On the Relationship between the Characteristics of Bilirubin Oxidases and O_2 Cathodes Based on Their "Wiring"

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The characteristics of a novel cathode on which O_2 is electroreduced to water under physiological conditions (pH 7.4, 0.15 M NaCl, 37.5 °C) are reported. The cathode was made by electrically connecting ("wiring") redox centers of bilirubin oxidase (BOD) from *Trachyderma tsunodae* (*Tt*) to carbon electrodes through a redox polymer. The cathode was more stable than that made by wiring BOD from *Myrothecium verrucaria* (*Mv*) (N. Mano et al., *J. Am. Chem. Soc*. **2002**, *124*, 6480−6486) (5% loss of current per day of operation vs 10%), and its operating potential at 3 mA cm^{-2} current density was −140 mV vs the potential of the reversible O_2/H_2O electrode, 50 mV higher than that of the *Mv*-BOD cathode. The improved stability is attributed to stronger electrostatic bonding in the adduct of *Tt*-BOD with the "wiring" redox polymer, and the higher operating potential to the replacement of methionine by phenylalanine in the axial position of the type 1 $Cu^{+/2+}$ center, which upshifts the center's potential.

Introduction

The four-electron electroreduction of O_2 to water under physiological conditions is of interest because a miniature biofuel cell with an O_2 electroreducing cathode and a glucose electrooxidizing anode[1−3] may power for about a week a sensor or an actuator implanted in the body. The blue copper-containing oxidases having four $Cu^{+/2+}$ centers catalyze the four-electron reduction of O_2 to water, without producing H_2O_2. Examples of such oxidases include laccases, bilirubin oxidases, ascorbate oxidase, and ceruloplasmine.[4−14] The crystal structures and the coordination chemistries of the copper sites of both native enzymes and of their mutants have been rigorously defined.[15] The catalytic reduction of O_2 to water depends on the coordination of the four $Cu^{+/2+}$ ions of the enzymes. The $Cu^{+/2+}$ ions are classified, by their ligands, into three "types".[4−7] Type 1 $Cu^{+/2+}$ centers show an intense Cys S to Cu(II) charge-transfer band at ∼600 nm; the site accepts electrons from an organic substrate, such as a phenol, ascorbate, or bilirubin, and relays the electrons to the O_2-reduction site. The O_2-reduction site is a trinuclear cluster, consisting of one type II $Cu^{+/2+}$ center and a pair of type III $Cu^{+/2+}$ centers, their spectrum showing a shoulder at 330 nm.[4,5,16−18]

The electrocatalysis of four-electron reduction of O_2 to water by laccases has been intensively studied.[19−24] Tarasevich et al. reported that laccase monolayers on vitreous carbon catalyze the four-electron electroreduction of O_2 to water. Their laccase cathodes operated at pH 5 at a current density of 175 $\mu A/cm^2$ when poised near the thermodynamic potential of the reversible O_2/H_2O electrode.[25] They also reported a 10 mA cm^{-2} cathode, made of a composite of high surface area porous carbon and an unidentified laccase, operating at pH 3.5 and at −0.2 V vs the reversible O_2/H_2O electrode potential, but did not provide enough detail to allow others to reproduce their experiment.[26] We reported earlier three "wired" laccase-based cathodes.[27−29] The first was based on the wiring of laccase from *Coriolus hirsutus*. It operated, when poised at −0.22 V vs the potential of the reversible O_2/H_2O electrode, at a current density of 5 mA cm^{-2} in a pH 5 chloride-free citrate buffer at 37.5 °C.[28] The second was based on the wiring of *Myrothecium verrucaria* (*Mv*) to redox centers of bilirubin oxidase (BOD) and operated under physiological conditions (pH 7.4, 0.15 M NaCl, 37.5 °C) at a current density of 5 mA cm^{-2} when poised at − 0.18 V vs the reversible O_2/H_2O electrode potential.[29] Our wiring of *Mv*-BOD followed the work of Tsujimura et al. whose cathode consisted of carbon-felt, immersed in a solution of *Mv*-BOD (0.11 μM) and ABTS (0.5 mM) in pH 7 phosphate buffer (0.1 M KCl). The dissolved enzyme cathode operated for 2 h and electroreduced O_2 at a current density of 0.5 mA cm^{-2} when it was poised at −0.17 V vs the reversible potential of the O_2/H_2O electrode.[30]

Bilirubin oxidase catalyzes the oxidation of bilirubin to biliverdin (eq 1) and to unindentified substances.[31−35] It is applied in the assay of bilirubin in serum and thereby in the diagnosis of jaundice.[36−39]

$$\text{bilirubin} + 1/2O_2 \rightarrow \text{biliverdin} + H_2O \qquad (1)$$

In our *Mv*-BOD cathode, the electrostatic adduct of the polyanionic enzyme and its "wire", the polycationic redox copolymer of polyacrylamide and poly(*N*-vinylimidazole) complexed with $[Os(4,4'\text{-dichloro-}2,2'\text{-bipyridine})_2Cl]^{+/2+}$, were co-immobilized on the electrode.[29] The current density of our rotating cathode was O_2 transport limited up to 8.8 mA cm^{-2}, and its kinetic limit was 9.1 mA cm^{-2}. When the electrode was rotated at 300 rpm and was poised at −256 mV vs the potential of the reversible O_2/H_2O electrode, its 2.4 mA cm^{-2} initial current density declined to 1.3 mA cm^{-2} upon 6 days of continuous operation at 37.5 °C.

In an effort to upshift the operating potential of the cathode and to improve its operational stability, we undertook the study of a second cathode, based on the wiring of *Trachyderma*

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tsunodae (*Tt*) BOD.[40] *Tt*-BOD, a monomeric protein with a molecular weight of 64 kDa,[41,42] is considerably larger than the 52 kDa *Mv*-BOD. Both BODs have one type I, one type II, and two type III $Cu^{+/2+}$ centers.[40,41] *Tt*-BOD was cloned by Iwamoto et al. who deduced its amino acid sequence and predicted, from homology of the amino acid sequences of ascorbate oxidase, the coordination of its type I, II, and III $Cu^{+/2+}$ sites.[43] In *Tt*-BOD all of the ligands of the type II and type III $Cu^{+/2+}$ centers are His, as they are also in ascorbate oxidase. While the type I $Cu^{+/2+}$ center of *Mv*-BOD has an axial, weakly coordinating methionine,[44−46] that of *Tt*-BOD has a noncooordinating phenylalanine. As a result of a similar difference, the redox potential of the type I $Cu^{+/2+}$ center of a laccase has been found to be ∼100 mV more oxidizing than that of its mutant.[10]

Here we compare the redox potentials, current densities, pH ranges, and the operational stabilities of the "wired" *Mv*-BOD and *Tt*-BOD cathodes. We propose that the observed differences derive from differences in the electrostatic adducts that the two polyanionic enzymes form with their wiring redox polymer and from the more oxidizing redox potential of the type I $Cu^{+/2+}$ center of *Tt*-BOD.

Experimental Section

Chemicals and Materials. Bilirubin oxidase (BOD) (EC 1.3.3.5, 1.3 $U.mg^{-1}$) from *Trachyderma tsunodae* was a gift from Amano, Lombard, IL. NaCl, NaOH, KCNS, KBr, $MgCl_2$, $CaCl_2$, and NaF were purchased from Sigma, St. Louis, MO. Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) was purchased from Polysciences Inc. (Warrington, PA). A fresh solution of BOD in pH 7.4 20 mM phosphate buffer (PB) was prepared daily. The uric acid was dissolved in dilute NaOH then neutralized with dilute H_3PO_4 to yield a 10 mM aqueous solution.[47] The electrochemical measurements were performed in pH 7.4 phosphate buffered saline (PBS, 20 mM phosphate, 0.15 M NaCl) except in the experiments where the pH and anion dependences of the steady-state O_2 electroreduction currents were determined. In these, borate, citrate, acetate, phosphate, and Tris buffers were employed. All solutions were made with deionized water, passed through a purification train (Sybron Chemicals Inc, Pittsburgh, PA). Carbon cloth (Toray TGPH-030) was received, as a sample, from E-TEK (Somerset, NJ). Ultrapure O_2 and argon were purchased from Matheson (Austin, TX). The synthesis of the BOD-wiring redox polymer PAA-PVI-[Os(4,4'-dichloro-2,2'-bipyridine)$_2$Cl]$^{+/2+}$ was previously reported.[29]

Electrodes. *Carbon Cloth.* The carbon cloth electrodes were made by the reported three-step procedure.[27,29] A deposition solution was prepared by mixing 10.3 μL of a 10 mg mL^{-1} aqueous redox polymer solution, 2 μL of PBS, and 1.7 μL of 55 mg mL^{-1} BOD in PBS and 2 μL of 7 mg mL^{-1} PEGDGE in water. A 5 μL aliquot of the mixed solution was pipetted onto the mounted hydrophilic carbon cloth, which was promptly wetted and penetrated by the solution. The electrodes were cured for at least 18 h at room temperature before they were used.

Carbon Fibers. Prior to their coating, the 7 μm diameter fibers (0.0044 cm^2) were made hydrophilic by exposure to 1 Torr O_2 plasma for 3 min.[48] The cathodic catalyst consisted of the cross-linked adduct of 44.4 wt % *Tt*-BOD, 49 wt % redox polymer, and 6.6 wt % PEDGE.

Instrumentation and Cell. The measurements were performed using a bipotentiostat (CH−Instruments, Austin, TX, Model #CHI832) and a dedicated computer. The temperature was controlled with an isothermal circulator (Fisher Scientific, Pittsburgh, PA). The dissolved O_2 concentration was monitored with an O_2-electrode purchased from BAS (West Lafayette, IN). The electrodes were rotated using a Pine Instruments rotator (Austin, TX). The measurements were carried out in a water-jacketed electrochemical cell at 37.5 °C containing 50 mL PBS. At the start of the experiments, argon was bubbled through the solution for at least 15 min, followed by oxygen. To maintain a fixed volume of solution in the cell, the bubbled gases were presaturated with water by passage through a bubbler, which also contained PBS. The potentials were measured vs a commercial Ag/AgCl (3 M KCl) reference electrode. The counter electrode was a platinum wire (BAS, West Lafayette, IN). In the coulometric measurements, the scan rate was 1 mV s^{-1}.

BOD Assay. The absorption spectra of the BOD solutions were measured at 25 °C with an Agilent 8453 UV−visible spectrophotometer following the procedure of Amano (Lombard, IL).

Results

The open circuit potential of the vitreous carbon electrode on which *Tt*-BOD (without redox polymer) was adsorbed was +440 mV vs Ag/AgCl in pH 7.4 PBS under argon at 37.5 °C (−116 mV vs the potential of the reversible O_2/H_2O electrode). The open circuit potential of a similar electrode, but with a thin adsorbed film of the electrostatic adduct of the redox polymer and *Tt*-BOD was remarkably higher, +530 mV vs Ag/AgCl (−26 mV vs the potential of the reversible O_2/H_2O electrode). No spectroscopic evidence was found for complex formation between the $Cu^{+/2+}$ ions of BOD and the redox polymer: When the water-soluble copolymer of acrylamide and *N*-vinylimidazole was added to a solution of *Tt*-BOD in PBS, the spectrum of the enzyme did not change.

Figure 1A shows the cyclic voltammogram of the composite wired *Tt*-BOD coated carbon cloth electrode under argon at 50 mV s^{-1} in PBS buffer at 37.5 °C. The coating of the electrode consisted of 44.4 wt % BOD, 49 wt % polymer, and 6.6 wt % PEDGE. Its voltammogram was characteristic of the osmium complex bound to the redox polymer, with an apparent redox potential of +350 mV vs Ag/AgCl. At 1 mV s^{-1} scan rate the voltammogram exhibited a symmetrical wave, with a 25 mV separation (ΔE_p) of the oxidation and reduction peaks. At 50 mV s^{-1} the separation increased to 110 mV. The width of the peaks at half-height, E_{whm}, was 113 mV, whereas in the wired *Mv*-BOD it was 90 mV, close to the theoretical width of 90.6 mV for an ideal nernstian one-electron-transfer reaction.[29] As observed earlier in wired laccases, but not in wired *Mv*-BOD, ΔE_p and E_{whm} increased with the PEDGE weight fraction in the catalyst. Upon 4-hour cycling of the potential at 50 mV s^{-1} between 0.0 V and +0.5 V vs Ag/AgCl, the heights of the voltammetric peaks of the electrodes rotating at 1000 rpm decreased only by less than 6% at 37.5 °C.

The reproducibility of the O_2 electroreduction currents of electrodes rotating at 1000 rpm under air, or under 1 am O_2 , was ±10% or better, both for different batches ($n = 4$) and within batches ($n = 12$). When the electrodes were rotated at 4000 rpm the reproducibility of their currents was ±15% between batches and ±11% within batches.

Because two-electron BOD-catalyzed electroreduction of O_2 to H_2O_2[49,50] might compete with the desired four-electron reduction, the solutions were tested for accumulation of H_2O_2. No trace of H_2O_2 was detected when they were assayed by established procedures.[29,51−54]

Figure 1B shows the dependence of the current *I* on the square root of the angular velocity of the rotating electrode, $\omega^{1/2}$. Up

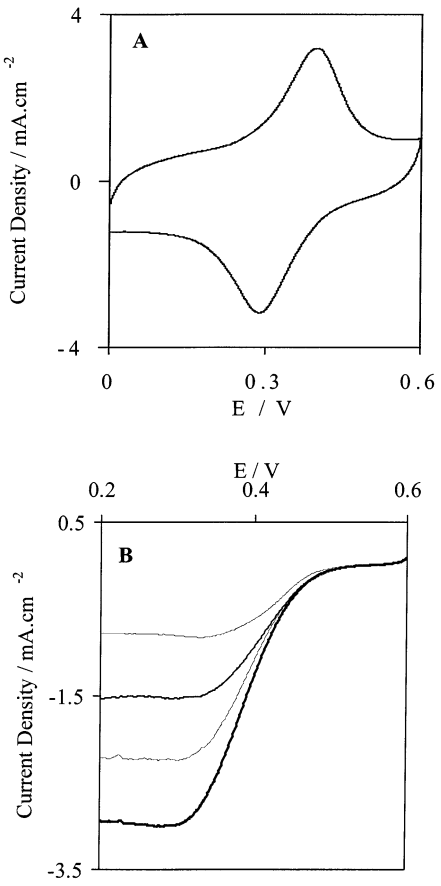

Figure 1. (A) Cyclic voltammogram of the wired *Tt*-BOD carbon cloth electrode under argon. PBS (0.15 M NaCl, pH 7.4; 20 mM phosphate) buffer, 37.5 °C, 50 mV s^{-1} scan rate. Total loading of the 44.4 wt % BOD, 49 wt % redox polymer, 6.6 wt % PEGDGE cross-linker composite is 0.6 mg cm^{-2}. The current density is based on the 0.126 cm^2 geometrical area of the 4-mm diameter carbon cloth disk. (B) Polarization of the cathode rotating at 500 rpm (top curve), 1000, 2000, and 4000 rpm (bottom curve), in PBS buffer at 37.5 °C, air, 1 mV s^{-1} scan rate. Other conditions as in Figure 1A.

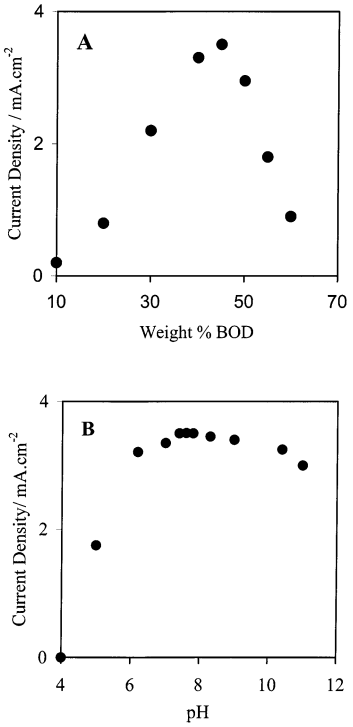

Figure 2. (A) Dependence of the current density on the *Tt*-BOD enzyme weight percentage. Electrode poised at +300 mV vs Ag/AgCl, 1 mV s^{-1} scan rate, 1000 rpm, 1 atm O_2. Other conditions as in Figure 1A. (B) pH dependence of the steady state current density under 1 atm O_2 for the electrode poised at +300 mV vs Ag/AgCl, 1000 rpm. Other conditions as in Figure 1A.

to 3000 rpm, where the current density reached 2.74 mA cm^{-2}, it increased linearly with $\omega^{1/2}$, precisely as predicted by the Levich equation, showing that the current was O_2 transport-limited in air when the electrode was poised at +300 mV vs Ag/AgCl. Above 3000 rpm the current continued to increase with $\omega^{1/2}$, but no longer linearly, until it reached 2.9 mA cm^{-2}. Under 1 atm O_2, at 1000 rpm, the current density reached 3.5 mA cm^{-2}, 30% less than reported for the *Mv*-BOD cathode,[29] under the same conditions. Under 1 atm O_2 and at 4000 rpm, where the current density was no longer mass transport limited, the current density was 4.6 mA cm^{-2} at +380 mV vs Ag/AgCl and reached its kinetic limit of 6.25 mA cm^{-2} at 300 mV vs Ag/AgCl. The kinetic limit of the *Tt*-cathode was ~30% lower than that of the *Mv*-cathode (9.1 mA cm^{-2}).

The optimal composition of the electrocatalyst was determined for electrodes rotating at 1000 rpm and poised at +300 mV vs Ag/AgCl. In the first group of experiments, the cross-linker (PEDGE) weight percentage was fixed at 6.6 wt % and the total loading of all film components was fixed at 0.6 mg cm^{-2}. Figure 2A shows the dependence of the current density on the wt % of *Tt*-BOD through the 20 to 60 wt % range. Between 10 wt % and 45 wt % the current density increased with the wt % of *Tt*-BOD, reaching 3.5 mA/cm^2 at 44.9 wt %. Above 47 wt % *Tt*-BOD the current density declined rapidly. At 50 wt % *Tt*-BOD the adduct of the enzyme (isoelectric point pH 4.1) and the redox polymer, a polycation, precipitated. The precipitation was similar to that observed in the case of glucose oxidase and other polyanionic enzymes and is attributed to the formation of an electrostatic adduct between the polycationic polymer and the polyanionic enzyme.[27,29,55−58] Above 55 wt % BOD, where the electrostatic adduct precipitated, the separation of the voltammetric peaks increased. These results differed sharply from those for *Mv*-BOD, where precipitation was observed only above 60 wt % and where the decline in current density above 60 wt % was not as sharp. In experiments where the weight percentage of the cross-linker was varied, with the *Tt*-BOD/redox polymer weight ratio fixed at 1:1, the optimal PEGDGE wt % was 6.6. Thus, at 0.6 mg cm^{-2} total loading, the optimized catalyst was composed of 44.4 wt % *Tt*-BOD, 49 wt % redox polymer, and 6.6 wt % PEDGE. Because it depended on the composition, the total loading was not optimized.

The pH dependence of the steady state current density of O_2 electroreduction was measured with the electrode poised at +300 mV vs Ag/AgCl in 0.15 M NaCl while the electrode rotated at 1000 rpm. Phosphate, borate, citrate, or Tris was added at 20 mM concentration to maintain the desired pH. The dependence of the current density on pH is shown in Figure 2B. As seen, the current density increased with pH until it reached a plateau at pH 7.5, then declined slightly above pH 10.5. In the pH 6 to 10.5 the range the current density was nearly independent of pH, varying by less than ±10%. Up to pH 10.5 there was no irreversible change in the current characteristics; above pH 11 the drop in the current was irreversible. The window of operation of the *Tt*-BOD cathode was upshifted relative to that of *Mv*-BOD, from 5 to 10 to 6 to 10.5.

Because copper-binding anions, particularly halide anions, inhibit the laccases and because serum and other physiological

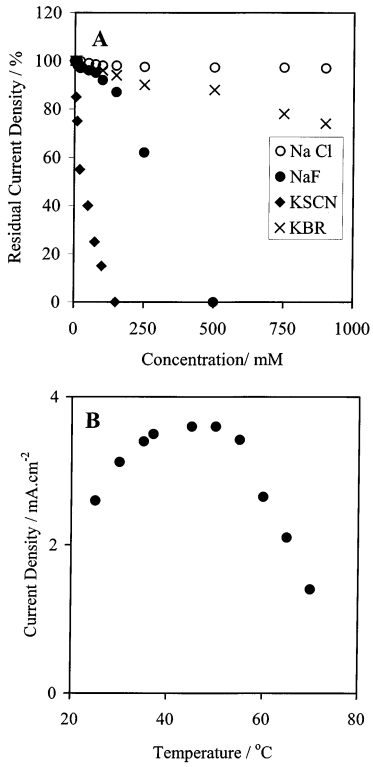

Figure 3. (A) Dependence of the current density on the concentrations of anions under 1 atm O_2. Electrode poised at +300 mV vs Ag/AgCl, 1000 rpm. Other conditions as in Figure 1A. (B) Temperature dependence of the current density: 1 atm O_2, electrode poised at +300 mV vs Ag/AgCl, 1000 rpm. Other conditions as in Figure 1A.

fluids contain 0.14 M chloride, the effect of added anions was investigated. In these experiments the electrode was rotated at 1000 rpm while it was poised at +300 mV vs Ag/AgCl in the pH 7.4, 20 mM phosphate buffer solution. The results are summarized in Figure 3A. The current density was nearly independent of the concentration of NaCl through the 0−1 M range. As found also in the case of *Mv*-BOD,[29] the current density declined above 1.5 M NaCl. When the concentration of KBr was raised from 0 to 1 M the current density declined monotonically; at 1 M KBr it was ∼20% lower than at 0 M KBr. A steeper decline was again observed at >1.5 M KBr concentration. Adding of fluoride (as NaF) inhibited the electroreduction of O_2, ∼30% of the current density being lost when the concentration of fluoride was raised from 0 to 0.25 M, and 100% being lost when the concentration was further raised to 0.5 M. Thiocyanate, added as KCNS, strongly inhibited the electroreduction, the current density dropping to nil at 0.135 M KCNS. The maximum current density was about the same when the solutions were buffered at pH 7 with Tris, acetate, or phosphate.

Figure 3B shows the temperature dependence of the current density of the electrode poised at +300 mV vs Ag/AgCl, rotating at 1000 rpm in PBS under 1 atm O_2. The current density increased with temperature up to 40 °C, then declined rapidly above 55 °C; with *Mv*-BOD the current density increased up to 60 °C, then declined rapidly. For both enzymes the increase was reversible only up to 50 °C, the enzymes being denatured at higher temperatures. For *Tt*-BOD, the Arrhenius plot (not shown) of the temperature dependence of the current yielded an activation energy E_{act} = 34.3 kJ mol^{-1}. The observed activation energy for the thermal denaturing of the enzyme was 88.2 kJ mol^{-1}. Repetitive cycling over a 4 h period at 37.5 °C in air or under 1 atm O_2, did not change the shape of the

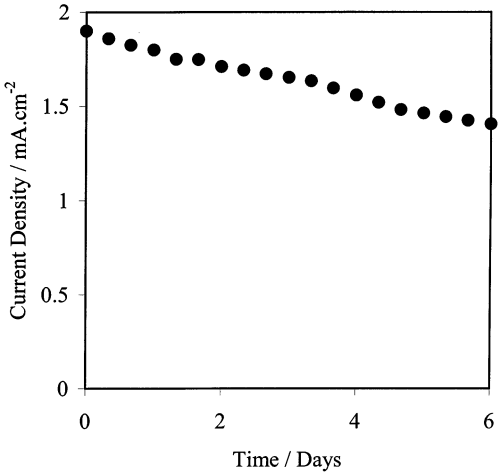

Figure 4. Stability of the wired enzyme carbon cloth electrode poised at +300 mV vs. Ag/AgCl under 1 atm O_2, 300 rpm. Other conditions as in Figure 1A.

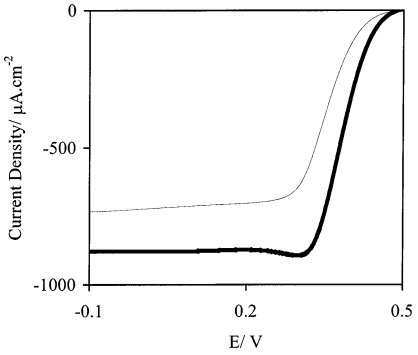

Figure 5. Polarization of 7 μm diameter, 2-cm long carbon fiber cathodes modified with wired *Mv*-BOD (fine line) and with wired *Tt*-BOD (heavy line). Quiescent solution, air, 37.5 °C, PBS buffer, 1 mV s^{-1}.

voltammograms of electrodes rotating at 1000 rpm, their peak currents decreasing only by less than 5%.

Consistent with earlier results,[29] the carbon cloth electrodes rotating at 4000 rpm (shear stress on the rim 0.7 N m^{-2}) were mechanically unstable. For this reason the extended stability tests were performed at 300 rpm, where the current was O_2 transport limited. At this angular velocity the shear stress acting on the rim of the 4 mm diameter electrode was 1.4 × 10^{-2} N m^{-2}. The time dependence of the current density of an electrode poised at +300 mV vs Ag/AgCl, rotating at 300 rpm in PBS under 1 atm O_2 at 37.5 °C, is shown in Figure 4. The current dropped by about 5% per day, the current density declining from 1.9 mA cm^{-2} to 1.4 mA cm^{-2} after six days of continuous operation. After storage of the dry electrodes for three weeks at 4 °C under air, 95 ± 3% of the initial current density was retained.

The presence of electrooxidizable serum constituents did not harm the electrodes. A transient 5% increase in the current density was observed when either 0.1 mM ascorbate or 0.1 mM ascorbate and 0.17 mM acetaminophen was added, and a transient 18% increase in current density was observed with 0.48 mM urate in the solution.

The polarization curves of miniature carbon fiber cathodes (7 μm diameter, 2 cm long) modified with wired *Mv*-BOD (light line) with wired *Tt*-BOD (heavy line) in a quiescent air solution at 37.5 °C in PBS are compared in Figure 5. On the *Mv*-BOD fiber O_2 was electroreduced at +0.25 V vs Ag/AgCl at a current

density of 0.73 mA cm^{-2}. On the *Tt*-BOD fiber O_2 was electroreduced at +0.3 V vs Ag/AgCl at 0.88 mA cm^{-2}.

Discussion

Open Circuit Potentials of the Nonwired BOD Electrodes under Argon. The open circuit potential of the electrode made by adsorbing nonwired *Tt*-BOD on vitreous carbon is, under argon, +440 mV vs Ag/AgCl, or −116 mV vs the potential of the reversible O_2/H_2O electrode, while that of *Mv*-BOD is +360 mV vs Ag/AgCl or −196 mV vs the potential of the reversible O_2/H_2O electrode. In the absence of redox polymer, the only redox center of BOD exchanging electrons with the carbon electrode is the type 1 $Cu^{+/2+}$ center. This center, unlike the cluster of type II and type III $Cu^{+/2+}$ centers, is close to the periphery of the globular proteins of the two BODs. The type I $Cu^{+/2+}$ center of *Mv*-BOD differs from that of *Tt*-BOD in the axial amino acid residue of its $Cu^{+/2+}$ center: In *Mv* the amino acid is methionine; in *Tt* it is phenylalanine.[40, 44−46] Palmer et al. found [10] that in the F463 M laccase mutant replacement of the noncomplexing axial phenylalanine by the weakly complexing methionine decreases the redox potential of the type I $Cu^{+/2+}$ center by 100 mV. Solomon et al.[10,14,59] also showed that in laccases the redox potential of the type I $Cu^{+/2+}$ center decreases by ∼100 mV when it is four-coordinated rather than three-coordinated. We attribute, therefore, the higher redox potential of *Tt*-BOD relative to that of *Mv*-BOD to the replacement of the methionine by phenylalanine in the type I $Cu^{+/2+}$ center.

Dependence of the Potential of the Type I BOD $Cu^{+/2+}$ Centers on pH in the Absence of O_2. For a typical nernstian electrode reaction in which a proton is consumed upon oxidation, the potential is upshifted at 25 °C by 59 mV when the pH is decreased by one unit. Hirose et al. measured under anaerobic conditions the redox potential of the type I $Cu^{+/2+}$ of *Tt*-BOD by titrating the centers with Li_2[Co(II)(2,6-pyridine-dicarboxylate)$_2$] at 25 °C. In pH 6.8, 50 mM phosphate buffer the potential was +400 mV vs Ag/AgCl and in pH 5.0, 0.2 M acetate buffer it was +442 mV. [40] The redox potentials of the copper proteins azurin and rusticyanin[60] also increase as the pH decreased. In contrast, the redox potential of *Mv*-BOD[29,44,45] decreases as the pH is lowered, possibly because of a structural change, leading to a change in the coordination of the $Cu^{+/2+}$.

Open Circuit Potentials of the Wired BOD Electrodes under 1 atm O_2. Because in both BODs the electroreduced O_2 is reversibly bound to the cluster of type II and type III $Cu^{+/2+}$ centers, this cluster is poised at or near the potential of the reversible O_2/H_2O electrode. At open circuit potential, where no current flows, it is not required that each of the four electrocatalytic reaction steps be fast. Specifically, it is not necessary that the redox potentials (which are the potentials where the concentrations of the oxidized and the reduced species are equal), increase in each of the steps of (a) electron transfer from the electrode to $Os^{2+/3+}$ centers of the redox polymer; (b) from the $Os^{2+/3+}$ centers to the type I $Cu^{+/2+}$ centers; (c) from the type I $Cu^{+/2+}$ centers to the type II and the type III $Cu^{+/2+}$ center-comprising clusters; and (d) from the clusters to their bound O_2 molecules. When no current flows, each of the centers can increase or decrease its potential, until the potentials are the same, by adjusting the concentration ratio of its oxidized and reduced species, $[Os^{2+}]:[Os^{3+}]$ or $[Cu^{+}]:[Cu^{2+}]$. For this reason the open circuit potentials of both the wired *Mv* and the *Tt* cathodes are, under 1 atm O_2, close to (within less than 30 mV of) the potential of the reversible O_2/H_2O electrode at pH 7.4.

Polarization. After the redox centers poise themselves near the open circuit potential by adjusting their $[Os^{2+}]:[Os^{3+}]$ or $[Cu^{+}]:[Cu^{2+}]$ ratios, the concentration of one or more of the electron donors or acceptors can be so far reduced that the reaction rate, which is the current, becomes very small. In the four-step cascade this will be avoided; the overall rate will remain high, when the $[Os^{2+}]:[Os^{3+}]$ and the $[Cu^{+}]:[Cu^{2+}]$ ratios of each of the centers are not much larger or much smaller than 1. When the ratio is not too far from 1, the potential of the redox center is, by definition, near its nernstian redox potential. Thus, the current will be high when the redox potentials of the centers are in the order O_2/H_2O > (type II and type III) $Cu^{+/2+}$ cluster > type I $Cu^{+/2+}$ center > wire $Os^{2+/3+}$ > electrode. The maximum current, which is the short circuit current, is reached when the rates of the four-electron transfer steps are equal. The equalization of the rates necessitates, however, $[Os^{2+}]:[Os^{3+}]$ and $[Cu^{+}]:[Cu^{2+}]$ ratios somewhat different than 1, because the centers differ in their self-exchange rates and because the concentration of $Os^{2+/3+}$ differs from that of the $Cu^{+/2+}$ centers. Upon adjusting the ratios, the rates of the four steps are equalized and the short circuit current reaches its maximum. Note that for ratios in the 0.1−10 range the potentials of the centers at short circuit will differ from their redox potentials by −59 to +59 mV.

As seen in Figure 5, O_2 was electroreduced on the *Mv*-BOD fiber at +0.25 V vs Ag/AgCl at a current density of 0.73 mA cm^{-2}, while on the *Tt*-BOD fiber it was electroreduced at +0.3 V vs Ag/AgCl at 0.88 mA cm^{-2}. The simultaneous gain in potential and current is attributed to the ∼100 mV higher redox potential of the type I $Cu^{+/2+}$ center of *Tt*-BOD relative to that of the *Mv*-BOD. The O_2 binding type II and type III $Cu^{+/2+}$ center-cluster is buried in the globular BOD protein and may not be directly reduced by electron transfer from an Os^{2+} complex of the redox polymer. It is the type I $Cu^{+/2+}$ center that accepts electrons from Os^{2+}, being nearer to the surface of the protein. When the rates of electron transfer between the type I center and the cluster and between the cluster and its bound O_2 are fast, then the current-controlling step is the transfer of electrons from Os^{2+} of the polymer to the type I $Cu^{+/2+}$ center. Increasing the redox potential of the type I $Cu^{+/2+}$ center increases the rate constant of electron transfer by increasing the transfer-driving potential difference. As a result, the rate of Os^{2+} → (type I Cu^{2+}) transfer can increase to match the (type I Cu^{+}) → (type II Cu^{2+}) rate at a lower $[Os^{2+}]:[Os^{3+}]$ ratio, and therefore when the redox polymer and the electrode are poised at a more oxidizing potential than in the *Mv* cathode where the redox potential of the type I center is ∼100 mV more reducing.

Composition. The key difference between the optimal *Tt* and *Mv* compositions is that the *Tt* films contain less enzyme. If the turnover rates of *Tt* and *Mv* were similar, the wt % of the 64 kDa *Tt* would be higher than the ∼50 wt %, the optimal composition of the 52 kDa *Mv* cathode. The higher wt % cannot be reached because of precipitation of the electrostatic adduct of the enzyme and the redox polymer above 55 wt % BOD. In absence of precipitation up to 60 wt % BOD, the *Mv* electrodes can have the optimal BOD wt %, which is the wt % where the rates of consumption of electrons by the enzyme and the rate of electron permeation from the electrode to the enzyme are equal. Because there is no difference between the isoelectric points (pH 4.1) of *Mv*-BOD and *Tt*-BOD, the abrupt precipitation of *Tt* at 55 wt % is attributed to electrostatic bonding of clusters of anions on the *Tt* globule and the redox polymer, leading to cross-linking. When the redox polymer is highly cross-linked, its segmental mobility, which underlies the

transport of electrons (by collisions between reduced and oxidized redox couple carrying segments) is reduced.[61] For this reason the wired *Tt*-BOD is a poorer electron conductor, even though the weight fraction of redox polymer in its adduct is higher. The poorer conductivity is evidenced by increased separation of the voltammetric peaks above 55 wt % *Tt*-BOD.

Cross-linking by PEGDGE further reduces the segmental mobility on which the electron conduction in the redox polymer film depends. Though high electron diffusivities are reached in films that are less cross-linked,[61] the poorly cross-linked films dissolve, or swell excessively, and are sheared off the rotating electrodes.[62] With 6.6 wt % PEDGE cross-linker, the films are mechanically stable at 300 rpm, where the maximum shear stress at the rims of the electrodes was 1.4×10^{-2} N m^{-2}. At this angular velocity the current density was 1.9 mA cm^{-2}.

As seen in Figure 5, the polarization and the current density of the wired *Tt*-BOD fiber-cathode are considerably improved over those of the wired *Mv*-BOD fiber cathode. In carbon cloth electrodes the 4.6 mA cm^{-2} current density at -176 mV vs the potential of the reversible O_2/H_2O electrode represents a 9-fold increase over that of the less stable and chloride-inhibited dissolved *Mv*-BOD based O_2 cathode of Tsujimura et al.[30]

Voltammetric Characteristics. When the weight fraction of *Tt*-BOD exceeded 55 wt %, the current was limited by the high electronic resistance of the film, caused by the redox polymer's electrostatic cross-linking by the polyanionic enzyme, leading to lesser mobility of the redox centers.[61] The $\Delta E_P = 25$ mV separation of the voltammetric peaks at 1 mV s^{-1} scan rate of the wired *Tt*-BOD cathode exceeded the 5 mV separation of the *Mv*-BOD cathodes.[29] The slower charge transport in the *Tt* electrode was a consequence of the reduced segmental mobility of in the *Tt* films.[61] The 113 mV width of the peaks at half-height also exceeded the 90 mV width of the *Mv* electrode,[29] also reflecting the loss of segmental mobility.[61]

Reproducibility. When the electrodes rotated at 1000 rpm, the apparent batch-to-batch reproducibility, as well as the within-the-batch reproducibility, of the O_2 electroreduction currents were better than $\pm$10%, as they were also for *Mv*-BOD.[29] This reproducibility does not necessarily imply that the composite catalysts are identical within $\pm$10%, because at 1000 rpm the current is limited by O_2 transport, not by the kinetics of the electrocatalyst. When the electrodes are rotated under O_2 at 4000 rpm, where the currents are limited by the kinetics of the electrocatalyst, their reproducibility between different batches of electrodes was $\pm$15% and within batches it was $\pm$11%. The spread was only half that between and within batches of *Mv* electrodes, where loss of the catalytic films by shearing worsened the reproducibility at high angular velocity (>1000 rpm).[29] The better reproducibility and stability (see below) are attributed to the enhanced electrostatic cross-linking of the enzyme and the redox polymer, which reduces the swelling and improves the mechanical stability of the sheared films.

Absence of Inhibition at Physiological pH And At Physiological Chloride Concentration. The dissolved *Tt*-BOD electrode retained its current through the pH 7$-$10 range,[30] the wired *Mv*-BOD electrode through the pH 5$-$10 range,[29] while the wired *Tt*-BOD electrode retained its current through the pH 6$-$10.5 range (Figure 2B). The decrease in current above pH 11 is irreversible. It is caused by the denaturation of the enzyme and is attributed in the case of *Mv*-BOD to the ionization of tyrosine residues.[63] The variation of the current is negligibly small across the pH range of human serum or blood.

Barton et al.[64] found that at pH 5 the current density of the wired *Coriolus hirsutus* laccase cathode declined by 60% when the chloride concentration was raised from nil to 0.1 M. Laccases are inhibited by halide anions,[46,65,66] including the chloride anion, because these anions bind with the type II $Cu^{+/2+}$ centers and slow the transfer of electrons from the type I $Cu^{+/2+}$ center to the type III $Cu^{+/2}$ pair.[33,40] The dissolved *Tt*-BOD cathode of Tsujimura et al.[30] is inhibited by chloride at 0.1 M concentration. The wired *Tt*-BOD cathode is the least inhibited by chloride, its current density declining only by 3% when the chloride concentration is raised from 0 to 0.1 M, while that of the wired *Mv*-BOD cathode declines by 6%. The loss remains small even at 1 M NaCl (Figure 3A). Significantly, the inhibition of the wired *Tt*-BOD cathode by chloride at its 0.096$-$0.106 M concentration in normal human blood is so small that physiological variation in chloride concentration will not affect the O_2 electroreduction current. Other copper-binding anions inhibit the electroreduction of O_2, the inhibition declining in the order $CNS^- > F^- \gg Br^- > Cl^-$. This order is only in partial agreement with the order $CNS^- \gg F^- \gg Br^- > Cl^-$ found for the wired *Mv*-BOD[29] and with the order $CNS^- > F^- \gg Cl^- > Br^-$ for the inhibition of dissolved *Tt*-BOD[40] and for laccases.[65] The order has been attributed, in the case of the dissolved enzymes, to differences in the blockage of the access to their type II and III $Cu^{+/2+}$ cluster.[40,65]

The observed partial loss of O_2 electroreduction current at very high chloride concentration (>1.5 M NaCl) is not attributed to specific binding of chloride to copper centers of the wired BOD, but to the screening of the charges of the electrostatically bound polycationic and polyanionic segments of the redox polymer$-$BOD adduct at high ionic strength, causing their dissociation and making the wiring of BOD less effective.[56,58,67]

Temperature Dependence and Stability of the Current. The O_2 electroreduction current increased with the temperature up to 60 °C when the rate of increase was about 10 °C h^{-1}, and declined rapidly when the temperature exceeded 60 °C (Figure 3B). The decline is attributed to denaturation of the enzyme. The wired *Tt*-BOD cathode was more stable than the cathode made of with the dissolved enzyme, for which an optimal temperature of 45 °C was reported. The apparent activation energy for O_2 electroreduction was $E_{act} = 34.3$ kJ mol^{-1} ($E_{act} = 28.2$ kJ mol^{-1} for *Mv*-BOD)[29] at pH 7.4 when the electrode rotated at 1000 rpm under 1 atm O_2, and the current was O_2 transport controlled. Because the current is O_2 transport limited at 1000 rpm, the 34.3 kJ mol^{-1} activation energy is that for diffusion of oxygen in the solution. Its value is consistent with the temperature dependence of the diffusivity of O_2.[68] That the thermal denaturation caused[69] 88.2 kJ mol^{-1} activation energy for current loss of the *Tt* cathode shows that the *Tt* cathode is more stable than the *Mv* cathode (77.2 kJ mol^{-1}),[29] explaining why the *Tt* cathode loses only 5% of its current per day of operation at 37.5 °C, while the *Mv* cathode loses 10%. The wired *Tt*-BOD cathode is much more stable than the cathode of Tanaka made with the dissolved enzyme, which had a half-life of 1 h in phosphate buffer at 37 °C.[33] It was also more stable than that of an immobilized BOD electrode used in the clinical monitoring of the concentration of bilirubin having a half-life of 17 h at 37 °C[70] and 8 h at 40 °C.[49]

At their physiological concentrations[71] the most readily electrooxidized constituents of blood urate, ascorbate, and acetaminophen do not damage the *Tt* cathode when poised at +300 mV Ag/AgCl in PBS at 37.5 °C. When 0.1 mM ascorbate or 0.1 mM ascorbate and 0.17 mM acetaminophen was added, the current transiently decreased by 5%, and when 0.48 mM urate was added, by 22%. While Binyamin et al.[47] showed that the electrooxidative polymerization of urate in a redox polymer

film with a higher density of cationic sites and thus a higher urate concentration in the film causes severe fouling, the present films were not fouled by urate electropolymerization.

Conclusion

"Wired" BOD films catalyze the electroreduction of O_2 to water under physiological conditions. When the O_2 electroreducing cathodes are poised at potentials that are only −136 mV reducing relative to the potential of the reversible O_2/H_2O, the current density of the wired *Tt*-BOD cathode exceeds 2 mA cm^{-2} at 37 °C. The cathode operates for 6 days, with 5% of the current being lost per day. The improvement in operating current density and potential is attributed to the higher redox potential of the type I $Cu^{+/2+}$ redox center of *Tt*-BOD and the improved stability to the enhanced electrostatic bonding of *Tt*-BOD and its polymeric "wire".

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