Supporting Information

Spectroscopic and Crystallographic Characterization of 'Alternative Resting' and 'Resting Oxidized' Enzyme Forms of Bilirubin Oxidase: Implications for Activity and Electrochemical Behavior of Multicopper Oxidases

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Experimental Procedures

Details about identification, cloning, and over-expression of the recombinant Bilirubin Oxidase (BOD) can be found elsewhere.[1]

EPR spectra were obtained with a Bruker EMX spectrometer, an ER 041 XG microwave bridge, and an ER4116DM cavity. A sample temperature of 77 K was maintained using a liquid nitrogen finger dewar. EPR settings were as follows: Freq. ≈ 9.6GHz, Power ≈ 10mW, Rec. gain = 5.02×10^3, Mod. Freq. = 100kHz, Mod. Amp. = 10.00G, Time

Constant = 327.68msec, Conversion time = 81.92msec, Sweep Time = 83.89sec. All spectra were averaged over 3scans. For spin quantitation of EPR spectra, a 1.0 mM $CuSO_4 \cdot 5H_2O$, 2 mM HCl, and 2 M $NaClO_4$ standard solution was used as reference, to determine the concentration of paramagnetic copper.[2]

UV−visible absorption (UVvis) spectra were obtained with an Agilent 8453 diode array spectrophotometer.

All spectroscopic data were obtained with BOD in 0.1M sodium phosphate buffer, pH = 6, unless otherwise stated.

Spectrographic graphite electrodes (SPGE) were from RingsdorffWerke GmbH, Bonn, Germany, (type RW001, 3.05 mm diameter and 13% porosity, http://www.sglcarbon.com). SPGE were prepared by wet polishing the end of a rod using water proof emery paper. The electrodes were then rinsed with Milli-Q water and allowed to dry in air. Subsequently 5 μL of enzyme solution (~0.2 mM) was placed on top of the polished rod and adsorption was allowed to occur. After 15 min the electrode was rinsed with Milli-Q water, inserted in a Teflon holder, and used as working electrode.

Voltammetric measurements were performed with a PINE potentiostat/galvanostat model WaveNow (Pine Research Instrumentation, 2741 Campus Walk Avenue, Durham, NC) using modified electrodes as the working electrode, a Ag|AgCl (3 M NaCl) reference electrode, and a platinum wire counter electrode. All potentials discussed in the main part are referred to the normal hydrogen electrode (NHE). The current densities were calculated with respect to the geometric electrode area.

ABTS activity of BOD, was determined by monitoring the increase in 420nm (ε = $36mM^{-1}cm^{-1}$) over time at 298K in a 1cm cuvette with stirring, in 0.1M sodium phosphate

buffer, pH=6. One unit is defined as the amount of enzyme that oxidizes 1μmol ABTS per minute.

Reduction of as-isolated BOD was performed by adding small increments of a 0.1M dithionite solution, to argon purged (~4h) enzyme, in a Vacuum Atmosphere glovebox (model: OMNILAB). Prior to reoxidation, excess dithionite was removed from the reduced enzyme, by buffer exchange into degassed sodium phosphate (0.1M) buffer, pH 6, using Amicon Ultra centrifugal filters (10K). Reoxidation was accomplished by addition of O_2-saturated sodium phosphate (0.1M) buffer, pH 6.

XAS: Cu K-edge X-ray absorption spectroscopy were measured at the Stanford Synchrotron Radiation Lightsource (SSRL) on the unfocused 20-pole, 2.0-T wiggler beam line 7-3 under storage ring parameters of 3 GeV and ~350 mA. A Rh-coated pre-monochromator, flat, bent mirror was used for harmonic rejection and vertical collimation. A Si(220) double crystal monochromator was used for energy selection. The protein samples were loaded into 2 mm Lucite XAS cells with 38 μm Kapton windows. The samples were maintained below 10 K during data collection using an Oxford Instruments CF 1208 continuous-flow liquid helium cryostat. A Canberra solid-state Ge 30-element array detector was used to collect Kα fluorescence data. Internal energy calibration was accomplished by simultaneous measurement of the absorption of a Cu-foil placed between two ionization chambers situated after the sample. The first inflection point of the foil spectrum was assigned to 8980.3 eV. The energy-calibrated data from 8675 to 9350 eV (k = 9.5 $Å^{-1}$) were processed by fitting a second-order polynomial to the pre-edge region and subtracting this from the entire spectrum as a background. A one-region spline of order 2 was used to model the smoothly decaying post-edge region

background. Normalization of the data was achieved by scaling the spline function and data such that the value of the spline equals 1.0 at 9000 eV. This background subtraction and normalization was done using PySpline.[3] For each sample, several scans from 8675 to 9350 eV were collected and normalized in PySpline. Several faster scans starting from 8970 eV with shorter energy range were also collected for the oxidized samples to reduce photoreduction in the beam. Subsequent scans collected over time on the same sample spot revealed an isosbestic point around ~8994 eV. The post-edge of the shorter scans was normalized in KaleidaGraph by maintaining the same isosbestic point as the data normalized using PySpline of the longer scans from 8675 to 9350 eV. The pre-edge of the shorter scans was processed to match the pre-edge of the longer PySpline-processed data. Data presented here are the first scans of each data set to eliminate spectral changes from photoreduction.

Crystallization: Purified BOD (concentration 18mg/ml) was initially screened against Hampton research Crystal screens I and II using the sitting-drop vapour diffusion method. The sitting drops consisted of 0.5 µl protein solution and 0.5 µl reservoir solution. They were equilibrated against 80 µl reservoir solution. Very small (70µ in the larger dimension) blue plate-shaped crystals were obtained after 2 days at room temperature using the reservoir composition 20% PEG 6K in 200 m*M* Na acetate pH 4.6. Crystallographic data were collected at 100K on Proxima I beamline at SOLEIL. Paratone-N oil was used as cryoprotectant. The data were processed using the XDS package software[4]. Data statistics are reported in table S2. The structure was solved by molecular replacement with MOLREP[5] using the PDB entry for BOD from *myrothecium verrucaria* (PDB: 3ABG) as the search model. The solution was consistent with the

expectation of one molecule in the crystal asymmetric unit. Interactive model refitting and molecular graphics analysis were performed using the coot software[6]. Buster TNT[7] from Global phasing was used to refine the model.

Figures

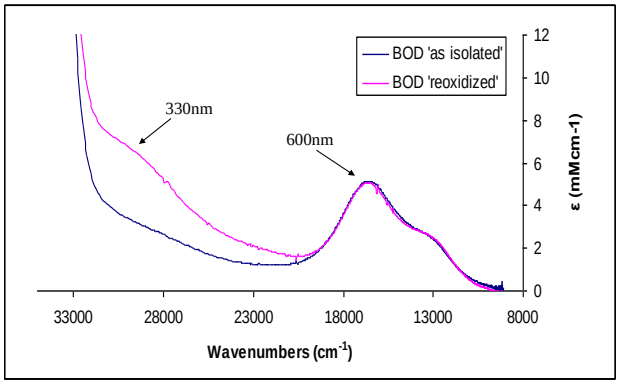

Fig. S1. UVvis absorption spectra of as-isolated (blue) and reoxidized (purple) forms of *M.oryzae* BOD.

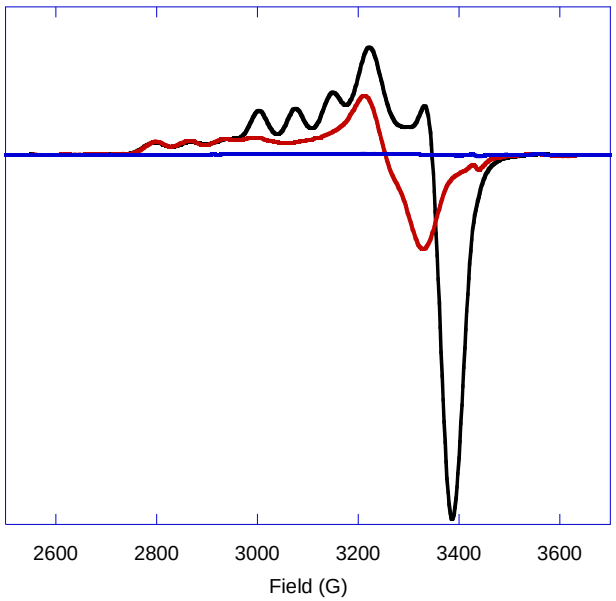

Fig. S2. EPR spectra of the as-isolated (black), partially reduced (red), and fully reduced (blue) BOD.

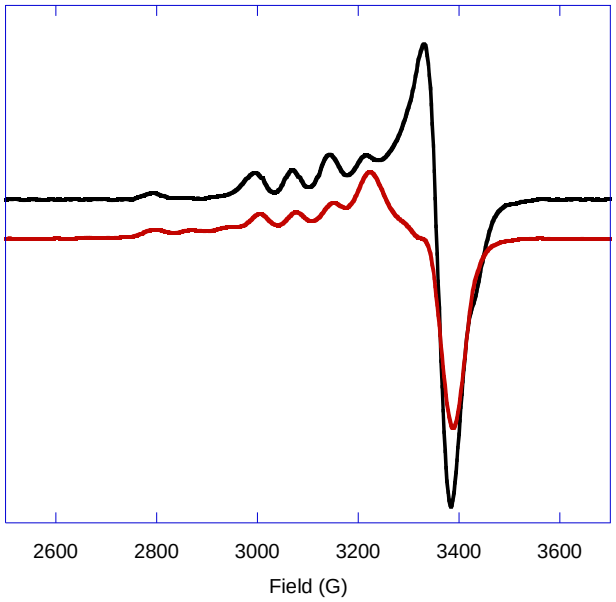

Fig. S3. EPR spectra of reoxidized (black) and reoxidized with 0.25M NaCl at 24h (red) BOD

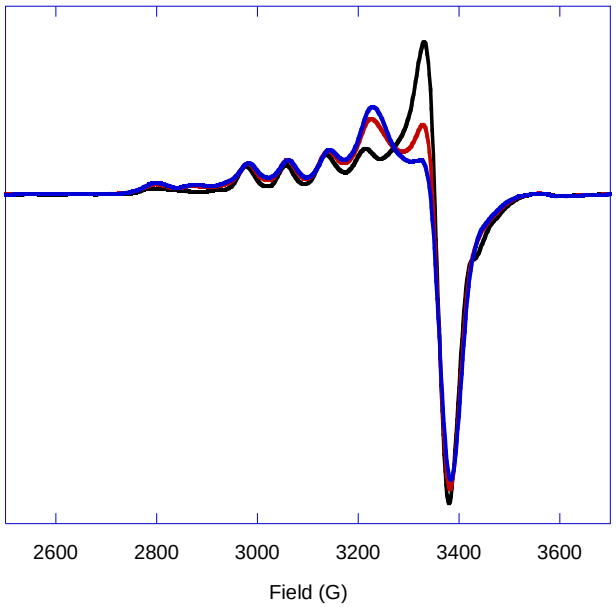

Fig. S4. EPR spectra of 'Amano 3' BOD from Amano Enzymes inc. dissolved in 0.1M NaCl containing sodium phosphate buffer (0.1M), pH=6, at 0h (black), 24h (red), and 48h (blue).

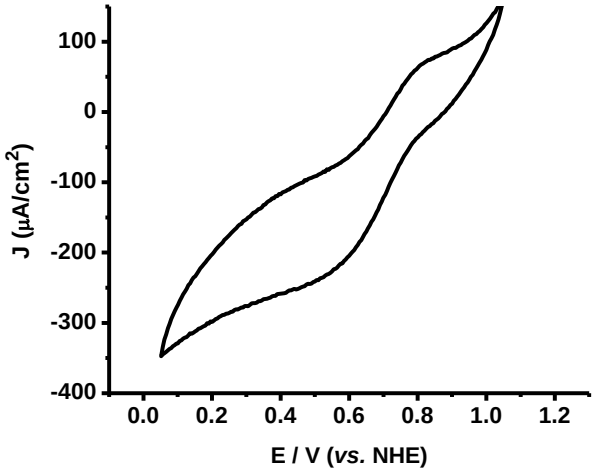

Fig. S5. First CV of RO BOD adsorbed on SPGE in air-saturated sodium phosphate buffer (0.1M), pH 6, at room temperature. Scan rate 5mVs^{-1}.

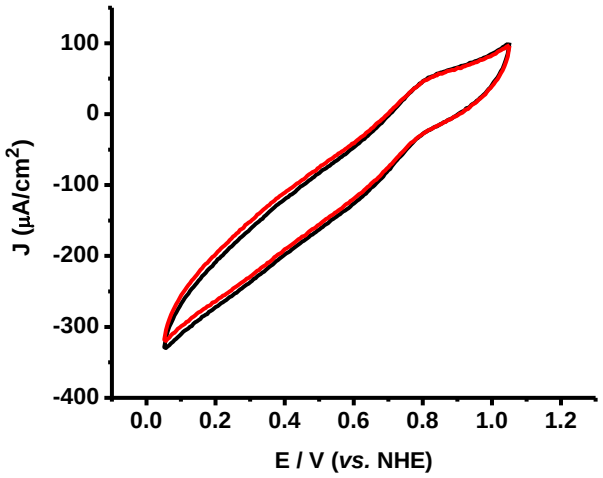

Fig. S6. CVs of RO BOD adsorbed on SPGE after 4h (black line) and after 8h (red line) in air-saturated sodium phosphate buffer (0.1M), pH 6, at room temperature. Scan rate 5 mVs^{-1}.

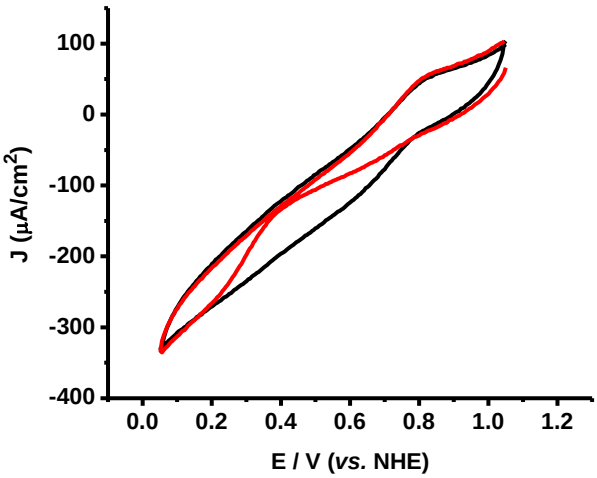

Fig. S7. CVs of RO BOD on SPGE after overnight incubation in 0.15M NaCl containing, air-saturated sodium phosphate buffer (0.1M), pH 6, at room temperature. First scan (red) and second scan (black). Scan rate 5 mVs^{-1}.

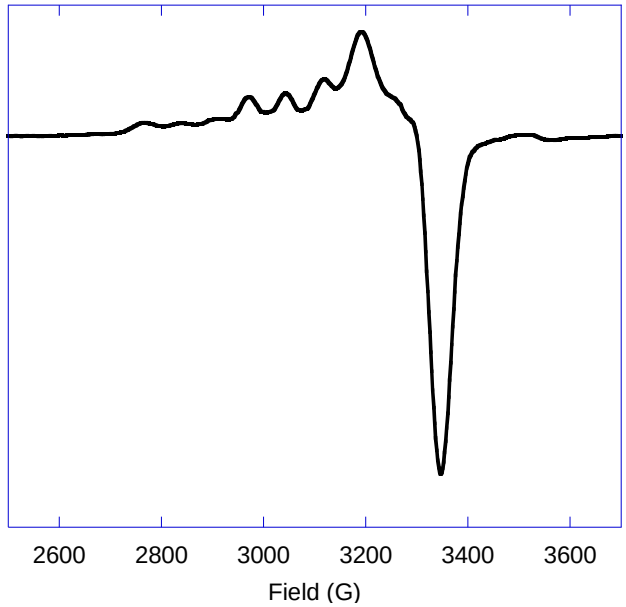

Fig. S8. EPR spectra of as-isolated *M.oryzae* BOD in Hampton Research acetate buffer, pH=4.6 at 96h

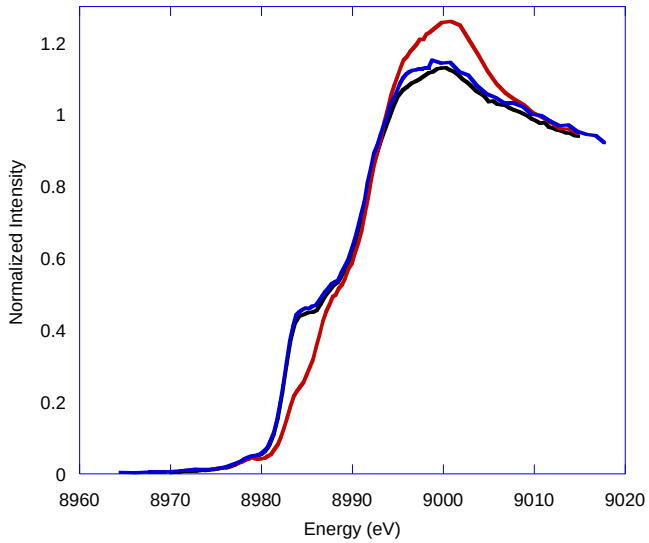

Fig. S9. Cu K-edge XAS spectra of the as-isolated AR (black), RO (red), and RO with 0.25M NaCl at 24h (blue).

Table S1. Structural parameters of TNC's in *M.oryzae* BOD and *T.versicolor* laccase

	∠T3α-O2-T3β	T3α-T3β	T3α-T2	T3β-T2	T2-O1	T2-O2	T3α-O2	T3β-O2	O2-O3
BOD	171°	5.0Å	4.1Å	4.1Å	2.8Å	3.4Å	2.6Å	2.4Å	3.2Å
TvL	133°	3.9Å	3.8Å	3.8Å	2.4Å	4.1Å	2.1Å	2.2Å	2.9Å

Table S2. Crystallographic data statistics measured on *Magnaporthe oryzae* BOD crystals

Data processing	XDS package
Beamline	Proxima I (SOLEIL)
Wavelength, Å	0.980110
Space group	C2
Cell parameters, Å	a=108.161 b=94.116 c=56.636; β= 96.62
Completeness, %	94.4 (70.1)
Redundancy	3.89 (2.42)
R_{sym}	0.1374 (0.4539)
R_{rim}	0.0748(0.2686)
I/σI	7.02(1.81)

Redundancy-independent merging *R* factor $R_{r.i.m.} = 100\sum_{hkl}[N/(N-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, with *N* being the number of times a given reflection *hkl* was observed;

References:

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