Journal of the Korean Electrochemical Society
Vol. 9, No. 3, 2006, 113-117

Electrochemical Behaviors of $ABTS^{2-}$ on the Thiol-modified Gold Electrodes

Hyug-Han Kim*

Department of Chemistry, Dankook University, Cheonan, 330-714, Korea

(Received July 7, 2006 : Accepted August 5, 2006)

Abstract : The electrochemical properties of the redox mediator, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{2-}$) were studied using cyclic voltammetry. The measured potentials ($E^{o'}$ vs SCE) of the two redox couples of ABTS are 0.45 V for $ABTS^{2-}/ABTS^{\bullet-}$ and 0.87 V for $ABTS^{\bullet-}/ABTS^{0}$. The rate constant for heterogeneous electron transfer and the diffusion coefficients for $ABTS^{2-}$ are 5×10^{-3} cm s^{-1} and 3.1×10^{-6} cm^2 s^{-1}, respectively. Our interest in $ABTS^{2-}$ stems from the fact that this molecule functions as a substrate to the copper oxidase, laccase, by providing the reducing equivalents necessary for the biocatalyzed reduction of dioxygen to water. Consequently, when laccase is tethered to an electrode surface or dissolved in solution, $ABTS^{2-}$ can be used to quantify enzyme activity electrochemically.

Key words : Laccase, ABTS, Redox mediator, Dioxygen, Cyclic voltammetry.

1. Introduction

In this paper, we describe electrochemical studies of laccase in solution and covalently confined to an electrode surface in the presence of the redox mediator, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) or $ABTS^{2-}$ (Fig. 1). We show that $ABTS^{2-}$ can function as an electrochemical reporter on the amount and activity of surface-confined laccase and demonstrate that amperometric measurements of the enzyme activity correspond well with traditional spectrophotometric assays.[1-3]

Electrochemical methodes of analysis are inexpensive and reproducible and can be highly sensitive. Azide shows directly electrochemically active at several electrodes such as glassy carbon, pyrolytic graphite, Pt and Au electrodes.[4] However there has been a few investigations of anions with laccase.[5,6] We illustrate how the amperometric measurement of enzyme activity can be used to monitor the concentration of azide in solution.

Laccase (oxygen oxidoreductase; EC 1.10.3.2) is a multi-copper oxidase that couples the oxidation of four equivalents of substrate to the four-electron reduction of dioxygen to water.[7-9] Laccase exhibits a low specificity for substrate and consequently will catalyze the oxidation of a number of substrates, including 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{2-}$) (Fig. 1). Previously, $ABTS^{-2}$ has been shown to be oxidized by inorganic peroxides[10] and by H_2O_2 in the presence of the enzyme peroxidase.[11,12] Spectroscopic and electrochemical studies of $ABTS^{2-}$ have been in addition to studies in the treatment of kraft pulp and lignin where $ABTS^{2-}$ was used as a redox mediator.[13,14]

The one-electron oxidation of $ABTS^{2-}$ produces $ABTS^{\bullet-}$, which can be oxidized further by one electron to $ABTS^{0}$ using an electrochemical or chemical methode.[14] The first oxidation product ($ABTS^{\bullet-}$) is a relatively stable radical and shows electrochemical reversibility at a variety of electrode surfaces. The second oxidation product ($ABTS^{0}$) is irreversible electrochemically at slow scan rates (< 50 mVs^{-1}) due to the rapid disproportionation reaction between $ABTS^{0}$ and $ABTS^{2-}$. We have found that an electrode modified with a self-assembled monolayer (SAM) inhibits the disproportionation reaction between $ABTS^{0}$ and $ABTS^{2-}$ at scan rates < 50 mVs^{-1}. The rate of this disproportionation reaction is reported for

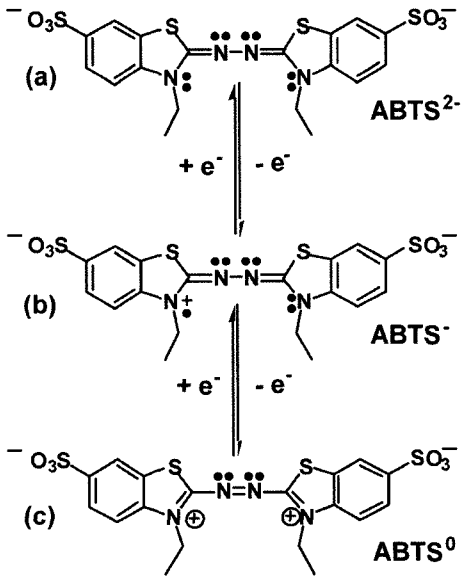

Fig. 1. The molecular structure of (a) $ABTS^{2-}$, (b) $ABTS^{-}$, and (c) $ABTS^{0}$.

*E-mail: hankim@dankook.ac.kr

bare and SAM-coated electrodes.

Previous studies on laccase confined to an electrode have focused on samples where the enzyme was adsorbed onto the surface of an electrode (e.g., gold, pyrolytic graphite, and carbon black)[15,16] or where it was co-immobilized with a redox mediator. In this paper, we describe our studies on the reaction between laccase and $ABTS^{2-}$ both when laccase is in a solution and when it is attached covalently to a gold electrode. We have monitored the change in the cathodic current at 255 mV in the cyclic voltammogram of $ABTS^{2-}$ in the presence of O_2 and laccase under conditions of saturation ($[ABTS^{2-}] \gg$ [laccase]).[17] The activity of laccase was determined from a plot of cathodic current vs. time and was found to be in agreement with that obtained using the more conventional spectrophotometric method.[18]

2. Experimental

Material. Fungal laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) from Pyricularia oryzae (144 or 389 U mg^{-1} protein using syringaldazine as substrate) was purchased from Sigma. ABTS [2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt] and chemicals used to prepare buffered solutions were purchased from Aldrich and used as received.

Modification of electrode surface. Gold electrodes (0.40 cm^2) were cleaned by soaking in dilute aqua regia (3 HCl: 1 HNO_3: 6 H_2O) for one minute at room temperature. Laccase was immobilized onto an electrode using the following procedure: A clean gold electrode was immersed in an aqueous solution of 10 mM 2-aminoethanethiol for 1 hour. Subsequently, the electrode was immersed in a 5% solution of glutaraldehyde for 1.5 hour. Finally, the electrode was immersed in the solution of laccase (33 μM) for 1 or 12 hours. Between each step in the procedure, the electrode was washed thoroughly with acetate buffer (pH 4.0).

Cyclic Voltammetry. Cyclic voltammograms were obtained using an EG&G Potentiostat/Galvanostat, Model 273A. A single-compartment cell was purged with nitrogen or oxygen gas for ten minutes prior to each measurement. The working electrode was a gold flag (0.40 cm^2), the counter and reference electrodes were platinum gauze (0.80 cm^2) and saturated calomel electrode (SCE), respectively. All reported potentials are in reference to the potential of SCE (0.241 V vs. NHE).

3. Results and Discussion

Electrochemical behavior of $ABTS^{2-}$. Shown in Fig. 2 are cyclic voltammograms of a 0.5 mM solution of $ABTS^{2-}$ in 0.2 M acetate buffer (pH 4.0) obtained using either a bare gold electrode (---) or a gold electrode modified with a thiol monolayer (—). The values for $E^{\circ\prime}_{ABTS^{2-}/ABTS^{\bullet-}}$ and $E^{\circ\prime}_{ABTS^{\bullet-}/ABTS^{0}}$ were determined by plotting i_p as a function of $v^{1/2}$. Taking the value of the extrapolated intercept at $v^{1/2}$ to be equal to zero, these values were found to be 0.45 and 0.87 V.[19] The cyclic voltammogram of ABTS obtained at a bare gold electrode contains a reversible wave at 0.45 V and an irreversible

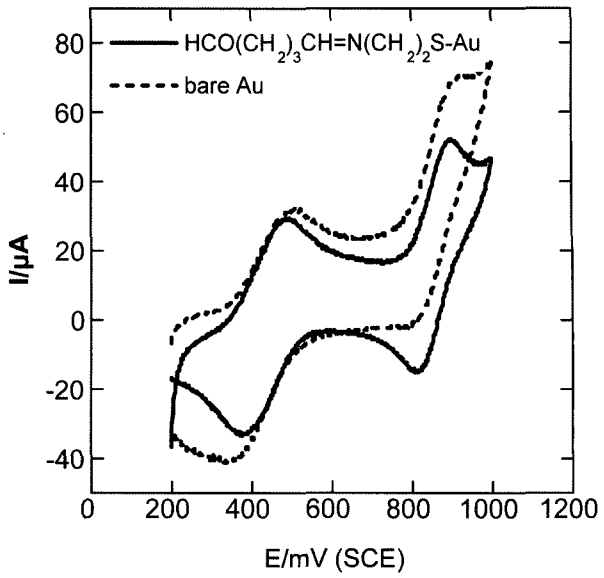

Fig. 2. Cyclic voltammograms of a 0.5 mM solution of $ABTS^{2-}$ at a bare gold electrode (---) and a gold electrode modified with ($HCO(CH_2)_3CH=N(CH_2)_2SH$) (—), the scan rate at 50 mVs^{-1}. The electrolyte was 0.2 M acetate buffer (pH 4.0).

wave at 0.87 V, confirming observations reported previously. The irreversible nature of the wave at 0.87 V is the result of a chemical reaction (disproportionation) between the electrochemically generated $ABTS^0$ and $ABTS^{2-}$ in the bulk solution (Eq. 1).

$$ABTS^0 + ABTS^{2-} \rightleftarrows 2ABTS^{\bullet-} \quad (1)$$

In contrast, the cyclic voltammogram of ABTS obtained at a gold electrode modified with a thiol monolayer contains two reversible one-electron waves at 0.45 V and 0.87 V. This result suggests that the thiol monolayers on the surface of the gold electrode inhibit the disproportionation reaction between the electrochemically generated $ABTS^0$ and the $ABTS^{2-}$ in bulk solution. The difference in the areas of the cathodic waves at 0.87 V corresponds to the amount of this inhibition, which approaches 100% at a scan rate of 50 mVs^{-1}.

Shown in Fig. 3 are cyclic voltammograms of a solution of $ABTS^{2-}$ (0.5 mM) as a function of scan rate obtained using a gold electrode modified with thiol monolayers. The CVs at scan rates faster than 50 mVs^{-1} have reversible waves at both 0.45 V ($ABTS^{2-}/ABTS^{\bullet-}$) and 0.87 V ($ABTS^{\bullet-}/ABTS^0$). At scan rates slower than 50 mVs^{-1}, the area of the cathodic peak at 0.87 V decreases, the amount corresponds to the increase in the area of the cathodic peak at 0.45 V. Thus, the ability of the thiol monolayer to inhibit the disproportionation reaction between $ABTS^0$ and $ABTS^{2-}$ occurs only at scan rates faster than 50 mVs^{-1}.

The rate equation for the disproportionation reaction between $ABTS^{2-}$ and $ABTS^0$ is given by Eq. (2) with an initial concentration of $ABTS^{2-}$ of 0.5 mM. The values for $d[ABTS^{\bullet-}]/dt$ and $[ABTS^0]$ are obtained from the difference of charge integration between the anodic and cathodic peaks

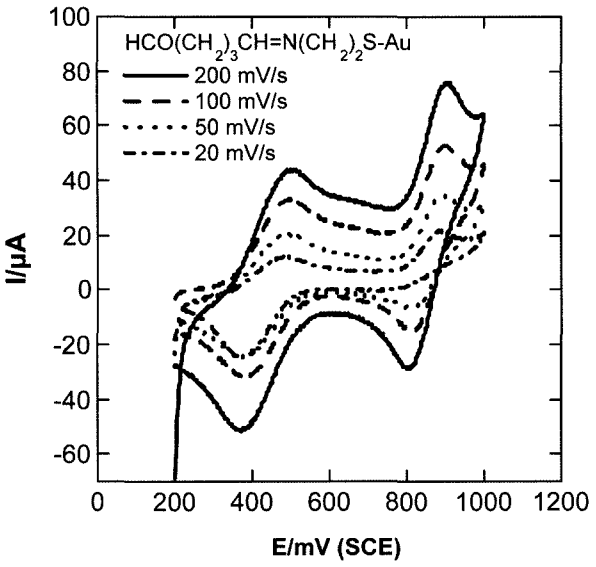

Fig. 3. Cyclic voltammograms of a 0.5 mM solution of $ABTS^{2-}$ as a function of scan rate. The working electrode was gold coated with a thiol monolayer. The electrolyte was 0.2 M acetate buffer (pH 4.0).

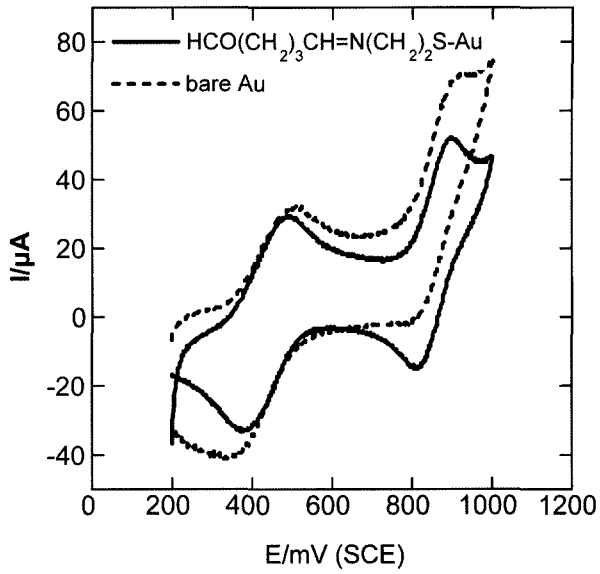

Fig. 4. Illustration of how a thiol monolayer might inhibit the disproportionation reaction between $ABTS^0$ and $ABTS^{2-}$. The neutral $ABTS^0$ is more soluble in the neutral monolayer than anionic $ABTS^{2-}$, and thus the collision frequency between $ABTS^0$ and $ABTS^{2-}$ is lower than when $ABTS^0$ is produced at a bare gold electrode.

at 0.45 V and from the charge integration in the cathodic peak at 0.87 V, respectively. When $ABTS^0$ is generated at a bare gold electrode, the rate constant for the disproportionation reaction between $ABTS^{2-}$ and $ABTS^0$ is 22.0±1.0 $M^{-1}s^{-1}$. When $ABTS^0$ is generated at a gold electrode modified with a thiol monolayer, however, the value for k_{dis} is 4.5±0.5 M^{-1} s^{-1}, a five-fold decrease in the rate of disproportionation.

$$\frac{d[ABTS^-]}{dt} = k_{dis}[ABTS^{2-}] \times [ABTS^0] \quad (2)$$

The decrease in the rate constant for disproportionation suggests that the neutral $ABTS^0$ is generated at the electrode within the monolayer and thus, reduces its interaction with $ABTS^{2-}$ in the bulk solution (Fig. 4). At scan rates slower than 50 mVs^{-1}, the area of the cathodic peak at 0.87 V is less than that of the anodic peak, indicating that at these scan rates $ABTS^0$ diffuses out of the monolayer and reacts with $ABTS^{2-}$.

The diffusion coefficient of ABTS was determined from cyclic voltammograms scanned between 0.2 and 0.6 V at different scan rates using a SAM/gold electrode (data not shown).[20] The peak currents of the cathodic ($i_{p,c}$) and anodic ($i_{p,a}$) waves in the cyclic voltammogram of ABTS vary linearly with $v^{1/2}$ (scan $rate^{1/2}$). Using the Randles-Sevcik equation (Eq. 3),[20]

$$i_{p,c} = (2.69\times10^5)n^{3/2}AD^{1/2}v^{1/2}C \quad (3)$$

where i_p is the value for peak current at a given scan rate, n is the number of electrons transferred, A is the area of the electrode measured in cm^2, v is the scan rate in units of Vs^{-1}, and C is the concentration of ABTS (5.0×10^{-7} mol cm^{-3}), we obtained a value for the diffusion coefficient of ABTS ($D = 3.1\times10^{-6}$ cm^2s^{-1}). The rate constant for heterogeneous electron transfer between an electrode and ABTS is determined using the method of Nicholson and Eq. (4),

$$\Psi = \Lambda\pi^{-1/2} = \frac{(D_o/D_r)^{\alpha/2}k_{het}}{[D_o\pi v(nF/RT)]^{1/2}} \quad (4)$$

where D_o and D_r are the diffusion coefficients of oxidized and reduced ABTS, respectively, v is the scan rate in units of Vs^{-1}, n is the number of electrons transferred in the rate determining step, F is the Faraday constant (9.6846×10^4 C equiv.$^{-1}$), α is the transfer coefficient and typically assigned a value of 0.5.[21,22] Assuming that D_o and D_r are equivalent, setting α=0.5, and rearranging Eq. (4) gives Eq. (5).

$$k_{het} = \Psi\left[D_o\pi v\left(\frac{nF}{RT}\right)\right]^{1/2} \quad (5)$$

The value for the kinetic parameter Ψ in Eq. (5) is dependent on $E_{p,a}$-$E_{p,c}$, and thus, the rate constant for electron transfer between ABTS and a gold electrode modified with a thiol monolayer is 4.5×10^{-3} cm s^{-1}. For comparison, the rate constant for electron transfer between an electrode surface and $K_3Fe(CN)_6$ is 4.4×10^{-2} cm s^{-1}.[23] Similarly, the rate constant for electron transfer between an electrode and cytochrome c, an oxidoreductase with a heme on the surface of the protein, k_{het} is 6×10^{-3} cm s^{-1}.[19] Catalytic behavior of laccase covalently attached to a gold electrode

Shown in Fig. 5 are the cyclic voltammograms of a 0.5 mM solution of ABTS. The cyclic voltammograms were obtained using a gold electrode modified with laccase in the absence (---) and presence (—, —•—) of dioxygen. In the absence of dioxygen, the area of the cathodic wave in the cyclic voltammogram of ABTS remains unchanged over

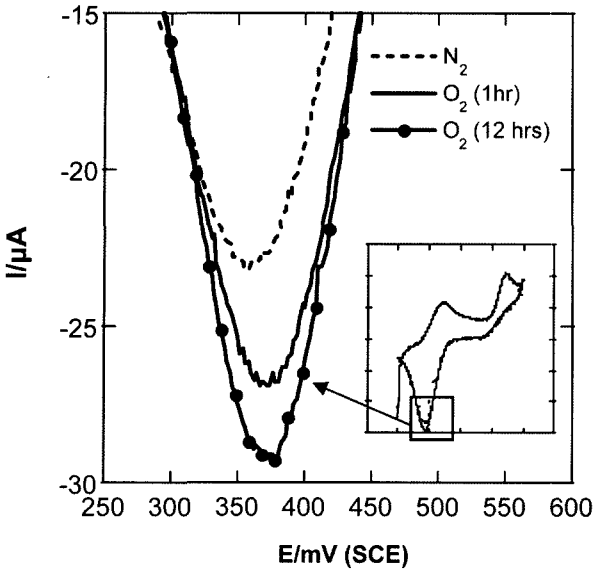

Fig. 5. Cyclic voltammograms of a 0.5 mM solution of ABTS using a gold electrode modified with laccase. Measurements were conducted in the absence (----) or presence (——,–•–) of dioxygen after the electrode was soaked in a 3.3 μM solution of laccase for one (----, ——) or twelve (–•–) hours, scan rate at 20 mVs^{-1}. The electrolyte was 0.2 M acetate buffer (pH 4.0).

time, indicating that laccase does not oxidize $ABTS^{2-}$ spontaneously under anaerobic conditions. In the presence of dioxygen, laccase oxidizes $ABTS^{2-}$ to $ABTS^{\bullet-}$, resulting in an increase in the concentration of $ABTS^{\bullet-}$ near the surface of the electrode during a stationary voltammetric experiment. The result is a change in the area of the cathodic peak in the cyclic voltammograms of ABTS taken at different times. This change in area corresponds to the amount of $ABTS^{\bullet-}$ produced during the biocatalytic reduction of dioxygen, and thus, the activity of laccase under conditions of saturation kinetics ($[ABTS^{2-}] \gg$ [laccase]).

Using these results, the activity of laccase confined to a gold electrode can be quantified from Eqs. (6) and (7),

$$\frac{d[ABTS^{2-}]}{dt} = \frac{\frac{d(\mu C)}{dt}}{96,500\frac{C}{mol}} = X\frac{mol}{min} \quad (6)$$

$$\text{activity of laccase} = \frac{\left(X\frac{mol}{min}\right)\left(10^{6}\frac{\mu mol}{min}\right)}{1.56\times10^{-4}\text{mg of laccase}} = \frac{Y\frac{\mu mol}{min}}{\text{mg of laccase}} = Y\frac{U}{\text{mg of laccase}} \quad (7)$$

where d(μC)/dt corresponds to the difference in charge represented by the cathodic waves in Fig. 5 with and without dioxygen present. It is interesting to note that laccase confined to a gold electrode is thirty times more active than the same laccase in solution (i.e., 1000 mU mg^{-1} protein vs. 33 mU mg^{-1} protein), suggesting that laccase is purified during the immobilization procedure.

The cyclic voltammograms in Fig. 5 also provide information about the amount of laccase confined to the surface of the electrode. Gold electrodes modified with a monolayer of thiol were soaked in a 33 μM solution of laccase for different time intervals. Identical cyclic voltammograms of a 0.5 mM solution of $ABTS^{2-}$ were obtained using electrodes soaked in laccase for twelve or more hours, indicating that the coverage of laccase on the electrode reaches peak after soaking for twelve hours. Based on the partial specific volume of laccase ($V = 0.73\ cm^3\ g^{-1}$), the maximum coverage (Γ_{max}) of laccase on a gold electrode is calculated to be 6.0×10^{-11} mol cm^{-2}.[24] Thus, when the electrode is soaked in a solution of laccase for only one hour, the coverage of laccase is 2.2×10^{-11} mol cm^{-2} or 37% of the maximum value.

4. Conclusions

The one-electron oxidation of $ABTS^{2-}$ produces a radical anion, $ABTS^{\bullet-}$, that is green in color and relatively stable. The one-electron oxidation of the radical produces a neutral species, $ABTS^0$ that will disproportionate with $ABTS^{2-}$ in the bulk solution to produce $ABTS^{\bullet-}$. This disproportionation reaction reduces the size of the cathodic wave at 0.87 V ($ABTS^{\bullet-}$/$ABTS^0$) in the cyclic voltammogram. We have shown that a gold electrode modified with a thiol monolayer inhibits the disproportionation reaction between $ABTS^0$ and $ABTS^{2-}$ by selectively protecting the neutral $ABTS^0$ within the monolayer, thereby reducing its exposure to the higher concentration of $ABTS^{2-}$ in the bulk solution. An analysis of the cyclic voltammograms of $ABTS^{2-}$ as a function of scan rate provides for the average rate of the disproportionation reaction between $ABTS^0$ and $ABTS^{2-}$.

Typically, the specific activity of an oxidoreductase is determined spectrophotometrically by monitoring the change in absorbance of substrate or product. Under certain circumstances, however, this method can be cumbersome, prone to error, and difficult to use with proteins adsorbed on surfaces. We have shown that an amperometric determination of the concentration of $ABTS^{2-}$ can be used to determine the specific activity of laccase both in solution and tethered to an electrode surface.

Acknowledgements

The present research was conducted by the research fund of Dankook University in 2004.

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