Spectroelectrochemistry of Redox Enzymes

Christenson, Andreas

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Redox potentials of the blue copper sites of bilirubin oxidases

Andreas Christenson a, Sergey Shleev a,⁎, Nicolas Mano b,c,⁎, Adam Heller b, Lo Gorton a

Abstract

The redox potentials of the multicopper redox enzyme bilirubin oxidase (BOD) from two organisms were determined by mediated and direct spectroelectrochemistry. The potential of the T1 site of BOD from the fungus Myrothecium verrucaria was close to 670 mV, whereas that from Trachyderma tsunodae was >650 mV vs. NHE. For the first time, direct electron transfer was observed between gold electrodes and BODs. The redox potentials of the T2 sites of both BODs were near 390 mV vs. NHE, consistent with previous finding for laccase and suggesting that the redox potentials of the T2 copper sites of most blue multicopper oxidases are similar, about 400 mV.

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Keywords: Bilirubin oxidase; Copper enzyme; Redox potential; T1; T2; T3 sites; Redox titration; Spectroelectrochemistry

1. Introduction

Bilirubin oxidase BOD (bilirubin:oxygen oxidoreductase, EC 1.3.3.5) is a multi-copper oxidase catalyzing the oxidation of tetrapyrroles, e.g., bilirubin to biliverdin, as well as of diphenols and aryl diamines, by molecular O2, which is reduced to water [1]. The primary structures of BODs from the fungi Myrothecium verrucaria, Pleurotus ostreatus, and Trachyderma tsunodae and from the bacterium (Bacillus subtilis) have been reported (see GenBank website). Of these, the BODs from M. verrucaria and T. tsunodae have been purified and biochemically characterized. The enzymes are glycosylated, have molecular weights of 52–64 kDa and absorb, like other multicopper oxidases, at 600 and 330 nm [2–6].

Though crystallographic data have not yet been published for the BODs, accumulated evidence shows that their catalytic centers comprise four copper ions, classified into three type of sites: type 1 (T1), type 2 (T2), and type 3 (T3) copper ions. This is also the case for laccase, ascorbate oxidase, and ceruloplasmin [1,7]. In all “blue” multicopper oxidases, including BOD, the T2 and T3 sites form trinuclear clusters, where molecular O2 is reduced to water [1].

The T1 center is the primary site for the oxidation of the electron donating substrate [1,6,8,9]. It absorbs intensely near 600 nm, the transition arising from a Cys S→Cu charge-transfer (CT), which displays a small hyperfine coupling-associated EPR signal [7,8]. The structure of the T1 site has been elucidated from spectral and biochemical data and from sequence analysis [6–12]. The ligands of the T1 copper of M. verrucaria BOD are identical with those of low redox potential multicopper oxidases [7–9,11,12], i.e., two histidines, a cysteine, and a methionine (see Table 1). The ligands of the T1 copper ions of T. tsunodae BOD are identical with those found in high redox potential laccases (e.g. Trametes versicolor and Trametes hirsuta [6–10,13–15]), i.e., two histidines, a cysteine, and a phenylalanine (see Table 1).

According to a recent proposal [16], the BODs from M. verrucaria and T. tsunodae should be classified, respectively, as low and high redox potential multicopper oxidases. As can be seen in Table 1 M. verrucaria BOD has a methionine axial ligand at the T1 site, whereas the axial ligand of the T1 site is phenylalanine in the BOD from T. tsunodae. One previous estimate of the value of the redox potential of the T1 site of M. verrucaria BOD (480 mV [17]) is consistent with the proposed
Recently, direct electron transfer (DET) between *M. verrucaria* BOD and spectrographic graphite was shown to take place under both aerobic and anaerobic conditions [20,21], and the kinetics of *M. verrucaria* BOD-catalyzed O₂ electro-reduction in direct electrical contact with carbon electrodes was studied [21,22]. The possibility of electrochemical control of the redox reactions of different multicopper oxidases at electrodes is needed both for fundamental understanding of the basis of biocatalysis and for their applications in biofuel cells. Various electrode materials, including gold, were used in biofuel cells. However, DET between BOD and gold, the focus of this report, has never been reported. High rate DET could be of relevance for electroreduction of O₂ to water near neutral pH, for which applications might exist [23–26].

### 2. Materials and methods

#### 2.1. Chemicals

Na₂HPO₄, KH₂PO₄, KCl, NaCl, and K₄[Fe(CN)₆], all of analytical grade, were obtained from Merck (Darmstadt, Germany). The buffers were prepared using water (18 MΩ) purified with a Milli-Q system (Millipore, Milford, CT, USA). K₄[Mo(CN)₈] was synthesized and purified as described previously [27].

#### 2.2. Enzymes

BOD from *Myrothecium verrucaria* was purchased from Sigma (St. Louis, MO, USA). The enzyme was additionally purified to homogeneity as described in [28] and the final specific activity was found to be 35 U per mg of protein. BOD from *Trachyderma tsunodae* (500 units/mg) was from Amano Enzyme, Inc. (Elgin, IL, USA). One unit of activity is defined as the amount of BOD oxidizing 1 μmol of bilirubin per min at pH 8.4 and 37 °C. The purified BOD preparations were homogeneous as judged from SDS-PAGE [29] and as confirmed by mass-spectrometry. They were stored at −18 °C until use. The concentration of BOD was determined spectrophotometrically at 600 nm using a ε of 4800 M⁻¹ cm⁻¹ [7].

#### 2.3. Spectroelectrochemical studies

The redox potentials of the T1 site of the two BODs were determined by mediated spectroelectrochemical redox titration, MRT, and by direct spectro-electrochemical redox titration, DRT [30–32]. The cell consisted of a 1-cm long gold capillary electrode with an I.D. of 350 μm, serving both as the working electrode and as the cuvette. The input and output optical fibers, respectively FCB-UV 400/050-2 and FC-UV 200, were purchased from Ocean Optics (Dunedin, Florida, USA) and were attached at the ends of the capillary. The system comprised a light source DH-2000, a spectrometer SD 2000 and an analogue to digital conversion board ADC-500 (Ocean Optics). The spectra were recorded with Spectra Win 4.2 software from TOP Sensor System (Erbwark, The Netherlands). The potential of the gold capillary electrode was controlled by a potentiostat (CV-50W, Bioanalytical Systems, BAS, West Lafayette, IN, USA). Two platinum wires served as counter electrodes and a home-made Ag/AgClKClsat (197 mV vs. NHE), separated from the enzyme solution by two ceramic frits and a buffer salt bridge, excluding chloride from the enzyme solution, was used as the reference electrode. The potential of the reference electrode was checked before and after each experiment versus a saturated calomel electrode (KClsat;+242 mV vs. NHE) from Radiometer (model K-401, Copenhagen, Denmark); its value remained within 1 mV before and after the experiments. The working gold capillary electrode was cleaned for approximately 10 h in freshly prepared 3:1 v/v 96% sulfuric acid, 37% H₂O₂ Piranha solution (Merck) as described in [15,31,32]. It should be noted that handling of the Piranha solution must occur under the most cautious circumstances. MRT and DRT were carried out according to a previously published protocol for laccase [15]. In the MRTs, initially 50 μM each of the reduced form of the two reduct mediators, K₄[Mo(CN)₈] and K₄[Fe(CN)₆], was used to enhance the communication between the enzymes and the electrode. It reduces the risk of hysteresis and a shift of the midpoint potential (Eₚₐ) of the titration curve, due to poor electronic contact, caused by protein insulation and slow heterogeneous electron transfer. A 50 μL aliquot of the BOD solution in 0.1 M phosphate buffer at pH 7.0 also containing the mixture of the two mediators was aspirated through the capillary to replace the buffer of the cell. Eₚₐ-values (midpoint potentials at pH 7) were determined by sequentially applying a series of potentials to the gold capillary electrode. Each potential was maintained until the Nernst equilibrium was reached (approximately 5 min) between the oxidized (Ox) and reduced (Red) forms of the mediators, the enzyme, and the poised electrode. The redox mediators were converted stepwise from one redox state to another by changing the applied potential, while the concentrations of the Ox and Red forms of the enzymes were determined from the spectra. Basic titration parameters, such as Eₚₐ, b (slope of the titration curve), n (number of electrons), and r (correlation coefficient) were determined from plots of the applied potentials (E_app) vs. log ([Ox]/[Red]).

In the case of DET the experimental procedure was identical to the one for MRT, but the titrations were performed without adding a soluble mediator. Moreover, the measurement time for each applied potential was increased from 5 to 15 min in order to assure that the enzyme redox centers and the electrode reach electrical equilibrium also while the electron path is more resistive. The entire cell and all solutions were deoxygenated by flushing with argon (AGA Gas AB, Sundbyberg, Sweden) before the DRT or MRT experiments. During the redox titrations argon was also flushed through an anaerobic box in which the spectroelectrochemical cell was placed. The K₄[Mo(CN)₈] solutions were prepared just prior to their use and protected from light to minimize photodecomposition of the oxidized Mo(V) mediator [33]. All reported potentials are referred to NHE and all redox titrations were performed in 0.1 M phosphate buffer at pH 7.0.

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2.4. Cyclic voltammetry measurements

Cyclic voltammograms (CVs) of the BODs on the capillary Au electrodes were recorded using the setup for redox titrations as previously described [15]. CVs of the mediator solutions were obtained with a planar Au electrode (BAS) in a 1 mL electrochemical cell with a Ag|AgCl|3 M NaCl reference electrode. The use of nitrogen as an atmosphere during the CVs was indispensable for obtaining the voltammograms. Both couples were recorded using the setup for redox titrations as described previously [15].

3. Results

3.1. Mediated redox titration

The $E_{m7}$-values of the redox couples K$_3$[Mo(CN)$_8$]/K$_4$[Mo(CN)$_8$] and K$_3$Fe[(CN)$_6$)/K$_4$Fe(CN)$_6$ were first determined using the spectroelectrochemical cell, then confirmed by cyclic voltammetry at a planar Au electrode. Their values were recorded using the setup for redox titrations as described previously [15].

Next, the $E_{m7}$-values of the T1 sites of M. verrucaria and T. tsunodae BODs were accurately measured by MRT. Each titration was carried out in both directions, i.e., from the fully oxidized to the fully reduced state of the enzyme (reductive titration) and vice versa (oxidative titration). Typical titration curves of M. verrucaria and T. tsunodae BODs are presented in Figs. 1A and 2A respectively.

The $E_{m7}$-values of the redox titrations of BODs from Trachyderma tsunodae and Myrothecium verrucaria are presented in Table 2. The values agree with those previously reported [33-36] and Table 2). Both couples of the mediators absorb strongly below 500 nm but are transparent above 500 nm.

Notes: “—” — information not available; in brackets recalculated values based on previously published data are shown; all redox potentials are given vs. NHE.

Table 2

<table>
<thead>
<tr>
<th>BOD</th>
<th>$E_{m7}$ (mV)</th>
<th>$b$ (mV)</th>
<th>$n$</th>
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<tbody>
<tr>
<td>Trachyderma tsunodae</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>660 (pH 7.0)</td>
<td>90</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>615 (pH 6.8)</td>
<td>76</td>
<td>1.5 (0.78)</td>
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<tr>
<td>642 (pH 5.0)</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>710 (pH 7.0)</td>
<td>–</td>
<td>3 (0.71)</td>
<td></td>
</tr>
<tr>
<td>Myrothecium verrucaria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>670 (pH 7.0)</td>
<td>74</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>490 (pH 5.3)</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>570 (pH 7.8)</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>660 (pH 7.0)</td>
<td>83</td>
<td>3 (0.71)</td>
<td></td>
</tr>
</tbody>
</table>

References:
1. S. Stuers, Cyclic voltammetry at a planar Au electrode. Their values were recorded using the setup for redox titrations as described previously [15].
2. D. C. H. Stowe, The redox properties of the blue copper centers of the fungal BODs. Their values were recorded using the setup for redox titrations as described previously [15].
3. D. C. H. Stowe, The redox properties of the blue copper centers of the fungal BODs. Their values were recorded using the setup for redox titrations as described previously [15].
points of the titrations did not coincide with each other (Figs. 1A and 2A). Moreover, a well-pronounced sigmoidal Nernst plot of the titration in the case of *T. tsunodae* could be clearly seen (Fig. 2B, insert). Similar, but a less pronounced behavior was observed in the titration curve of the *M. verrucaria* BOD.

### 3.2. CV measurements

The possibility for DET between *M. verrucaria* and *T. tsunodae* BODs and gold under aerobic and anaerobic conditions was investigated using the bare capillary gold electrode. Cyclic voltammograms recorded at sweep rates varying from 1 to 1000 mV s⁻¹ did not reveal any clear redox transformation of either enzyme in the potential range between −500 mV and +1000 mV vs. NHE. Changing the pH from 3 to 9 did not lead to the appearance of a clearly traceable faradaic current in the voltammograms. Nevertheless, DET between the bare gold electrode and the copper sites of either of the BODs was confirmed by the much slower, only very low current, spectroelectrochemical measurements (*vide infra*).

### 3.3. Direct (mediatorless) redox titrations

Spectroelectrochemical data for solutions containing BOD without mediators and under anaerobic conditions in the Au capillary clearly show that the blue color vanishes when the applied potential is switched from +1000 mV to +50 mV vs. NHE. The fading of the colour can only be explained by the direct reduction of the blue copper sites at the gold capillary electrode. Typical absorbance spectra of the oxidized, partly reduced, and fully reduced forms of *M. verrucaria* and *T. tsunodae* BODs in the absence of any mediators are presented in Figs. 3B and 4B, respectively. The redox reactions were reversible and both BODs were reoxidized either by applying an oxidizing potential (Figs. 3 and 4) or by O₂ (data not shown). Moreover, the first and the last points of the titrations perfectly coincided with each other (cf. curves 1 and 2 in Figs. 3A and 4A). In contrast, as mentioned above, a significant decrease in the absorbance of the enzymes after titration of both BODs was observed in the MRT experiments (*vide supra*).

![Fig. 1. MRT of Myrothecium verrucaria BOD in 0.1 M phosphate buffer, pH 7.0.](image1)

**Fig. 1.** MRT of *Myrothecium verrucaria* BOD in 0.1 M phosphate buffer, pH 7.0. (A) Potentiometric titration curves (curve 1—oxidative titration, curve 2—reductive titration). (B) Spectra from the titrations, corresponding to oxidized BOD (800 mV), partly reduced BOD (675 mV), and fully reduced BOD (475 mV). Insert: a typical Nernst plot of the dependence of the applied potential (E) versus the absorbance at 600 nm and averaged parameters calculated from the titrations.

![Fig. 2. MRT of Trachyderma tsunodae BOD in 0.1 M phosphate buffer, pH 7.0.](image2)

**Fig. 2.** MRT of *Trachyderma tsunodae* BOD in 0.1 M phosphate buffer, pH 7.0. (A) Potentiometric titration curves (curve 1—oxidative titration, curve 2—reductive titration). (B) Exemplary spectra from the titration corresponding to the oxidized BOD (875 mV), partly reduced BOD (650 mV), and fully reduced BOD (500 mV). Insert: Typical Nernst plot of the applied potential (E) dependence of the absorbance at 600 nm and averaged parameters calculated from the titrations.
The spectroelectrochemical data provide evidence of direct, unmediated heterogeneous ET between both of the BODs and gold. However, the mechanism of this process seems to be more complex than the mechanism of the mediated process. The complexity is reflected in the spectroelectrochemical titration curves (Figs. 3A and 4A). The reductive and oxidative titration curves do not overlap (cf. curves 1 and 2 in Figs. 3A and 4A) and only a single well-pronounced ET process with a low $E_{m}^{\prime}$ value ($320 \text{ mV}$) was seen when titrating $T. \text{tsunodae}$ BOD (Fig. 4A, curve 1), whereas two ET processes, one in the low and one in the high potential range ($460 \text{ mV}$ and $805 \text{ mV}$, respectively) were seen in the oxidative titration of $M. \text{verrucaria}$ BOD (Fig. 3A). Additionally, the reductive titration curve of $M. \text{verrucaria}$ BOD was similar to the oxidative titration curve of $T. \text{tsunodae}$ BOD (cf. Figs. 3A and 4A).

4. Discussion

The data reveal without any doubt that DET between a gold electrode and both BODs can be established. It is, however, evident from the absence of pronounced redox peaks in the CVs that the rate of DET is very low.

Even though redox potentiometry is now a routine technique widely used in studies of biological ET processes [37], large discrepancies were reported between the $E_{m}$-values for identical proteins. The $\sim 180 \text{ mV}$ difference between the reported $E_{m}$-values of the T1 sites of the same BOD (Table 2) is an example of such a discrepancy. According to the Nernst equation, $b$ values higher than $59 \text{ mV}$ ($25^\circ \text{C}$) would imply the physically impossible transfer of a fraction of the charge of the electron [37]. The reported slope of $83 \text{ mV}$ in the titration curve of $M. \text{verrucaria}$ BOD corresponds to an $n$ value of 0.71, and the $76 \text{ mV}$ slope in the titration curve of $T. \text{tsunodae}$ BOD implies an $n$ value of 0.78 (Table 2). Obviously, these $n$ values, deviating from the expected integers by more that 10%, should be candidates for re-evaluation [38]. Two frequent causes of erroneously low $n$ values (less than 1.0) are incomplete equilibration in the redox titration and/or the presence of multiple potential-wise closely spaced redox couples. The low reported values may well have resulted from the latter (Table 2). As early as in 1970 Wilson and Dutton [37,38] have

![Fig. 3. DRT of Myrothecium verrucaria BOD in 0.1 M phosphate buffer, pH 7.0. (A) Potentiometric titration curves (curve 1—oxidative titration, curve 2—reductive titration) with two midpoint potentials ($E_{m}^{\prime}$) of pronounced ET processes during the reductive titration. (B) Some spectra from the titration corresponding to the oxidized BOD (980 mV), partly reduced BOD (550 mV), and fully reduced BOD (100 mV).](image)

![Fig. 4. DRT of Trachyderma tsunodae BOD in 0.1 M phosphate buffer, pH 7.0. (A) Potentiometric titration curves (curve 1—oxidative titration, curve 2—reductive titration) with a midpoint potential ($E_{m}^{\prime}$) of pronounced ET processes. (B) Exemplary spectra from the titration corresponding to the oxidized BOD (850 mV), partly reduced BOD (350 mV), and fully reduced BOD (50 mV).](image)
pointed out that two independent, closely spaced one-electron redox pairs can yield a sigmoidal Nernst plot with apparent $n$ values between 0.5 and 1.0. As seen in Figs. 1B and 2B, this could be the case for the MRTs of M. verrucaria and T. tsunodae BOD. As reported by Wilson and Dutton [39,40], the presence of two chemically different cytochrome $b$ constituents results in a sigmoidal Nernst plot yielding an apparent $n$ value close to 0.5. In the present set of experiments the homogeneity of both BODs was confirmed by SDS-PAGE and by mass-spectrometry. Nevertheless cyano-copper “multiforms” of the enzyme can be formed in the presence of cyanide-containing mediators [41], the cyanide changing particularly the coordination of the T2/T3 Cu centers [15]. While this could explain the small difference between the first and the last points of the titration curves observed in the MRT of either BOD, it does not explain the still unacceptable $n$ values obtained from MRTs of T. tsunodae BOD performed using non-cyanide redox couples (Table 2). Furthermore, MRTs with cyano-metal complexes yielded excellent values for laccase and ceruloplasmin [20,35,42,43]. Thus, it is unlikely that the use of cyanide-based mediators is responsible for the scatter in the MRT results of the BODs.

With the results shown above one suggestion could be the T. tsunodae BOD has two different T1 sites. Human ceruloplasmin has three different T1 sites, T1A, T1B, and T1 PR with redox potentials of $-490 \text{ mV}$, $580 \text{ mV}$, and $-1000 \text{ mV}$ [16]. As shown by Deninum and Vännärd [43], MRT of the T1A and T1B sites of human ceruloplasmin resulted in a well-pronounced sigmoidal Nernst plot. Moreover, their titration curve presented in [43] is very similar to the one we obtain for T. tsunodae MRT (Fig. 2) and the parameters of both redox titrations are also close ($n \approx 0.65$, $b \approx 91 \text{ mV}$). This is, however, unlikely to be the case for M. verrucaria BOD, where all previously published primary isoenzyme structures contain only one cysteine residue, a mandatory amino acid for coordination of the T1 copper (GeneBank accession numbers BAA02123, BAA03166, B48521, and Q12737). Additional computer analysis of the primary structure of T. tsunodae BOD shows only one typical T1 site subsequence ($CHX$; where X is an axial ligand of the T1 site, i.e. F, L, or M) in analogy with all others blue multicopper oxidases (Table 1). Nevertheless, a hypothetical possibility of the presence of a second “abnormal” T1 site in T. tsunodae BOD cannot be ruled out because the enzyme contains five cysteine residues (GenBank Accession number BAA28668).

Though that the T2 copper cannot be detected spectrophotometrically and that the bi-nuclear T3 copper only displays a spectral absorbance shoulder near $330 \text{ nm}$, we observe for both BODs changes between 450 and $800 \text{ nm}$ (Figs. 3B and 4B). The broad band does not depend on the source of the enzyme and accounts for about 1/5th of the absorbance. Recently, a red-absorbing chromophore, considered to be one of the copper sites of the T2/T3 cluster, probably the T2 site, was reported for T. tsunodae BOD, where all previously published primary isoenzyme structures contain only one cysteine residue, a mandatory amino acid for coordination of the T1 copper (GeneBank accession numbers BAA02123, BAA03166, B48521, and Q12737). Additional computer analysis of the primary structure of T. tsunodae BOD shows only one typical T1 site subsequence ($CHX$; where X is an axial ligand of the T1 site, i.e. F, L, or M) in analogy with all others blue multicopper oxidases (Table 1). Nevertheless, a hypothetical possibility of the presence of a second “abnormal” T1 site in T. tsunodae BOD cannot be ruled out because the enzyme contains five cysteine residues (GenBank Accession number BAA28668).

The midpoint potential of the T1 site of M. verrucaria BOD is close to $670 \text{ mV}$ vs. NHE, whereas a much broader potential range, between 650 and $750 \text{ mV}$, is estimated for the $E_m$-value of the T1 site of T. tsunodae BOD. A long-range, but a very slow electron transfer between gold and either of the BODs was observed. The earlier suggestion that the redox potentials of the T2 copper sites in all blue multicopper oxidases are similar, i.e. approximately $400 \text{ mV}$, holds for the BODs.

### 5. Conclusions

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### Acknowledgment

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### References


