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Redox potentials of the blue copper sites of bilirubin oxidases

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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 O_2, 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 O_2 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

Abbreviations: BOD, bilirubin oxidase; DET, direct electron transfer; ET, electron transfer; DRT, direct redox titration; MRT, mediated redox titration; E_m, midpoint redox potential; E_{m7}, midpoint potential at pH 7.0

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Table 1
Comparison of the midpoint redox potentials, E_m, and the amino acid subsequences for low and high redox potential multicopper oxidases

Multicopper Oxidase		Subsequence	E_m, T1 site (mV)
High redox potential	*Trachyderma tsunodae* BOD	… ^{474}H C H I D F H L **E** A G F^{485}… GenBank Accession number BAA28668	≥650*(pH 7.0)
	Trametes hirsuta laccase	… ^{474}H C H I D F H L **E** A G F^{485}… GenBank Accession number Q02497	780 (pH 6.0) [16,20]
Low redox potential	*Myrothecium verrucaria* BOD	… ^{494}H C H N L I H A D H D M^{505} … GenBank Accession numbers BAA02123, BAA03166, B48521, Q12737	≈ 670* (pH 7.0)
	Rhus vernicifera laccase	… ^{492}H C H F E R H T T E G M^{503} … GenBank Accession number BAB63411	430 (pH 7.5) [41,42]
	Zucchini ascorbate oxidase	… ^{506}H C H I E P H L H M G M^{517} … GenBank Accession number A51027	340 (pH 7.0) [51]
	Human ceruloplasmin (T1A or T1B site)	… ^{506}H C H V T D H I H A G M^{517} … GenBank Accession number NP_000087	490 (pH 5.5) [43]

Notes. "*"—present study, underlined—ligand to the T1 copper; all redox potentials are given vs. NHE.

classification [16], but two other studies reported more positive redox potentials for this site (see Table 2) [7,18]. The latter are close to, or even higher than, the redox potential of the T1 site of *T. tsunodae* BOD (Table 2) [7,18,19]. This distinction was not sufficiently considered in recent studies of the redox states of the T1 site in BODs [7,8,11]. One of our objectives was, therefore, to carefully determine the redox potentials of the T1 sites of both BODs and to reconcile the differences in the reported values.

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 O_2 electroreduction 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 O_2 to water near neutral pH, for which applications might exist [23–26].

2. Materials and methods

2.1. Chemicals

Na_2HPO_4, KH_2PO_4, KCl, NaCl, and $K_4[Fe(CN)_6]$, 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). $K_4[Mo(CN)_8]$ 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 at 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 an 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 spectroelectrochemical 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 (Eerbreek, 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 $Ag|AgCl|KCl_{sat}$ (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 ($Hg|Hg_2Cl_2|KCl_{sat}$;+242 mV vs. NHE) from Radiometer (model K401, 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% H_2O_2 Piranha solution (Merck) as described in [15,31,32]. It should be noted that handling of the Piranha solution must occur under the most cautions 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 redox mediators, $K_4[Mo(CN)_8]$ and $K_4[Fe(CN)_6]$, 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 (E_m) 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. E_{m7}-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 E_{m7}, *b* (slope of the titration curve), *n* (number of electrons), and *r* (correlation coefficient) were determined from plots of the applied potentials (E_{appl}) 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 $K_4[Mo(CN)_8]$ 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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Table 2
Comparison of reported parameters of redox titrations of BODs from *Trachyderma tsunodae* and *Myrothecium verrucaria*

BOD	E'_m, T1 site (mV)	*b* (mV)	*n*	Mediator system(s); E_m (mV)	Reference
Trachyderma tsunodae	660 (pH 7.0)	90	0.66	$K_3[Mo(CN)_8]/K_4[Mo(CN)_8]$; 780 mV	Present study
				$K_3Fe[(CN)_6)]/K_4[Fe(CN)_6]$; 435 mV	
	615 (pH 6.8)	76	1.5 (0.78)	$Co(III)(2,6\text{-}PA)_2/CoII(2,6\text{-}PA)_2$; 747 mV	[6]
	642 (pH 5.0)	–	–	$Co(III)(2,6\text{-}PA)_2/CoII(2,6\text{-}PA)_2$; 747 mV	[6]
	710 (pH 7.0)	–	3 (0.71)	$K_3[Mo(CN)_8]/K_4[Mo(CN)_8]$; 780 mV	[18]
				$K_3Os[(CN)_6)]/K_4[Os(CN)_6]$; 640 mV	
				$K_3[W(CN)_8]/K_4[W(CN)_8]$; 520 mV	
Myrothecium verrucaria	670 (pH 7.0)	74	0.80	$K_3[Mo(CN)_8]/K_4[Mo(CN)_8]$; 780 mV	Present study
				$K_3Fe[(CN)_6)]/K_4[Fe(CN)_6]$; 435 mV	
	490 (pH 5.3)	–	–	NaI_3/NaI; 536mV or $K_3Fe[(CN)_6)]/K_4[Fe(CN)_6]$; 433 mV	[17]
	570 (pH 7.8)	–	–	$K_3Fe[(CN)_6)]/K_4[Fe(CN)_6]$; 435 mV	[7]
	660 (pH 7.0)	83	3 (0.71)	$K_3[Mo(CN)_8]/K_4[Mo(CN)_8]$; 780 mV	[18]
				$K_3Os[(CN)_6)]/K_4[Os(CN)_6]$; 640 mV	
				$K_3[W(CN)_8]/K_4[W(CN)_8]$; 520 mV	

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

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 described previously [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 (BAS) and a Pt counter electrode. The Au electrode was polished with a DP suspension (0.25 μm; Stuers, Copenhagen, Denmark), followed by an alumina FF slurry (0.1 μm; Stuers), rinsed with Millipore water, and sonicated between and after polishing for 10 min. Then the electrode was kept in concentrated H_2SO_4 with 10% H_2O_2 for 1 h, subjected to 30 cycles in 0.5 M H_2SO_4 and rinsed with Millipore water before use.

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 $K_3Fe[(CN)_6)]/K_4[Fe(CN)_6]$ were first determined using the spectroelectrochemical cell, then confirmed by cyclic voltammetry at a planar Au electrode. Their values were respectively 780 mV and 435 mV vs. NHE (0.1 M phosphate buffer, pH 7.0, 25 °C) for the $K_3[Mo(CN)_8]/K_4[Mo(CN)_8]$ and for the $K_3[Fe(CN)_6)]/K_4[Fe(CN)_6]$ couples and did not depend on the direction of the scan (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 they are transparent above 500 nm.

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 spectra of the BODs were recorded at redox equilibrium and spectra of the oxidized, partly reduced, and fully reduced *M. verrucaria* and *T. tsunodae* BODs are presented in Figs. 1B and 2B, respectively. Equilibration of the blue copper center at each applied potential was apparent from the stabilization of the absorbance at 600 nm. Because the two redox mediators are transparent above 500 nm, the spectral changes at 600 nm were attributed to the blue copper centers of the fungal BODs.

Least-squares linear regression analysis of the 600-nm Nernst plots provided an E_{m7}-value of 670 mV vs. NHE and a slope of 74 mV for *M. verrucaria* BOD (Fig. 1B, insert; Table 2). Similar calculations for *T. tsunodae* BOD resulted in an E_{m7}-value of 660 mV vs. NHE and a slope of 90 mV (Fig. 2B, insert; Table 2). The linear correlation coefficients were higher, in both instances, than 0.99. In addition to the least-squares regression analyses, the titration curves were analyzed by direct data fitting [30]. The results are presented in Figs. 1A and 2A for each titration curve.

The differences between the reductive and oxidative titration curves were very small, less than 12 mV for both BODs (Figs. 1A and 2A). The enzymes could be reversibly cycled between their fully oxidized and fully reduced states and the E_{m7}-values calculated for the two BODs were practically independent of the direction of the potential scans. However, significant fading of the blue color of the enzymes (up to 10%) after even a single titration cycle (oxidized BOD → reduced BOD → oxidized BOD) was observed for both BODs , i.e., the first and the last

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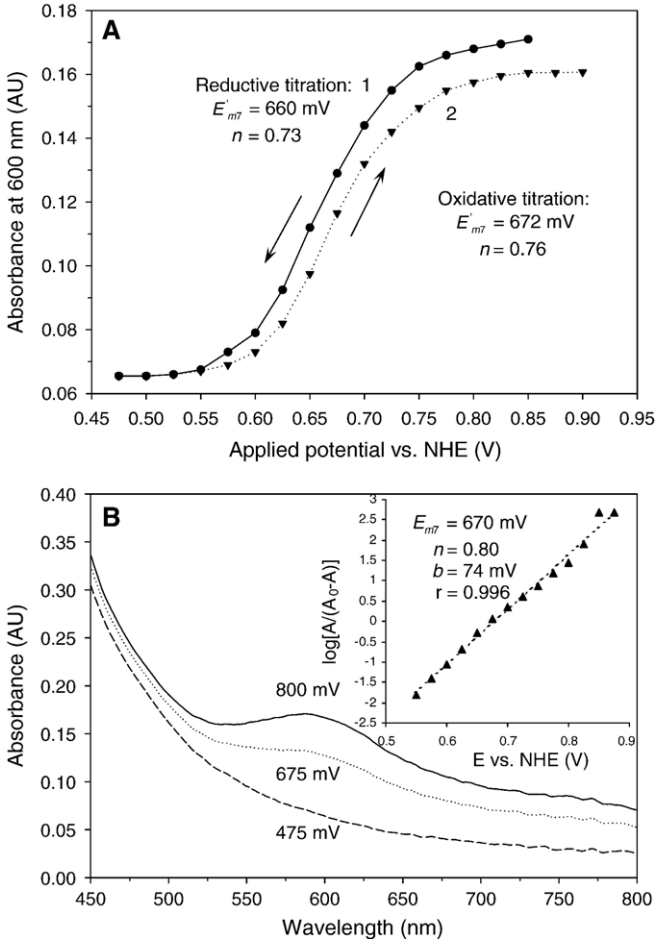

Fig. 1. MRT of *Myrothecium verrucaria* BOD in 0.1 M phosphate buffer, pH 7.0. (A) Potentiometric titration curves (curve 1—oxidative titration, curve 2—reductive titration). (B) Spectra from the titrations, corresponding to oxidized BOD (800 mV), partly reduced BOD (675 mV), and fully reduced BOD (475 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.

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 O_2 (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*).

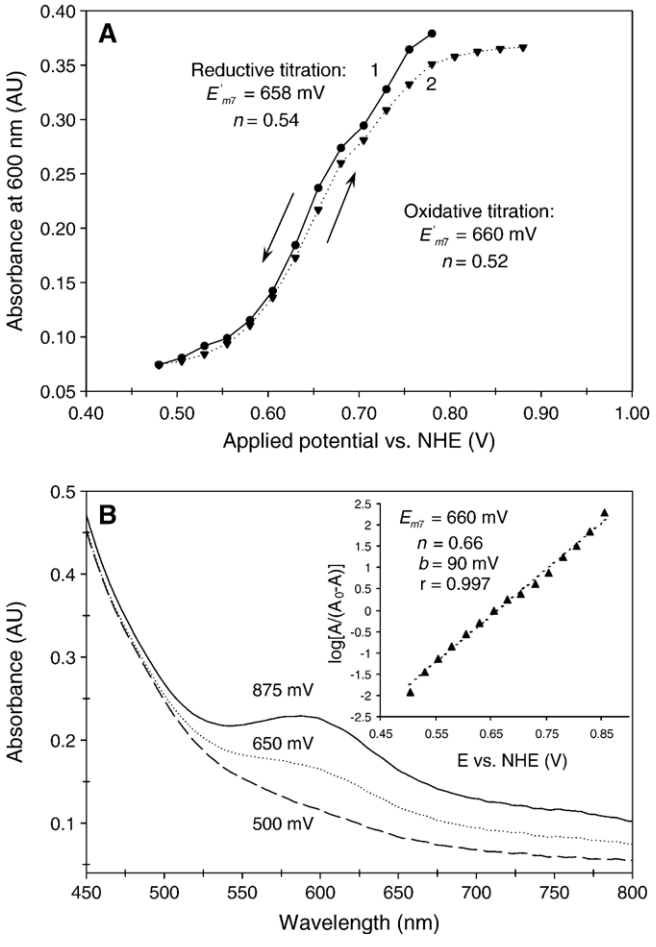

Fig. 2. MRT of *Trachyderma tsunodae* BOD in 0.1 M phosphate buffer, pH 7.0. (A) Potentiometric titration curves (curve 1—oxidative titration, curve 2—reductive titration). (B) Exemplary spectra from the titration corresponding to the oxidized BOD (875 mV), partly reduced BOD (650 mV), and fully reduced 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.

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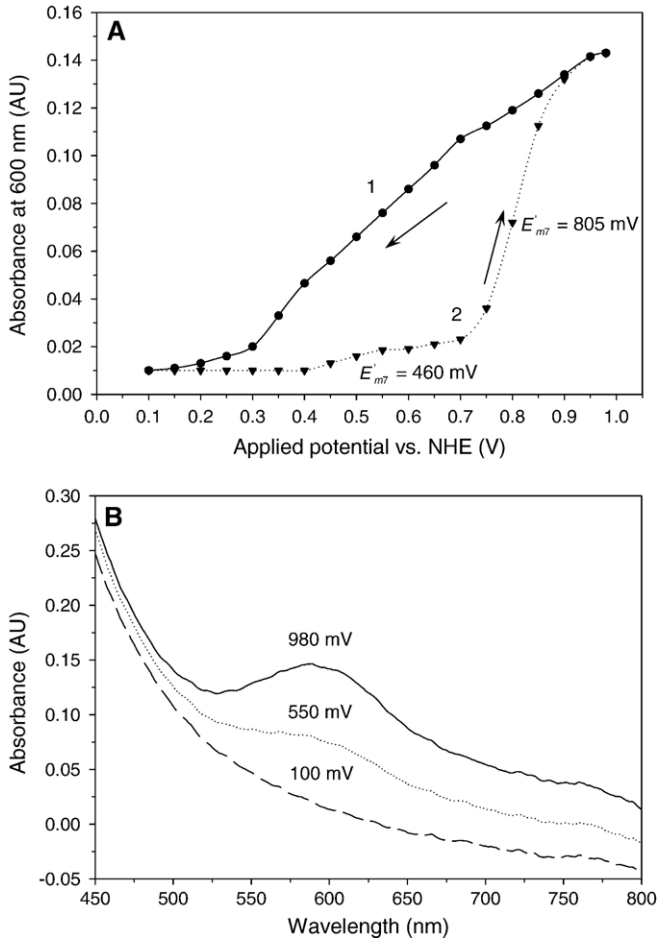

Fig. 3. DRT of *Myrothecium verrucaria* BOD in 0.1 M phosphate buffer, pH 7.0. (A) Potentiometric titration curves (curve 1—oxidative titration, curve 2—reductive titration) with two midpoint potentials (E'_m) of pronounced ET processes during the reductive titration. (B) Some spectra from the titration corresponding to the oxidized BOD (980 mV), partly reduced BOD (550 mV), and fully reduced BOD (100 mV).

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-value (320 mV) was seen when titrating *T. tsunodae* BOD (Fig. 4A, curve 1), whereas two ET processes, one in the low and one in the high potential range (460 mV and 805 mV, respectively) were seen in the oxidative titration of *M. verrucaria* BOD (Fig. 3A). Additionally, the reductive titration curve of *M. verrucaria* BOD was similar to the oxidative titration curve of *T. 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 ~180 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 mV (25 °C) would imply the physically impossible transfer of a fraction of the charge of the electron [37]. The reported slope of 83 mV in the titration curve of *M. verrucaria* BOD corresponds to an *n* value of 0.71, and the 76 mV slope in the titration curve of *T. 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

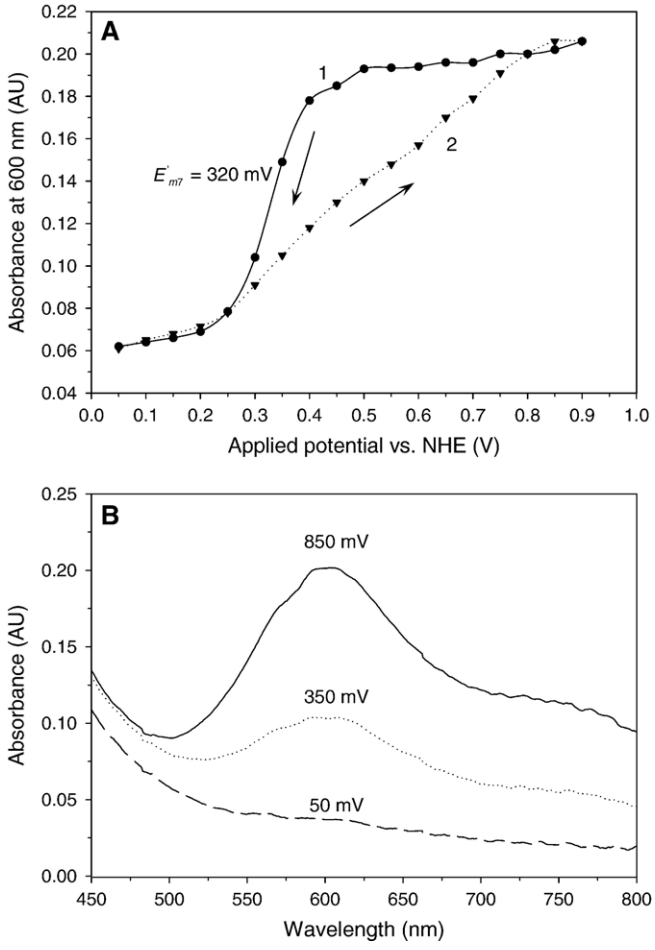

Fig. 4. DRT of *Trachyderma tsunodae* BOD in 0.1 M phosphate buffer, pH 7.0. (A) Potentiometric titration curves (curve 1—oxidative titration, curve 2—reductive titration) with a midpoint potential (E'_m) of pronounced ET processes. (B) Exemplary spectra from the titration corresponding to the oxidized BOD (850 mV), partly reduced BOD (350 mV), and fully reduced BOD (50 mV).

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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 mV, 580 mV, and ~1000 mV [16]. As shown by Deninum and Vänngård [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 ($n \approx 0.65$, $b \approx 91$ 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 (*C H X*; 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 nm, we observe for both BODs changes between 450 and 800 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 coppers of the T2/T3 cluster, probably the T2 site, was reported for *Trametes hirsuta* laccase [15]. Thus, any of several of the chromophores might be responsible for the blue color of the enzyme, and following conclusions can be drawn: Because the extinction coefficients of the individual copper sites are unknown, the redox potentials of the T1 centers of *T. tsunodae* and *M. verrucaria* BODs cannot be spectrophotometrically resolved. For this reason, only the lowest value for the T1 site of *T. tsunodae* BOD is presented (Table 1). Its estimate is based on interpretation of the titration curves of Fig. 2A. Nevertheless, for *M. verrucaria* BOD, the parameters of the redox titration are those expected for a Nernstian couple and the data agree well with results of Ikeda et al. [18]. Thus, we can consider the new values presented in Tables 1 and 2 to be the best values of the redox potential of the T1 site estimates to date.

Recent reviews link the observed high redox potentials of the T1 site, which we also observe, to the methionine-binding of their copper [16,44–46]. The *M. verrucaria* BOD is an interesting exception to this "rule".

As seen from Figs. 3A and 4A, a pronounced low redox potential ET process is observed during DRTs of both BODs and the middle E'_{m7}-value between the two ET processes is at 390 mV. We proposed, in view of earlier findings [15] that underlying this value is the redox potential of all T2 copper sites of all multicopper oxidases, near 400 mV vs. NHE. The similarity of the titration curves of Figs. 3A and 4A and of previously obtained curves for fungal laccase [15] also points to the fact that one of the redox potentials of the T2/T3 cluster of either of the BODs, most likely the potential of T2, is close to 400 mV.

From the applied point of view, non-mediated BOD-based O_2 cathodes with Au electrodes would be much too slow. Methods to overcome the sluggish DET include "wiring' of the BOD in a redox hydrogel [28,47,48], use of conducting nanoparticles, or simply by orientation in thiol based self-assembled monolayers [49,50].

5. Conclusions

The midpoint potential of the T1 site of *M. verrucaria* BOD is close to 670 mV vs. NHE, whereas a much broader potential range, between 650 and 750 mV, is estimated for the E_{m7}-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 mV, holds for the BODs.

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