Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 367 (2008) 457–461
BBRC
www.elsevier.com/locate/ybbrc

Elucidation of the factors affecting the oxidative activity of *Acremonium* sp. HI-25 ascorbate oxidase by an electrochemical approach

Kenichi Murata, Nobuhumi Nakamura *, Hiroyuki Ohno

Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16 Nakacho, Koganei, Tokyo 184-8588, Japan

Received 20 December 2007
Available online 4 January 2008

Abstract

Steady-state kinetics of *Acremonium* sp. HI-25 ascorbate oxidase toward *p*-hydroquinone derivatives have been examined by using an electrochemical analysis based on the theory of steady-state bioelectrocatalysis. The electrochemical technique has enabled one to examine the influence of electronic and chemical properties of substrates on the activity. It was proven that the oxidative activity of ascorbate oxidase was dominated by the highly selective substrate-binding affinity based on electrostatic interaction beyond the one-electron redox potential difference between ascorbate oxidase's type 1 copper site and substrate.

Keywords: Ascorbate oxidase; Multicopper oxidase; Bioelectrocatalysis; Enzyme kinetics; *p*-Hydroquinone derivatives

Ascorbate oxidase (EC 1.10.3.3) is a member of the multicopper oxidase family, which has been obtained from higher plants, such as green zucchini squash (*Cucurbita pepo medullosa*) and cucumber (*Cucumis sativus*), and fungal *Acremonium* sp. HI-25. It catalyzes the one-electron oxidation of ascorbate with the concomitant four-electron reduction of dioxygen to water [1]. Ascorbate oxidase from green zucchini squash (ZAO) was the first of the multicopper oxidases to be crystallized and characterized by X-ray diffraction [2,3]. ZAO has been shown to exist as a dimer of identical subunits, which are folded into three interacting domains, all of a similar Greek key β-barrel type structure (cupredoxin fold), distantly related to small blue copper proteins. Each subunit has four copper ions which can be classified into Type 1 copper (T1Cu), Type 2 copper (T2Cu), and Type 3 copper (T3Cu) by their spectroscopic and magnetic properties. Ascorbate is bound in the cavity nearby the T1Cu site and oxidized to the semidehydroascorbate radical, which then spontaneously dismutates in solution. Electrons donated from ascorbates are transferred to the T2/T3 trinuclear cluster, where dioxygen is reduced to water. It has been found that these structural features are similar to almost all multicopper oxidases, although other multicopper oxidases exist as a monomer.

Multicopper oxidases can be divided into two classes according to substrate specificity [4]; the first, a laccase group including laccase, bilirubin oxidase, and ascorbate oxidase, oxidizes organic substrates, and the second, represented by metallo-oxidases including CueO, Fet3p, and MnxG, oxidizes Cu (I), Fe (II), and Mn (II), respectively. Elucidation of the factors affecting the substrate specificity of multicopper oxidases has attracted considerable attention [4], and is important for applications of these enzymes. It has been found that the differences in substrate specificity are attributable to the protein fold around the T1Cu sites. For example, the substrate cavity nearby the T1Cu site in *Trametes versicolor* laccase is shallow with few steric constrains for binding substrates, and organic substrates bind to one of the T1Cu histidine ligands, which is exposed to solvent, via a hydrogen bond [5]. This structural characteristic contributes to the broad specificity of fungal laccases toward organic substrates, and the activity (k_{cat}/K_m) is mainly affected by the difference in redox potential between T1Cu ($E_{T1Cu} = +270$ to $+580$ mV) [6] and a substrate ($\Delta E_{T1Cu-sub} = E_{T1Cu} - E_{substrate}$) [7]. On the other hand,

* Corresponding author. Fax: +81 42 388 7482.
E-mail address: nobu1@cc.tuat.ac.jp (N. Nakamura).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.12.159

the cavity in Fet3p is more structurally defined, particularly by acidic amino acid residues which construct the Fe (II) ion substrate-binding site [8,9]. The acidic amino acid residues in the metal ion substrate-binding site mask the two histidine ligands of T1Cu, and prevent the metallo-oxidase from binding organic compounds as substrates.

AO has high substrate specificity for ascorbate, although it belongs to the laccase–type multicopper oxidases which oxidize organic compounds. The high specificity is attributable to the smaller substrate cavity, and two tryptophans and a histidine inside the cavity stabilize the lactone ring of ascorbate [3]. For ZAO, studies of substrate specificity using ascorbic acid derivatives [10], leuco-2,6-dichlorophenolindophenol and *p*-hydroquinone derivatives [11], and of inhibition using substituted phenols [12], have showed that the enzyme tend to more efficiently bind the deprotonated anionic form of a substrate and inhibitor than the protonated neutral form. In addition, the low redox potential of T1Cu (ASOM, +197 mV [13]; ZAO, +139 mV [14]) could be one of the factors affecting the substrate specificity of ascorbate oxidases. However, the relationship between oxidase activity and ΔE has not been examined and the dominant factor affecting the activity of ascorbate oxidase has been unclear.

Ascorbate oxidase from *Acremonium* sp. HI-25 (ASOM) is monomeric with a molecular mass of about 80 kDa and thermostability at up to 60 °C, while higher plant ascorbate oxidases are homo-dimers with a monomer of about 70 kDa and thermolabile properties. For applications of ascorbate oxidases, thermostability is important and therefore we have focused on the kinetics of ASOM.

Here, we evaluated steady-state kinetic parameters of ASOM for *p*-hydroquinone derivatives, using an electrochemical analysis based on the theory of steady-state bioelectrocatalysis [15] in order to elucidate the factors affecting the oxidative activity of ASOM. The electrochemical method allows us to directly evaluate the correlation between oxidase activity and $\Delta E_{\mathrm{T1Cu-sub}}$ whereas the other analytical methods need to measure and evaluate the oxidative activity and $\Delta E_{\mathrm{T1Cu-sub}}$ separately. Additionally, by using *p*-hydroquinone derivatives as substrates with little alteration of the steric hindrance effect, we can examine the influence of electronic and chemical properties of substrates including redox potential and acid dissociation constant (pK_a) on the activity. Hence, we can evaluate the effects on the oxidative activity of ASOM, which is derived from both $\Delta E_{\mathrm{T1Cu-sub}}$ and the pK_a of the substrate.

Materials and methods

Reagents. Ascorbate oxidase from *Acremonium* sp. HI-25 (ASOM) was purchased from Asahi Kasei Co., Japan. The concentration of ASOM in a stock solution was determined spectrophotometrically with an absorption coefficient of 4270 $M^{-1}\,cm^{-1}$ for LMCT transition at 600 nm [16]. The pK_a value of 9.3 for 2-chloro-5-methyl-*p*-hydroquinone, calculated with the Advanced Chemistry Development software Solaris V8.14, was obtained from Sci Finder Scholar.

Electrochemical measurements. Linear sweep voltammetry (LSV) was performed using a BAS electrochemical analyzer in a three-electrode system with a glassy carbon electrode, an Ag/AgCl (3 M NaCl) electrode, and a Pt wire as the working, reference, and counter electrodes, respectively. Voltammetric responses were recorded in a buffer solution dissolved with the oxidized substrate and ASOM. The buffer solution was 0.1 M phosphate buffer (pH 6–8) and 0.1 M boric acid–Na_2CO_3 buffer (pH 8–9), which were saturated with air by bubbling for 30 min.

Under the condition $K_{O_2} \ll [O_2]$, the nonlinear dependence of I_s^{lim} on [M] was analyzed by Eq. (1) to evaluate k_{cat} and K_m [15].

$$\frac{I_s^{lim}}{n_M F A \sqrt{(n_{O_2}/n_M) D_M k_{cat} K_m [E]}} = \sqrt{2\left[\frac{[M]}{K_m} - \ln\left(1+\frac{[M]}{K_m}\right)\right]} \quad (1)$$

where *F, A,* D_M, n_{O_2} and n_M are Faraday's number, the electrode surface area, the diffusion coefficient of a mediator, the number of electrons for the substrate dioxygen, and the mediator (substrate) in the reaction, respectively.

Results and discussion

p-Benzoquinone derivatives should be used as substrate precursors in electrochemical measurements. The *p*-hydroquinone derivatives which are generated at the electrode's surface by the reduction of *p*-benzoquinone act as a substrate. The *p*-benzoquinones used in this study exhibit a reversible redox response with pH-dependent redox potential and function as a mediator of the electron transfer between ASOM and the electrode's surface. Without the enzyme at pH 7.0, the substrates showed a reversible redox response with different redox potential values; 2-chloro-5-methyl-*p*-benzoquinone: +187 mV, *p*-benzoquinone: +144 mV, 2-chloro-*p*-benzoquinone: +132 mV, 2,6-dichloro-*p*-benzoquinone: +117 mV (in this communication, potentials refer to Ag/AgCl (3 M NaCl), whose potential is +205 mV vs. NHE, unless stated otherwise). Fig. 1 (inset) shows linear sweep voltammograms of 2,6-dichloro-*p*-benzoquinone, as an example of analyses among several *p*-benzoquinone derivatives, in the presence of ASOM and saturated air. The steady-state limiting catalytic current (I_s^{lim}), which is obtained by linear sweep vol-

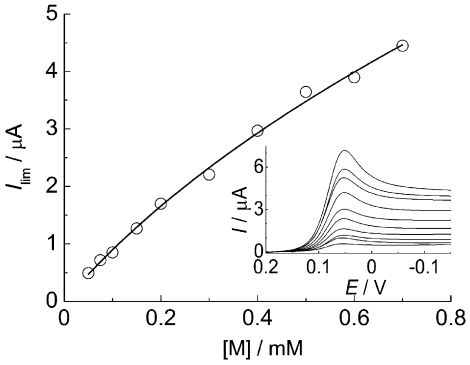

Fig. 1. Dependence of the steady state catalytic current I_s^{lim} on the 2,6-dichloro-*p*-hydroquinone concentration. The solid curve represents a nonlinear regression curve obtained based on Eq. (1). The inset shows the linear sweep voltammograms of various concentrations of 2,6-dichloro-*p*-benzoquinone, in the presence of 0.02 μM ASOM under the saturated air condition. Scan rate: 2 mV s^{-1}.