to the T2/T3 cluster site [3-5], it is thought that the more positive one (i.e., 0.50 V) of two $E^{0'}$ values of BOx obtained under Ar atmosphere corresponds to the redox reaction of the T1 site; $E^{0'}$(T1) = 0.50 V and another $E^{0'}$ value (0.29 V) is ascribed to that of the T2/T3 cluster site; $E^{0'}$(T2/T3) = 0.29 V. Thus, the overall electron transfer process in the absence of redox mediators can be generally illustrated as Case A in Scheme 2, in which the BOx is oriented by its T1 site to the electrode. The obtained two $E^{0'}$ values ($E^{0'}$(T1) = 0.70 V vs. NHE and $E^{0'}$(T2/T3) = 0.49 V vs. NHE) are close to those ($E^{0'}$(T1) = 0.69 V vs. NHE and $E^{0'}$(T2/T3) = 0.39 V vs. NHE) reported for the BOx (from *Trachyderma tsunodae*) adsorbed at bare spectrographic graphite in 0.1 M PBS (pH 7.0) [2]. As mentioned above, the orientation of BOx to the electrode by the T2/T3 site (Type B in Scheme 1) and the electron transfer like Case B may be also considered.

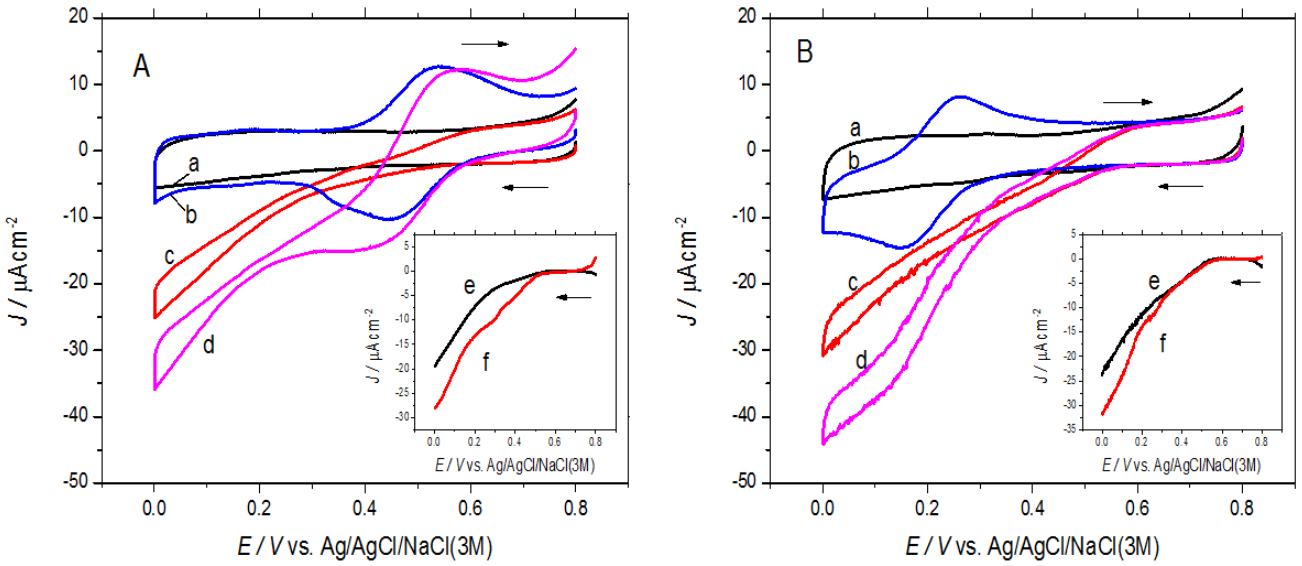
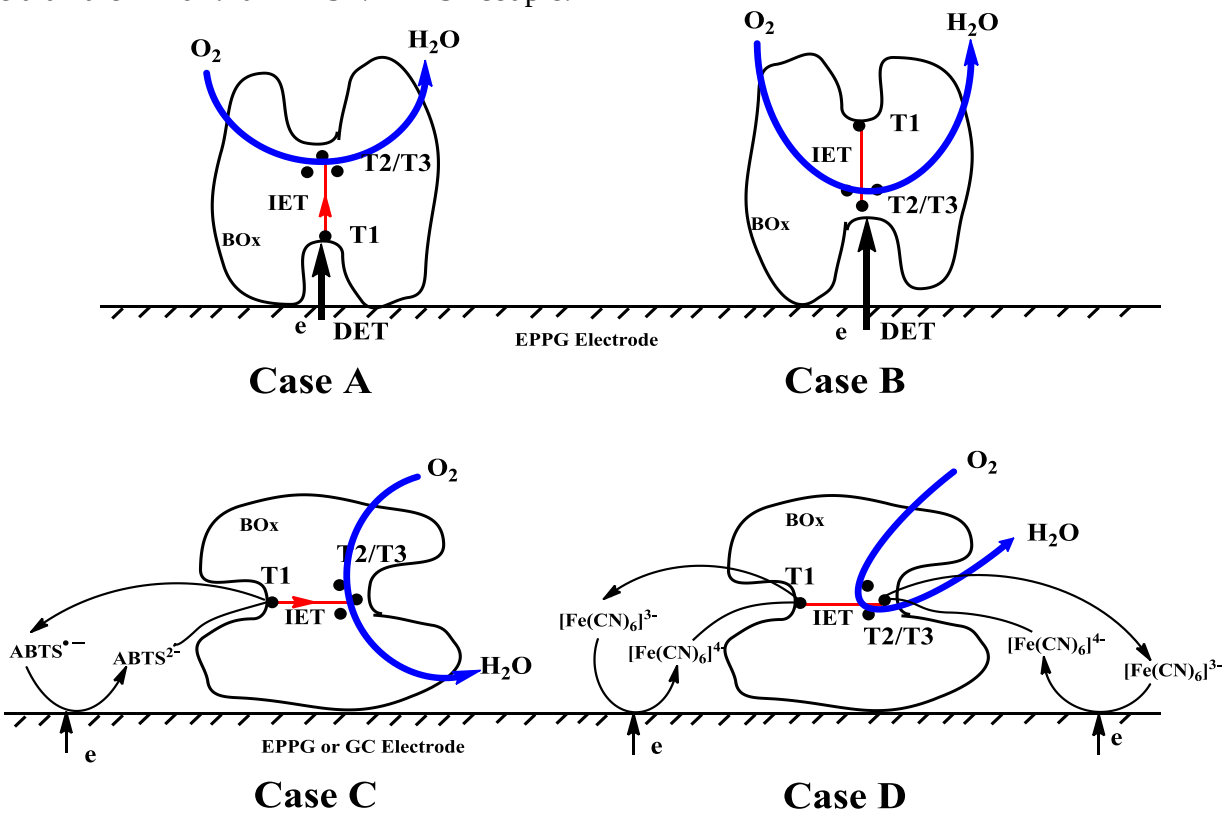


Figure 2. CVs obtained at BOx-adsorbed EPPG electrode in 0.05 M PBS (pH 5.0) in the absence (a,c,e) and the presence of (b,d,f) of (A) 0.1 mM ABTS and (B) 0.1 mM $[Fe(CN)_6]^{3-}$ under (a,b) Ar and (c,d) O_2 atmosphere. Potential scan rate: 10 mVs^{-1}. The insets e and f in each case of A and B were obtained by subtracting the currents a and b obtained in the cathodic scan under Ar atmosphere from the cathodic currents c and d obtained under O_2 atmosphere, respectively.

In the presence of redox mediators, ABTS and $[Fe(CN)_6]^{3-}$, in both cases, the ORR current is increased (see the insets of Figs. 2A and 2B), reflecting a so-called redox mediation effect, that is, some BOx molecules, which are adsorbed on the electrode, but the DET of which does not occur, also can function as a bioelectrocatalyst based on their original enzymatic activity, i.e., they can catalyze the reduction of O_2 to H_2O. In the case of ABTS, the increased ORR current resulting from the redox mediation by ABTS begins to flow at a potential at which the reduction of $ABTS^{\bullet-}$ commences, because the $E^{0'}$ value of the $ABTS^{\bullet-}/ABTS^{2-}$ couple is almost the same as the $E^{0'}$(T1) (0.50 V). $ABTS^{\bullet-}$ is reduced at the EPPG electrode to generate the one-electron reduced form ($ABTS^{2-}$) and $ABTS^{2-}$ diffuses to the T1 site and reduces it, i.e., the T1 site accepts an electron from $ABTS^{2-}$ and then

an electron is transferred to the T2/T3 cluster site (as IET) at which O_2 is reduced to H_2O (Case C). Here, it should be noted that the cyclic voltammogram obtained in the presence of ABTS (Fig.3A(d)) resembles that which might be obtained for the ORR if it is redox-mediated by ABTS, but ABTS itself can neither catalyze nor mediate the ORR actually (see the inset Fig.3A). In addition, it is to be noted that in this case $ABTS^{2-}$ can not reduce the T2/T3 site, because the $E^{0'}$(T2/T3) (0.29 V) is more negative than the $E^{0'}$ of the $ABTS^{\bullet-}/ABTS^{2-}$ couple.



Scheme 2. Probable overall bioelectrocatalytic ORR mechanisms on BOx-adsorbed EPPG and GC electrodes in the absence of redox mediators (Case A and B) and the presence of ABTS (Case C) and $[Fe(CN)_6]^{3-}$ (Case D). In Cases A, B and C and D, BOx is adsorbed on the electrodes with the orientation of Types A, B and C, respectively, in Scheme 1.

In the case of $[Fe(CN)_6]^{3-}$ also a similar redox mediation effect on the ORR is observed (Fig.2B). The inset of Fig.2B shows that the ORR current is almost same in the absence and the presence of $[Fe(CN)_6]^{3-}$ in the potential range between the E_{onset}(0.50 V) and ca. 0.3 V and at more negative potential than 0.3 V it is larger in the presence of $[Fe(CN)_6]^{3-}$ than in its absence, being different from the case of ABTS in which the redox-mediated ORR current begins to flow at the E_{onset} obtained in the absence of ABTS under O_2 atmosphere (see the inset of Fig.2A). This result confirms that the overall ORR is enhanced as a result of the redox mediation by the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ couple. Before the reduction of $[Fe(CN)_6]^{3-}$ commences, no redox mediation takes place actually and only the DET-based bioelectrocatalytic ORR occurs (Cases A and B), but when its reduction commences at ca. 0.3 V, the increased ORR current resulting from this redox mediation also starts to flow. At < ca. 0.3 V, thus, the overall ORR takes place via both DET-based (Cases A and B) and redox

mediator ($[Fe(CN)_6]^{3-}$)-assisted (Case D) bioelectrocatalytic electron transfer reactions. In this case, $[Fe(CN)_6]^{4-}$ can reduce both the T1 site and the T2/T3 cluster site.

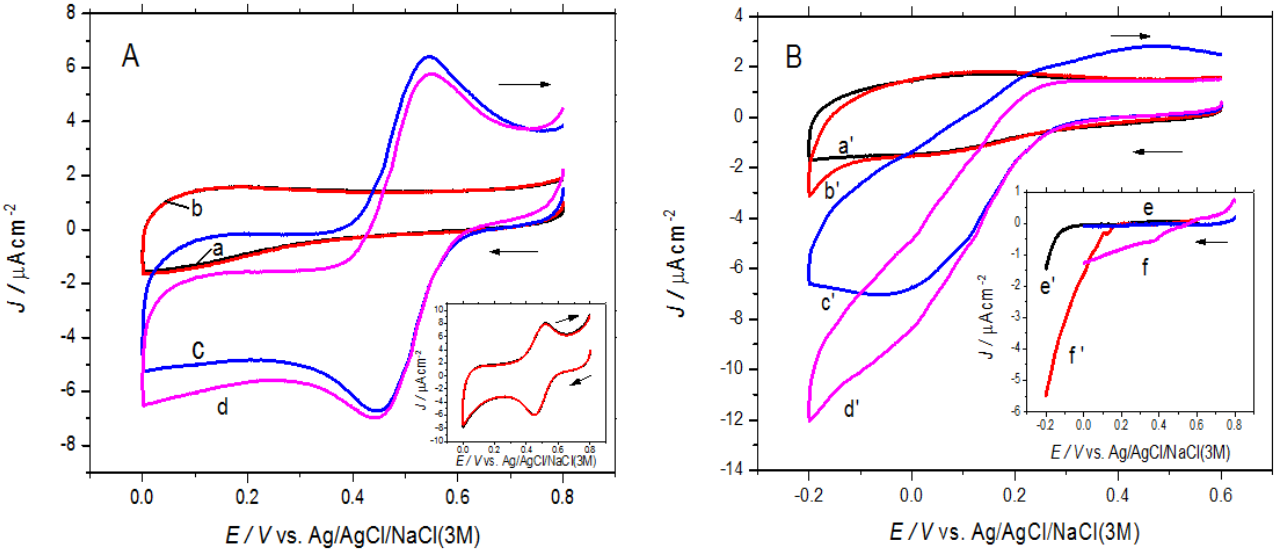


Figure 3. CVs obtained at BOx-adsorbed GC electrode in 0.05 M PBS (pH5.0) in the absence (a,c,e) and the presence (b,d,f) of (A) 0.1 mM ABTS or in the absence (a′,c′,e′) and the presence (b′,d′,f′) of (B) 0.1 mM $[Fe(CN)_6]^{3-}$ under (a,a′,c,c′) Ar and (b,b′,d,d′) O_2 atmosphere. Potential scan rate: 10 mVs^{-1}. The inset in Fig.3A shows CVs obtained at GC electrode in 0.05 M PBS (pH 5.0) containing 0.1 mM ABTS under (black line) Ar and (red line) O_2 atmosphere at 10 mVs^{-1}. The insets e and f (e′and f′) in Fig.3B were obtained by subtracting the currents a and c (a′and c′) obtained in the cathodic scan under Ar atmosphere from the cathodic currents b and d (b′and d′) obtained under O_2 atmosphere, respectively.

As mentioned above (Fig.1), no DET was observed for the BOx adsorbed on the GC electrode and the adsorption of Type C was assumed as a probable orientation of BOx. In this case, a reduction current was not recorded under O_2 atmosphere (Figs.3A(b) and 3B(b′)). However, in the presence of redox mediators, ABTS and $[Fe(CN)_6]^{3-}$, a redox mediator-assisted reduction current was observed clearly (Figs.3A(d) and 3B(d′)) and, as expected, the ORR current starts to flow at different potentials depending on the mediator used, i.e., ca. 0.55 and 0.25 V in the presence of ABTS and $[Fe(CN)_6]^{3-}$, respectively (see the inset of Fig.3B). The overall ORR mechanisms for both cases may be illustrated as Cases C and D in Scheme 2. In Case D, the T1 site may be more favorably reduced by $[Fe(CN)_6]^{4-}$ than the T2/T3 site from the redox potential differences between $E^{0\prime}$(T1) or $E^{0\prime}$(T2/T3) and $E^{0\prime}$($[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$), but once $[Fe(CN)_6]^{4-}$ is generated electrochemically, both the T1 and T2/T3 sites can be reduced by it. Therefore, a higher ORR current may be obtained in the presence of $[Fe(CN)_6]^{3-}$ compared with the case of ABTS.

Interestingly, the above-obtained values of $E^{0\prime}$(T1) and $E^{0\prime}$(T2/T3) (0.50 V and 0.29 V, respectively) mean that the IET is an uphill reaction over 0.21 V barrier between the T1 site and the T2/T3 cluster site (Cases A and C), as suggested by Ramírez et al. for the first time for the ORR biocatalyzed by BOx (from *Trachyderma tsunodae*) adsorbed on bare spectrographic graphite in 0.1 M PBS (pH 7.0) under O_2 atmosphere [2]. They also proposed a possibility of DET via the T2/T3 site and

further experimentally observed the participation of the T2/T3 cluster (oriented to the electrode) in the O_2 bioelectroreduction. In the present case, no experimental observation reflecting such a possibility was obtained, but the adsorption of BOx like Case B and the ORR via the BOx can not be denied. In Cases B and D, the situation for an uphill IET reaction seems more or less different from that in Cases A and C, because in Case B the T2/T3 site can accept an electron from the electrode and an electron can be transferred to the T1 site as a downhill reaction, and in Case D the electrochemically produced $[Fe(CN)_6]^{4-}$ can reduce both the T1 and T2/T3 sites and thus it is considered that the essential IET from the T1 site to the T2/T3 cluster site is not necessarily required for the biocatalytic ORR.

4. CONCLUSIONS

We have demonstrated that DET of BOx takes place on the EPPG electrode, but not on the GC electrode in 0.05 M PBS (pH 5.0), suggesting the different orientation of the adsorption of BOx on both electrodes. The formal potentials ($E^{0\prime}$) of the T1 and T2/T3 sites were estimated to be 0.50 and 0.29 V vs. Ag/AgCl/NaCl(3 M), respectively. The bioelectrocatalytic ORR by the BOx depends on the electrode substrate and the redox mediator, e.g., the onset potentials of the ORR on the EPPG electrode are ca. 0.55 V irrespective of the mediators, while those on the GC electrode are ca. 0.55 V and 0.25 V in the presence of ABTS and $[Fe(CN)_6]^{3-}$, respectively, because in the former case the DET takes place, but does not in the latter one. The net ORR current is larger in the presence of the redox mediators, compared with their absence, indicating a redox mediation effect, that is, some BOx molecules which are adsorbed on the electrode, but the DET of which does not occur, also can function as a bioelectrocatalyst based on their original enzymatic activity in the presence of the redox mediators, i.e., they can catalyze the reduction of O_2 to H_2O. An uphill IET reaction from the T1 site to the T2/T3 site during the catalytic ORR is also suggested.

ACKNOWLEDGEMENTS
This research was financially supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan and Tokyo Institute of Technology Global COE Program for Energy Science. A scholarship to D. Li from the Mitsubishi UFJ Trust Scholarship Foundation is gratefully acknowledged.

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