Biochemistry and Biophysics Reports 5 (2016) 396–400

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Isothermal titration calorimetry uncovers substrate promiscuity of bicupin oxalate oxidase from *Ceriporiopsis subvermispora*

Hassan Rana [a], Patricia Moussatche [b], Lis Souza Rocha [a], Sofiene Abdellaoui [c], Shelley D. Minteer [c], Ellen W. Moomaw [a,*]

[a] Department of Chemistry and Biochemistry, Kennesaw State University, Kennesaw, GA 30144, USA
[b] Foundation for Applied Molecular Evolution Alachua, FL 32615, USA
[c] Department of Chemistry, University of Utah, Salt Lake City, UT 84112, USA

ARTICLE INFO

Article history:
Received 30 September 2015
Received in revised form
30 December 2015
Accepted 28 January 2016
Available online 4 February 2016

Keywords:
Oxalate oxidase
Isothermal titration calorimetry
Enzyme kinetics
Cupin

ABSTRACT

Isothermal titration calorimetry (ITC) may be used to determine the kinetic parameters of enzyme-catalyzed reactions when neither products nor reactants are spectrophotometrically visible and when the reaction products are unknown. We report here the use of the multiple injection method of ITC to characterize the catalytic properties of oxalate oxidase (OxOx) from *Ceriporiopsis subvermispora* (CsOx-Ox), a manganese dependent enzyme that catalyzes the oxygen-dependent oxidation of oxalate to carbon dioxide in a reaction coupled with the formation of hydrogen peroxide. CsOxOx is the first bicupin enzyme identified that catalyzes this reaction. The multiple injection ITC method of measuring OxOx activity involves continuous, real-time detection of the amount of heat generated (dQ) during catalysis, which is equal to the number of moles of product produced times the enthalpy of the reaction (ΔH_{app}). Steady-state kinetic constants using oxalate as the substrate determined by multiple injection ITC are comparable to those obtained by a continuous spectrophotometric assay in which H_2O_2 production is coupled to the horseradish peroxidase-catalyzed oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) and by membrane inlet mass spectrometry. Additionally, we used multiple injection ITC to identify mesoxalate as a substrate for the CsOxOx-catalyzed reaction, with a kinetic parameters comparable to that of oxalate, and to identify a number of small molecule carboxylic acid compounds that also serve as substrates for the enzyme.

1. Introduction

Cereporiopsis subvermispora is a white rot basidiomycete fungus that efficiently depolymerizes lignin [1] and is of interest in degrading biomass for the production of ethanol [2], sugar [3], and methane [4]. Oxalate oxidase (OxOx) catalyzes the cleavage of the carbon–carbon bond of oxalate to yield two moles of carbon dioxide as dioxygen is reduced to hydrogen peroxide [5]. While not fully understood, the role of oxalate oxidase in *C. subvermispora* (CsOxOx) has been proposed to provide a source of extracellular hydrogen peroxide for manganese peroxidase to oxidize Mn^{2+} to Mn^{3+} [6, 7], which is a robust and diffusible oxidant able to degrade many components of lignin [8]. Oxalate oxidase activity has been found in a number plant species including rice [9], wheat [10], barley [11,12,13], beet [14,15], and sorghum [16,17] where it has been shown to play roles in signaling and in the defense against pathogenic microbes [18]. Plant OxOx enzymes possess a single cupin (β-barrel) domain containing a single manganese ion and are, therefore, structurally characterized and classified as monocupins [19,20,21,22]. Sequence analysis indicates that CsOxOx is the first manganese-containing bicupin enzyme identified that catalyzes the oxidation of oxalate [23,24,25].

Oxalate oxidase is of commercial interest for a number of applications including the determination of oxalate levels in blood and urine [26,27], the protection of plants against pathogens, the production of transgenic plants with reduced levels of oxalate [19,28], pulping in the paper industry [19,29,30,31], and as a component of enzymatic biofuel cells [32,33]. The utility of OxOx as a biocatalyst for anodic electrode reactions in biofuel cells motivates efforts to tailor the properties of the enzyme to this application through directed evolution and/or rational design [34,35]. Understanding the degree of promiscuity (or fidelity) of CsOxOx is an important

Abbreviations: OxOx, oxalate oxidase; CsOxOx, OxOx from *Ceriporiopsis subvermispora*; HRP, horseradish peroxidase; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid).

* Corresponding author.
E-mail address: emoomaw@kennesaw.edu (E.W. Moomaw).

http://dx.doi.org/10.1016/j.bbrep.2016.01.016

endeavor as it may provide a basis for these modifications. Previous enzymatic characterization of CsOxOx employing a continuous spectrophotometric assay in which H_2O_2 production is coupled to the horseradish peroxidase (HRP) catalyzed oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) determined that acetate and other small molecule carboxylic acid compounds (malonate, malate, glycolate, glyoxylate, and pyruvate) reduced the rate of oxalate oxidation at low concentrations of oxalate. Uninhibited maximal reaction rates could, however, be achieved at high substrate concentrations, suggesting that these molecules were competitive inhibitors [24]. Using the HRP coupled assay, none of these molecules served as substrates.

Isothermal titration calorimetry (ITC) may be used to determine the kinetic parameters of enzyme catalyzed reactions even if the identity of the products are not known [36,37,38]. The multiple injection ITC method of measuring OxOx activity involves continuous, real-time detection of the amount of heat generated (dQ) during catalysis, which is equal to the number of moles of product produced times the enthalpy of the reaction (ΔH_{app}). Determination of the kinetic parameters of a reaction using this method, therefore, requires two experiments 1) determination of the enthalpy of the reaction from the complete conversion of substrate to product, and 2) determination of the differential power effects from the continuous conversion of substrate to product. We report here the use of ITC to characterize the catalytic properties of oxalate oxidase through the direct and continuous detection of the amount of heat generated. Furthermore, we used multiple injection ITC to identify mesoxalate (oxopropanedioic acid) as a substrate for CsOxOx with a kinetic parameters comparable to that of oxalate and to identify other small molecule carboxylic acids (that were previously shown to be competitive inhibitors) as substrates for CsOxOx.

2. Materials and methods

2.1. Materials

Recombinant oxalate oxidase from *C. subvermispora* was expressed and purified as a secreted soluble protein using a *Pichia pastoris* expression system as previously described [24].

Reagents were of the highest purity available and were purchased from either Sigma-Aldrich or Fisher Scientific unless otherwise stated. A modified Lowry assay (Pierce) was used to determine protein concentration using bovine serum albumin as a standard [39].

2.2. Coupled steady-state kinetic assay

The oxalate oxidase-catalyzed oxidation of oxalate was measured using a continuous, coupled spectrophotometric assay in which the formation of hydrogen peroxide is coupled to the HRP-catalyzed oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) [13]. Each assay contained 25 U HRP, 5 mM ABTS, 50 mM potassium oxalate, and 1 μM CsOxOx dissolved in the indicated buffer, pH 4.0 (total volume 1.0 mL) and was monitored at 650 nm. An extinction coefficient of 10,000 M^{-1} cm^{-1} for the ABTS radical product was used in the rate calculations. Control samples without HRP were carried out in order to distinguish between H_2O_2 production and any oxalate-dependent dye oxidation activity by CsOxOx. Reactions were carried out at specific substrate and enzyme concentrations in duplicate, and data were analyzed to obtain the values of V_{max} and V_{max}/K_m by standard computer-based methods [40].

2.3. Isothermal titration calorimetry

Calorimetric measurements were performed using a Nano ITC Low Volume (TA Instruments) equipped with a 24 K gold reaction cell (190 μL volume). Degassed solutions were equilibrated at 25.0 °C with stirring at 125 rpm. A 50 μL stirred syringe inserted into a buret handle was used to inject substrate into the sample cell. Water was used in the reference cell. Heat (Q) produced from the chemical reaction between enzyme and substrate was measured by the continuous supply of instrumental thermal power (dQ/dt) to the sample cell, which maintains isothermal condition between the sample cell and reference cell. Thermal power relates to enzyme reaction rate:

$$\frac{dQ}{dt} = \frac{d[P]}{dt} \times V \times \Delta H \quad (1)$$

where V is the volume of the solution in the sample cell, ΔH is the apparent enthalpy, and $(d[P])/dt$ is the enzyme reaction rate. When Eq. (1) is solved for $(d[P])/dt$, the resulting equation is:

$$\frac{d[P]}{dt} = \frac{1}{V \times \Delta H} \times \frac{d[Q]}{dt} \quad (2)$$

Protein samples were exhaustively dialyzed into the reaction buffer (25 mM sodium succinate, pH 4.0) and substrate solutions were prepared in the resulting dialysate. Enzyme concentrations for the rate determinations ranged from 112 nm to 456 nM as described in the Two blank reactions were performed for each condition tested: 1) the injection of substrate into buffer and 2) the injection of buffer into enzyme solution. These blank heat effects were subtracted to yield the corrected heat rate of reaction. NanoAnalyze (TA Instruments Inc.) was used to transform the raw ITC data into reaction rates according to Eqs. (1) and (2) above and to obtain the values of k_{cat} and K_m. The instrument is an overflow calorimeter and the volume, therefore remains constant, but the number of moles does change as volume is displaced. This is tracked in the NanoAnalyze software.

2.4. Calorimetric determination enthalpies of reaction

The apparent molar enthalpies of oxalate and other possible substrates were determined by measuring the power required to maintain constant temperature in conditions where the reaction proceeded to completion. These conditions required the use of higher enzyme concentrations (1 μM) and lower substrate concentrations (2 mM) than the corresponding rate determination experiments. Upon complete consumption of substrate, the power returned to baseline. The experimental enthalpy relates to the area under the curve (less the blank heat of the mixing event) according to Eq. (3).

$$\Delta H_{app} = \frac{1}{[S]*V} \int_{t=0}^{t=\infty} \frac{dQ(t)}{dt} dt \quad (3)$$

3. Results and discussion

3.1. Direct detection of CsOxOx activity

Direct measurement of enzymatic reaction rates has numerous advantages over a coupled assay. Previously, we reported the use of membrane inlet mass spectrometry (MIMS) as a direct and continuous method to measure oxalate oxidase activity [41]. In the MIMS assay method, ^{13}C doubly labeled oxalate is used to distinguish the CO_2 generated by CsOxOx from adventitious CO_2 dissolved in the reaction mixtures. Since the use of labeled substrates