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Bilirubin oxidase from *Bacillus pumilus*: A promising enzyme for the elaboration of efficient cathodes in biofuel cells

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ABSTRACT

A CotA multicopper oxidase (MCO) from *Bacillus pumilus*, previously identified as a laccase, has been studied and characterized as a new bacterial bilirubin oxidase (BOD). The 59 kDa protein containing four coppers, was successfully over-expressed in *Escherichia coli* and purified to homogeneity in one step. This 509 amino-acid enzyme, having 67% and 26% sequence identity with CotA from *Bacillus subtilis* and BOD from *Myrothecium verrucaria*, respectively, shows higher turnover activity towards bilirubin compared to other bacterial MCOs. The current density for O_2 reduction, when immobilized in a redox hydrogel, is only 12% smaller than the current obtained with *Trachyderma tsunodae* BOD. Under continuous electrocatalysis, an electrode modified with the new BOD is more stable, and has a higher tolerance towards NaCl, than a *T. tsunodae* BOD modified electrode. This makes BOD from *B. pumilus* an attractive new candidate for application in biofuel cells (BFCs) and biosensors.

1. Introduction

Enzymatic miniature membrane-less glucose/O_2 biofuel cells (BFCs) are of particular interest because they may power implanted medical devices, in the near future. (Barton et al., 2004; Heller, 2004) Despite the continued improvement of the performance of enzyme-based biofuel cells, the power output and lifetime are still not sufficient for direct applications. (Osman et al., 2011) In most of the BFCs, these limitations are generally attributed to the cathode (Gallaway and Barton, 2008). At the cathode, two enzymes in particular have been used for bioelectrocatalysis: laccases and bilirubin oxidases (BOD). Both enzymes belong to the multicopper oxidase (MCO) family which utilize a minimum of four copper ions to catalyze the four-electron reduction of O_2 to water, without release of H_2O_2 (Solomon et al., 1996). The copper ions of these enzymes are classified into three types depending on their optical and magnetic properties. Type 1 (T1) copper has an intense Cys to Cu(II) charge transfer absorption band around 600 nm. This copper center accepts electrons from the electron-donating substrate, e.g. phenols or metal ions, and relays these to the O_2 reduction site. This site is a tri-nuclear cluster (TNC), consisting of an EPR active type 2 (T2) or "normal" copper ion, and a type 3 (T3) pair of copper ions, antiferromagnetically coupled via a bridging hydroxide, with a characteristic 330 nm absorption shoulder.

High potential laccases, e.g. *Trametes versicolor*, are characterized by high T1 Cu redox potentials and high catalytic activities at low pH (4–5). They have proven to be efficient electrocatalysts for the electroreduction of O_2, (Gallaway et al., 2008; Hussein et al., 2011; Mano, 2008; Rengaraj et al., 2011a,b; Rincón et al., 2011) although poor stability in the presence of chloride and/or at neutral pH, limit their utilization, especially in implantable devices, even though different strategies are developed to overcome both issues. (Beyl et al., 2011; Kavanagh et al., 2008) More recently, bilirubin oxidases have attracted attention because, unlike laccases, they show high activity at neutral pH and high tolerance towards NaCl. In addition to more traditional laccase substrates such as syringaldazine (SGZ), 2,6-dimetoxyphenol (DMP) or 2,2′-azino-di-[3-ethylbenzthiazoline-6-sulphonic acid] (ABTS), BODs also catalyze the oxidation of bilirubin to biliverdin which can be used for the diagnosis of jaundice and hyperbilirubinemia. (Doumas et al., 1999; Kirihigashi et al., 2000; Kosaka et al., 1987; Kurosakaa et al., 1998) Fungal BOD from *Myrothecium verrucaria* (Guo et al., 1991) and *Trachyderma tsunodae* (Hiromi et al., 1992) have been widely used for the elaboration of biocathodes, either immobilized in redox polymer or directly connected to electrode surfaces, mainly because of their commercial availability. (Gupta et al., 2011; Mano et al., 2003; Ramirez et al., 2008; Tsujimura et al., 2004; Wang et al., 2012; Zloczewska et al., 2011) These enzymes, however, have

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significant limitations including low turnover activity (*M. verrucaria*) and instability (*T. tsunodae*), which have motivated the search for new BODs with improved properties.

Recently, Reiss et al. identified a new CotA enzyme from *Bacillus pumilus* and classified it as a laccase (Reiss et al., 2011). In their study, however, the spectroscopic properties and bilirubin oxidation were not investigated.

We now present the spectroscopic properties of the new CotA enzyme from *B. pumilus*, which are consistent with features observed, in general, for MCOs. Also, the activity towards bilirubin, allows us to assign this enzyme as a bilirubin oxidase. Furthermore, biochemical and biophysical evaluations show that this enzyme is an excellent candidate for the elaboration of cathodes for biofuel cells operating under physiological conditions, due to their high electrocatalytic currents at neutral pH, thermostability, and tolerance towards NaCl.

2. Materials and methods

2.1. Chemicals

IPTG (Isopropyl β-D-1-thiogalactopyranoside) was purchased from Euromedex (Souffelweyersheim, France) and all other chemicals were of analytical grade or higher and purchased from Sigma (Sigma–Aldrich, Saint-Louis, MO). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). Oligonucleotides were provided by Genecust (Genecust Europe, Luxembourg). All solutions were made with deionized water passed through a AQ 10 Milli-Q purification system from Millipore (Molsheim, France). AKTA purifier 10 UV 900, HisPrep FF 16/10 column, containing Ni Sepharose 6 fast flow medium, were from GE Healthcare bio-sciences AB (Uppsala, Sweden). Amicon ultra ultrafiltration columns were from Millipore (Molsheim, France). Polymerase chain reactions were carried out in an automated thermal cycler (VWR). DNA was introduced into *Escherichia coli* by electroporation using a Eppendorf Eporator system (Eppendorf, France).

2.2. UV–vis and electron paramagnetic resonance

Spectroscopic UV–vis measurements were performed on a Cary 100 system from Varian, Inc. (Palo Alto, CA), equipped with a peltier thermostable multicell holder. X-band EPR spectra were obtained at 77 K using a Bruker EMX spectrometer equipped with an ER051QR microwave bridge and a dual mode ER4116DM cavity. Purified enzyme was added to a 4 mm Wilmad EPR tube, and frozen in liquid nitrogen. The following parameters were applied: 9.65 GHz microwave frequency, 10 mW microwave power, 10 G modulation amplitude, 100 kHz modulation frequency, 328 ms time constant, and 82 ms conversion time.

2.3. Resonance Raman

Resonance Raman spectra were obtained in a ∼135° backscattering configuration using a coherent I90C-K Kr^+ CW ion laser. The 568.2 nm laser line with an incident power of 25 mW was used as the excitation source. Scattered light was dispersed through a Spex 1877 CP triple monochromator with 1200, 1800, and 2400 grooves/mm holographic gratings and detected with an Andor Newton charge-coupled device (CCD) detector cooled to −80 °C. Samples were contained in a 4 mm NMR tube immersed in a liquid nitrogen finger dewar for measurements. The Raman energy scale was calibrated using Na_2SO_4 and citric acid. Background spectra were obtained using ground, activated charcoal at 77 K in an equivalent NMR tube. Frequencies are accurate to within 2 cm^{-1}.

2.4. Electrochemical experiments

Measurements were performed using a bipotentiostat (CH Instruments, model CHI 842B, Austin, TX, USA) with a dedicated computer. A platinum spiral wire was used as counter electrode and all potentials were referred to a Ag/AgCl (3 M NaCl) electrode (BAS, West Lafayette, IN). All electrochemical measurements were performed in a water-jacket electrochemical cell in 100 mM sodium phosphate buffer (PB) at pH 7.2 at 37 °C. The 3 mm diameter glassy carbon electrodes were rotated using a pine instruments rotator and the temperature was controlled by an isothermal circulator (Lab Companion, FR).

2.5. Preparation of the modified electrodes

Modified electrodes were prepared as previously described. (Mano et al., 2003) The deposition solution for *B. pumilus* electrodes consisted of l.26 μL of polymer (PAA-PVI-{Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl}$^{+/2+}$ at 10 mg/mL, 1 μL of Tris–H_2SO_4 50 mM buffer at pH7.5, l.24 μL of BOD from *B. pumilus* at 5 mg/mL in the same buffer, and 0.75 μL of poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) at 2 mg/mL. 1.48 μL were deposited on the 3 mm diameter glassy carbon electrodes for a total loading of 100 μg cm^{-2}. For *T. tsunodae* electrode, the proportions were 1.25 μL redox polymer, 1.2 μL water, 0.49 μL BOD at 5 mg/mL and 0.6 μL PEGDGE at 2 mg/mL, from which 1.55 μL were deposited on the electrode to obtain a total loading of 100 μg cm^{-2}. After deposition, the electrodes were cured, protected from dust, during 18 h in an oven at 25 °C.

2.6. Enzyme assay and kinetic measurements

The enzyme activity was determined spectrophotometrically at 37 °C by following the oxidation of ABTS at 420 nm ($\varepsilon_{420\,nm}$ = 36 mM^{-1} cm^{-1}) in a McIlvaine's citrate–phosphate buffer pH 3.2. Syringaldazine at 22 μM (SGZ, $\varepsilon_{530\,nm}$ = 64 mM^{-1} cm^{-1}) and 2,6-dimethoxyphenol at 1 mM (DMP, $\varepsilon_{468\,nm}$ = 14.8 mM^{-1} cm^{-1}) were used to measure protein activity at pH 6.2 and 6.8, respectively, in McIlvaine's citrate–phosphate buffers. Unconjugated bilirubin and conjugated bilirubin were used to assay the bilirubin activity at 37 °C and were measured spectrophotometrically at 450 nm and 440 nm ($\varepsilon_{450\,nm}$ = 32 mM^{-1} cm^{-1} and $\varepsilon_{440\,nm}$ = 25 mM^{-1} cm^{-1}) in sodium phosphate 50 mM pH 7.2 and in a McIlvaine's citrate–phosphate 0.1 M buffer pH 4.8, respectively. Due to low solubility at low pH, the oxidation of unconjugated bilirubin can only be measured for pH > 7. One unit was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute. K_M and k_{cat} were determined in the 2.5 μM–5 mM concentration range for ABTS, 2.5–300 μM for SGZ, 2.5 μM–4 mM for DMP and 2.5–150 μM for conjugated bilirubin by fitting the data with the simple Michealis–Menten model by non-linear regression.

McIlvaine's citrate–phosphate 0.1 M buffers at pH 3–7.5 and Tris–H_2SO_4 50 mM at pH above 7.5 were used to study the influence of pH on substrate oxidation by the enzyme. McIlvaine's citrate–phosphate 0.1 M buffer at pH 4 was used to study the influence of temperature on ABTS oxidation by the enzyme. The effect of NaCl on the enzyme activity in solution was studied at 25 °C in the presence of 0.05 mM SGZ in McIlvaine's citrate–phosphate 0.1 M buffer pH 7. The thermostability of the enzymes was investigated by incubating the proteins for different times in the absence of substrate in a McIlvaine's citrate/phosphate 0.1 M buffer pH 7 at 80 °C and/or 37 °C, followed by activity measurement, determined with 1 mM of ABTS at 37 °C in a McIlvaine's citrate/phosphate 0.1 M buffer pH 4 at 420 nm.