

Unmediated heterogeneous electron transfer reaction of ascorbate oxidase and laccase at a gold electrode

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The unmediated electrochemistry of two large Cu-containing proteins, ascorbate oxidase and laccase, was investigated by direct-current cyclic voltammetry. Rapid heterogeneous electron transfer was achieved in the absence of promoters or mediators by trapping a small amount of protein within a solid, electrochemically inert, tributylmethyl phosphonium chloride membrane coating a gold electrode. The problems typical of proteins in solution, such as adsorption on the electrode surface, were avoided by this procedure. In anaerobic conditions, the cyclic voltammograms, run at a scan rate of up to 200 mV/s, showed the electron transfer process to be quasi-reversible and diffusion-controlled. The pH-dependent redox potentials (+360 mV and +400 mV against a normal hydrogen electrode at pH 7.0 for ascorbate oxidase and laccase respectively and +390 mV and

+410 mV at pH 5.5) were similar to those of the free proteins. The same electrochemical behaviour was recorded for the type 2 Cu-depleted derivatives, which contain reduced type 3 Cu, whereas the apoproteins were electrochemically inactive. Under aerobic conditions the catalytic current intensity of holoprotein voltammograms increased up to approx. 2-fold at a low scanning rate, with unchanged redox potentials. The voltammograms of type 2 Cu-depleted proteins and of apoproteins were unaffected by the presence of oxygen. This suggests that electron uptake at the electrode surface involves type 1 Cu and that only in the presence of oxygen is the intramolecular electron transfer to other protein sites rapid enough to be observed. The analogy with available kinetic results is discussed.

INTRODUCTION

Protein–electrode electron transfer (ET) has become the focus of recent research efforts because it might provide information on the thermodynamics and kinetics of redox reactions and represent the first step for the realization of suitable sensors. The electrochemistry of biological macromolecules is complicated by several factors, namely the low diffusion rate to and from the electrode, the adsorption on the electrode surface with consequent structural 'degradation' and loss of activity, and the fact that the active site is often buried within the molecule interior [1–3]. Direct electrochemistry has been achieved for low-molecular-mass redox proteins (5–20 kDa) such as cytochrome *c* [4], ferredoxin [5] and the blue single-copper proteins [1,6–8]. It often occurs in the presence of promoters, substances that enhance the ET rate by favouring the proper orientation of the diffusing biological macromolecules at the electrode. With few exceptions, as for instance cytochrome *c* oxidase [9], electrochemical measurements on large enzymes require the use of redox mediators [1–3]. As these substances can interfere in the determination of kinetic and thermodynamic properties such as the ET rate or the value of the redox potentials, an important task is to establish the conditions for direct heterogeneous ET. A worthwhile strategy is the trapping of proteins within a solid matrix to avoid problems related to proteins in solution. The immobilized system should satisfy some fundamental requirements, the first of which is to provide the ET pathway typical of the protein in the native conformation. The incorporation of biological systems within films of different nature and their deposition on solid electrodes has already been described [10–13]. In this paper we report the

direct (unmediated) electrochemistry at a gold electrode of laccase and ascorbate oxidase, two high-molecular-mass Cu proteins, trapped in a polymeric film coating the electrode surface.

Laccase (EC 1.10.3.2) and ascorbate oxidase (AO; EC 1.10.3.3) form, together with caeruloplasmin, a small group of enzymes known as blue copper oxidases [14], which catalyse the reduction of O₂ to water by sequential one-electron uptake from a reducing substrate such as polyphenols, ascorbate, organic radicals and hexacyanoferrate(II) [15].

In resting proteins the minimum catalytic unit consists of four Cu ions bound to three distinct sites: one type 1 Cu (or blue Cu) with an intense absorption band at approx. 600 nm (ϵ 5000–6000 M⁻¹·cm⁻¹) and a narrow A_{II} in the EPR spectrum (3.8–9.0 mT); one type 2 Cu with undetectable visible absorption and normal EPR spectrum (A_{II} 18–20 mT); and one type 3 Cu pair of magnetically coupled cupric ions, undetectable by EPR and associated with an absorption band at 330 nm ($\Delta\epsilon$ 2800–3300 M⁻¹·cm⁻¹) [14]. All spectroscopic features are bleached by Cu reduction, allowing the oxidation–reduction processes of each Cu type to be monitored.

Laccase is formed by a highly glycosylated single peptide chain (110 kDa, 45% carbohydrate content) containing one set of Cu centres [16]. AO is a dimer of identical 70 kDa subunits, each containing one set of Cu centres and 3% carbohydrate content. The 1.9 Å X-ray structure of AO from green zucchini (courgette) shows that each subunit is composed of three domains showing similar β -barrel folding, distantly related to the small blue single-Cu proteins plastocyanin and azurin [17,18]. Type 1 Cu is bound inside domain 3 by one cysteine thiolate, one methionine sulphur, and two histidine residues, as in plastocyanin. Type 2 Cu and

Abbreviations used: AO, ascorbate oxidase; DC, direct current; ET, electron transfer; T2D, type 2 Cu-depleted; TBMP, tributylmethyl phosphonium chloride.

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type 3 Cu form a trinuclear cluster at the interface between domain 1 and 3, which each contribute four histidine Cu ligands. The two type 3 Cu ions and the type 1 Cu are connected through a His-Cys-His stretch, which has been suggested to provide a possible direct through-bond pathway for intramolecular ET. A similar three-domain structure has been proposed for laccase, on the basis of an alignment of the amino acid sequences [19] and the similar thermal behaviour recorded by differential scanning calorimetry [20]. The trinuclear Cu cluster has also been implicated in laccase by spectroscopic studies [21–23]. It probably represents the oxygen-binding site and electron storage unit required for O₂ reduction, whereas type 1 Cu is the entrance gate for electrons from reducing substrate as indicated by kinetics [18].

The voltammetric measurements described in this paper show that protein trapping considerably enhances the heterogeneous ET rate and allows kinetic and thermodynamic parameters for the electron exchange reaction, such as the ET rate constant and the value of the redox potential, to be determined. Further, voltammetric results indicate that in both proteins type 1 Cu is the only metal ion involved in the redox reaction at the electrode in the absence of oxygen.

EXPERIMENTAL

Enzymes

Laccase was purified from the acetone-dried powder of the lac of the Japanese lacquer tree *Rhus vernicifera* (Saito, Osaka, Japan), by the procedure of Reinhammar [16]. AO was purified from courgette peelings by the method of Avigliano et al. [24]. Type 2 Cu-depleted (T2D)-laccase and T2D-AO were prepared as described by Morpurgo et al. [25]. The visible–near-UV spectrum of the T2D derivatives showed only native 600 nm absorption because of the reduction of the type 3 Cu [26]. Fully Cu-depleted apo-laccase and apo-AO were prepared by the method of Savini et al. [27]. The concentrations of native and T2D proteins were measured with identical results by absorbance at 280 nm (ϵ_{280} 93 500 M⁻¹·cm⁻¹ for laccase and 240 000 M⁻¹·cm⁻¹ for ascorbate oxidase) and at 614 nm for laccase (ϵ_{614} 5700 M⁻¹·cm⁻¹) and at 610 nm for AO (ϵ_{610} 10 000 M⁻¹·cm⁻¹). The ϵ_{280} for the native proteins was also used for the apo-derivatives. The Cu content was measured by the biquinolyl method [28]. The numbers of atoms of Cu per molecule obtained for native laccase and ascorbate were 4.0 ± 0.1 and 7.8 ± 0.3 respectively, and for T2D-laccase and T2D-AO were 3.0 ± 0.1 and 5.5 ± 0.2 respectively. The Cu content of the apoproteins was undetectable with the above procedure. Less than 5% residual activity was measured for the T2D and apo-derivatives as described previously [25]. Optical measurements were recorded on a Lambda 18 UV/visible Perkin-Elmer spectrophotometer. All chemicals were reagent grade and were used without further purification.

Electrode modification

Proteins were embedded within a polymeric film of an anionic exchange resin containing tributylmethyl phosphonium chloride (TBMPC) bound to polystyrene, cross-linked with 1% (w/v) divinylbenzene (Fluka, Buchs, Switzerland). The trapping procedure consisted of mixing the protein (50–100 μ M in 50 mM phosphate buffer, pH 7.0) with a solution of the TBMPC resin (6/1 or 3/1, v/v). An appropriate amount of the resin had been dissolved in DMSO (high-grade purity, from Aldrich) to obtain a final concentration of 0.07%. This procedure did not alter the catalytic or optical properties of the proteins in solutions of up to 30% (v/v) DMSO. The electrode modification was achieved

by drying 5 μ l of the solution on the electrode surface for 48 h at 4 °C. The TBMPC membrane, without protein, showed no sign of electrochemical activity.

Electrochemical measurements

Direct current (DC) cyclic voltammograms were run in 50 mM phosphate buffer, pH 7.0, or in 50 mM acetate buffer, pH 5.5. In the experiments performed under anaerobic conditions, the electrode was introduced into the electrochemical cell after O₂ had been removed with a gentle flow of high-purity grade N₂. A gold electrode (AMEL, Milan, Italy) was the working electrode, a saturated calomel electrode (+244 mV against a normal hydrogen electrode, at 25 °C; AMEL) was the reference and a platinum ring the counter-electrode. A multipolarograph Amel 433 interfaced with a PC as the data processor was employed for cyclic and differential-pulse voltammetry measurements. All the redox potentials reported in the text refer to the normal hydrogen electrode.

RESULTS

Preliminary voltammetric measurements showed that neither AO nor laccase solutions produce a signal at a naked gold electrode. A rapid ET rate was observed when the proteins were embedded in a solid, inert TBMPC membrane coating the electrode, as described in the Experimental section. Under anaerobic conditions, the DC cyclic voltammograms of membrane-trapped AO run at a gold electrode in 50 mM phosphate buffer, pH 7.0 (Figure 1), showed well-defined electrochemistry in the scan rate range 50–200 mV/s. At lower scan rates the current intensity was too small to be quantified. Anodic and cathodic peak currents were almost identical, with an intensity ratio (i_{pa}/i_{pc}) close to unity. The linear dependence of

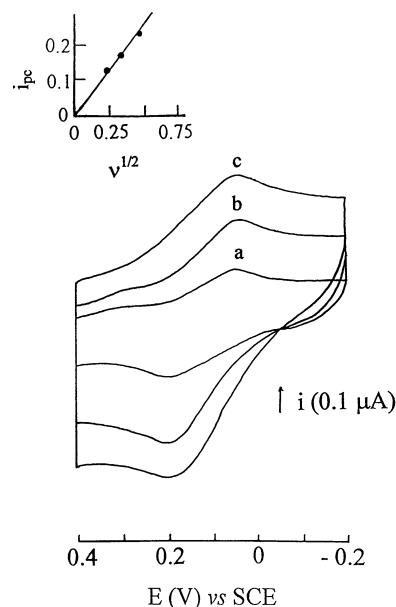


Figure 1 DC cyclic voltammograms of AO embedded within a TBMPC membrane at a gold electrode

The experiments were performed under anaerobic conditions in 50 mM phosphate buffer, pH 7.0, at 25 °C. Scan rates were: 50 mV/s (trace a), 100 mV/s (trace b) and 200 mV/s (trace c). Scans were typically recorded on the fifth cycle. Inset: i_p plotted against the square root of the scan rate. Abbreviation: SCE, standard calomel electrode.

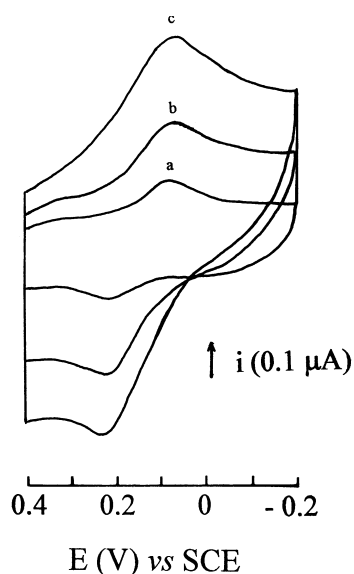


Figure 2 DC cyclic voltammograms of AO embedded within a TBMPC membrane at a gold electrode at acidic pH

Experimental conditions were as described in the legend to Figure 1, with the exception that 50 mM acetate buffer, pH 5.5, was used. Abbreviation: SCE, standard calomel electrode.

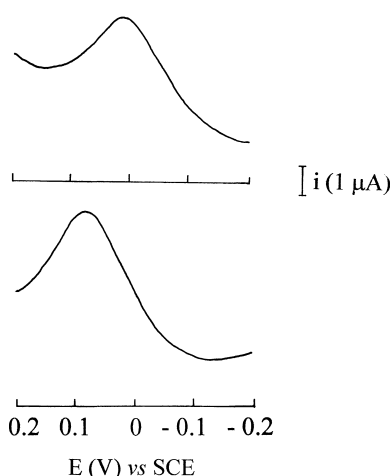


Figure 3 Differential-pulse voltammograms of AO embedded within a TBMPC membrane at a gold electrode

The experiments were performed under anaerobic conditions in 50 mM phosphate buffer, pH 7.0 (upper panel) and in 50 mM acetate buffer, pH 5.5 (lower panel), at 25 °C. The scan rate was 20 mV/s, the pulse amplitude was 50 mV and the pulse time was 10 ms. Abbreviation: SCE, standard calomel electrode.

the voltammetric peak current on the square root of the scan rate (Figure 1, inset) indicates that the redox process was diffusion-controlled. The observed ΔE_p values of approx. 150 mV were larger than expected for a reversible one-electron reaction (ΔE_p , 57 mV [29]), indicating that the ET rate was low, i.e. that the process was quasi-reversible ([30], chapter 6). The estimated redox potential, $E_{1/2}$, at $+360 \pm 5$ mV (S.D.) was close to the value measured potentiometrically under similar solvent conditions [31]. The heterogeneous ET rate constant, k_s , was calculated to be $(1.2 \pm 0.3) \times 10^{-4}$ cm/s at 25 °C, by taking n (i.e. the number of

Table 1 Thermodynamic and kinetic parameters for heterogeneous ET reaction of TBMPC-embedded AO and laccase at a gold electrode

Values are the averages for at least three measurements. Potentials are relative to a normal hydrogen electrode.

pH	Holo- and T2D-AO		Holo- and T2D-laccase	
	$E_{1/2}$ (mV)	$10^4 k_s$ (cm s ⁻¹)	$E_{1/2}$ (mV)	$10^5 k_s$ (cm s ⁻¹)
7.0	$+360 \pm 5$	1.2 ± 0.3	$+400 \pm 5$	5.3 ± 0.3
5.5	$+390 \pm 5$	1.3 ± 0.3	$+410 \pm 5$	4.3 ± 0.3

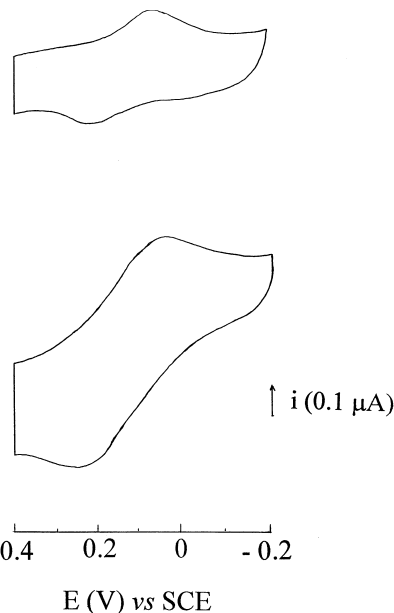


Figure 4 Effect of oxygen on DC cyclic voltammograms of AO embedded within a TBMPC membrane at a gold electrode

The scan rate was 50 mV/s. Other experimental conditions were as described in the legend to Figure 1. Abbreviation: SCE, standard calomel electrode.

electrons involved in the ET process) to be 1 and the diffusion coefficient, D_o , to be $(3.0 \pm 0.5) \times 10^{-8}$ cm²/s ([30], chapter 6). The DC cyclic voltammograms run at pH 5.5 (Figure 2) had a similar shape and intensity to those run at neutral pH. The value of k_s , $(1.3 \pm 0.3) \times 10^{-4}$ cm/s, was almost identical with the neutral pH value, whereas $E_{1/2}$, at $+390 \pm 5$ mV, was approx. 30 mV more positive (means \pm S.D. in each case).

Figure 3 shows the differential-pulse voltammograms of embedded AO run at pH 7.0 (upper panel) and pH 5.5 (lower panel). Both voltammograms show a single wave, centred on an E_p of $+355 \pm 5$ mV at pH 7.0 and $+375 \pm 5$ mV at pH 5.5, in good accord with the respective $E_{1/2}$ values obtained from cyclic voltammograms.

The electrochemical behaviour of TBMPC-embedded T2D-AO at a gold electrode was similar to that of the holoprotein. The DC cyclic voltammograms (results not shown) had the same shape and intensity as those illustrated in Figures 1 and 2. $E_{1/2}$ values were also identical with those of the holoprotein (Table 1). Apparently the removal of the type 2 Cu did not influence the

electrochemical behaviour. The TBMPC-embedded apo-AO, in contrast, caused no electrochemical signals.

Under aerobic conditions, the current intensity of native AO cyclic voltammograms increased significantly without a change in the peak potentials. Figure 4 shows that the current intensity was about twice that under anaerobic conditions, at a low scan rate of 50 mV/s. At higher scan rates the current increase was smaller: 1.5 at 200 mV/s. Similar results were obtained at pH 7.0 and 5.5. In contrast, no change was observed in the voltammograms of T2D-AO.

The electrochemistry of TBMPC-embedded laccase was similar to that of AO, both in the holoprotein and T2D derivative, under both anaerobic and aerobic conditions, confirming the close structural and functional similarities of the two enzymes. The DC cyclic voltammograms at the gold electrode (results not shown) were typical of a quasi-reversible heterogeneous ET reaction. Minor differences, namely a smaller definition of the voltammograms and a larger peak separation at both pH values, clearly showed that the system was less reversible than in AO and that the heterogeneous ET rate at the electrode was slower. This might be related to a negative influence on ET of the high carbohydrate content of the protein. The overall similarity of laccase and AO voltammetric behaviour is reflected by the kinetic and thermodynamic parameters reported in Table 1. Only the change in pH seems to have a slightly different effect on laccase k_s and E_p values with respect to AO. Apo-laccase, similarly to apo-AO, was electrochemically silent.

DISCUSSION

The results of this study demonstrate that the TBMPC membrane considerably enhances the ET reaction of trapped large proteins, such as AO or laccase, at a gold electrode in the absence of promoters or mediators. The physical trapping probably facilitates the ET by permitting macromolecules to come into contact with the electrode surface, in the absence of the adsorption phenomena typical of proteins in solution. The electrochemical process was diffusion-controlled, suggesting that rehydration of the membrane occurs on insertion of the electrode into the buffer, permitting the appropriate orientation of the embedded protein. An electrochemical effect of either the polymeric film or the protein amino acid backbone was excluded because no signal was present when apoproteins were used. The unmediated ET was more rapid in AO than in laccase (Table 1), as fast as or slower than in small blue single-copper proteins (k_s 3.3×10^{-4} and $(2-5) \times 10^{-3}$ cm/s for stellacyanin and plastocyanin respectively [8]).

Only one peak was detected under all conditions in the cathodic and anodic waves of the two proteins, although two redox centres are present in laccase and in each AO subunit, i.e. the mononuclear type 1 Cu and the trinuclear type-2-type-3 Cu cluster. This can be attributed either to a similar redox potential of the Cu ions or to the occurrence of ET between the electrode and one specific Cu, most probably the type 1 Cu. The second hypothesis is supported by the finding that the disruption of the trinuclear Cu centre on removal of the type 2 Cu and reduction of the type 3 Cu pair did not affect the electrochemical behaviour. The only apparent difference between the voltammograms at a given pH of either protein was the doubling in intensity under aerobic conditions compared with anaerobic conditions (Figure 4).

In favour of the conclusion above is the agreement between the oxidation-reduction potentials measured in the present study (Table 1) with published results for the type 1 Cu of both AO and

laccase. In these proteins, the redox potential of all Cu types is higher than that of common low-molecular-mass Cu complexes. At pH 7.0–7.5, the AO type 1 Cu and type 3 Cu have the same redox potential of +345 mV, measured at 25 °C by a potentiometric method [31], whereas the type 2 Cu has a similar or a slightly lower value [25]. The reported redox potentials of laccase type 1 Cu and type 3 Cu are in the range +394 to +434 mV, depending on experimental conditions [32,33], and that of the type 2 Cu is +390 mV [32]. The slightly higher values measured at pH 5.5, by 30 mV in AO and 10 mV in laccase (Table 1), agree with the redox potential increase of 32 mV previously observed for the type 1 Cu of T2D-laccase when the pH was lowered from 7.4 to 5.3 [34]. These effects might be associated with the protonation of either a histidine ligand, as in plastocyanins [35], or a residue close to the active site. The instability of AO and laccase at pH < 5 prevented an extension of the investigation to lower pH values [36].

The crystal structure of AO displays a pocket near the type 1 Cu as the possible binding site for L-ascorbate and the entry point of electrons [18]. Three residues in the pocket seem to be of particular importance, namely Trp-163, His-512 (a type 1 Cu ligand) and Trp-362, the side chains of which might form a staggered system of aromatic molecules with the lactone ring of ascorbate. In addition to ascorbate, several one-electron reductants, such as hexacyanoferrate(II) and organic radicals generated by pulse radiolysis and flash photolysis, are able to interact with AO. The ET from these molecules might not necessarily occur along the physiological pathway. Organic radicals (irradiated metronidazole [37], Methyl Viologen and lumiflavin [38,39]) have been shown to reduce AO by interacting with the type 1 Cu, at a similar rate to ascorbate ($k \approx 3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$), without non-specifically reducing the polypeptide moiety. A sequential intramolecular ET from type 1 Cu to the trinuclear Cu centre then provides the necessary four electrons for O_2 reduction. The rate constant of intramolecular ET, which is $7.5 \times 10^9 \text{ s}^{-1}$ under steady-state conditions, is considerably lower in the absence of oxygen [40]. A value of 120–160 s^{-1} was measured in the one-electron reduced enzyme [38,39,41]. A lower value of 25 s^{-1} was obtained with hexacyanoferrate(II) [15,42], which seems to be a non-specific substrate.

In a stopped-flow study on the anaerobic reduction of laccase, it was concluded that reduction occurs via the type 1 Cu [43]. Subsequently type 2 Cu is reduced and only at this stage can two electrons be simultaneously transferred from the type 1 and type 2 Cu atoms to the type 3 Cu, in a relatively slow process. In a subsequent study on the reduction of laccase by metronidazole radicals produced by pulse radiolysis [42], it was found that bleaching of the type 1 Cu requires the uptake of two electrons in the native protein and only one in the T2D derivative. It was concluded that the slow intramolecular ET to type 3 Cu could not be observed. The reduction of native and T2D-laccase at the membrane-coated electrode, involving in air two electrons and one electron respectively, suggests that ET operates by a very similar mechanism. In the absence of oxygen, the identity of native and T2D-laccase voltammograms suggests a slower intramolecular ET from type 1 to type 2 Cu.

In AO the ET seems to be strongly dependent on the nature of the electron donor. When the reducing molecule can fit into the cavity near the type 1 Cu, e.g. in the case of ascorbate or the metronidazole radical, both the ET to type 1 Cu and the intramolecular ET are fast and AO is able to accept approx. 1 electron per copper [37]. When this is not possible, as in the reaction at the electrode or with hexacyanoferrate(II), ET occurs with the same mechanism and rate constants as in laccase, probably at the protein surface.

The results described above show the great potential value of the protein-trapping procedure for basic and applied biochemistry. The method is relatively simple and does not produce alterations in the protein's functional properties. The TBMPc membrane, in combination with a high chemical inertia, shows low ohmic resistance and permits a relatively rapid ET at the electrode/film interface.

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