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Mechanistic studies of formate oxidase from *Aspergillus oryzae*: A novel member of the glucose-Methanol-choline oxidoreductase enzyme superfamily that oxidizes carbon acids

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ABSTRACT

Formate oxidase (FOX) from *Aspergillus oryzae* is the only GMC member that oxidizes a carbon acid rather than alcohols; thus, its catalytic mechanism may be different from that of other GMC members. We have used pH, solvent viscosity, and deuterium kinetic isotope effects, to investigate the catalytic mechanism of FOX. The enzyme followed a Bi-Bi sequential steady-state kinetic mechanism. The k_{cat} value was pH-independent between pH 2.8 and 6.8, suggesting a lack of ionizable groups in kinetic step(s) that limit the overall turnover of the enzyme. The $k_{cat}/K_{formate}$ value decreased from a value of 10,000 $M^{-1}s^{-1}$ at low pH with a pK_a value of 4.4, consistent with the requirement of a protonated group for substrate binding. An inverse viscosity dependence on the $k_{cat}/K_{formate}$ value indicated an isomerization of the Michaelis complex. The k_{cat}/K_{oxygen} value was 340,000 $M^{-1}s^{-1}$ and pH independent up to pH 6.0. The $^{D}k_{cat}$ and $^{D}(k_{cat}/K_{formate})$ values were 2.5 and 1.9, respectively, indicating that substrate CH bond cleavage is rate-limiting for FOX catalysis. Analytical ultracentrifugation indicated a concentration dependence of the oligomeric state of FOX. The $^{app}k_{red,H}$ value was ~75% that of $k_{cat,H}$, indicating that the anaerobic reduction of FOX was dependent on the oligomeric state of FOX.

1. Introduction

Formates are a toxic class of compounds that occur ubiquitously in nature through the microbial degradation of organic compounds as well as in methanol and formaldehyde metabolism [1]. Enzymes that are capable of oxidizing formate to carbon dioxide are classified as formate dehydrogenases (FDHs;[1] E.C. 1.2.1.2) or formate oxidases (FOXs; E.C. 1.2.3.1) [1–5]. The primary difference between these two groups of enzymes is the identity of the electron acceptor required to oxidize formate to CO_2; FDHs are dependent on NAD(P)$^+$ or a cytochrome to serve as an electron acceptor, while FOXs utilize molecular oxygen. A variety of plants, bacteria, and fungi have been shown to consume formate through various metabolic pathways, of which most involve the well-characterized FDH. FDHs comprise an extensively studied, heterogeneous group of enzymes that range from complex, metal containing enzymes with broad acceptor specificity to simpler non-metal containing, NAD(P)$^+$ dependent enzymes with lower affinity towards formate [6]. In methylotrophic organisms, NAD$^+$-dependent FDHs catalyze the terminal step in the catabolism of C1 compounds, thus supplying these organisms with energy and reducing equivalents [6]. In contrast, little is known about the oxidative route of formate metabolism via the recently identified FOX.

FOX has been isolated from the formaldehyde-resistant fungi *Aspergillus nomius* IRI013, *Paecilomyces variotii* IRI017, *Debaryomyces vanrijiae* MH201, and *Aspergillus oryzae* RIB40, suggesting that its primary role is to detoxify formate from the organism [1,2,7,8]. Previous reports demonstrated that FOX displays oxidase activity towards formate, but did not present detailed biochemical or kinetic data for the enzyme reaction [1–3,5,7,8]. The crystal structure of FOX from *Aspergillus oryzae* was reported to 2.2 Å resolution; analyses of the amino acid

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sequence and the three-dimensional structure identified the presence of a *p*-hydroxybenzoate hydroxylase fold, an FAD-binding domain containing an 8-formyl FAD, and a conserved histidine residue consistent with that found in members of the Glucose-Methanol-Choline (GMC) oxidoreductase enzyme superfamily [9–13]. The best characterized members of GMC oxidoreductase family, choline oxidase (CHO; EC 1.1.3.17), aryl-alcohol oxidase (AAO; EC 1.1.3.7), pyranose-2-oxidase (P2O; EC 1.1.3.10), alcohol oxidase (AO; EC 1.1.3.13), cholesterol oxidase (CO; EC 1.1.3.6), and glucose-1-oxidase (GO; EC 1.1.3.4) contain FAD as the coenzyme and typically oxidize alcohol substrates to aldehydes or ketones [13–23]. In contrast, FOX was shown to act on formate, a carbon acid, and to contain a non-covalently bound 8-formyl FAD. As a result, FOX is the first reported member within the GMC oxidoreductase superfamily that oxidizes a carbon acid rather than an alcohol substrate [13].

Previous kinetic characterization studies with FOX focused on establishing relative enzymatic activity over a range of pH and temperature values, but they did not establish the kinetic mechanism or demonstrate a mechanistic role for the 8-formyl FAD in catalysis [1,2,5,7]. The current study serves to establish the kinetic mechanism for FOX catalysis as well as the rate limiting step therein through the use of steady-state kinetics, deuterium kinetic isotope effects (KIE), viscosity effects, and rapid-reaction kinetic analysis. These combined results reveal a rate-limiting step dependent on FOX concentration as well as a unique pH effect on the kinetic parameters of the enzyme within the GMC oxidoreductase enzyme superfamily.

1.1. Experimental procedures

Materials. Sodium chloride, potassium phosphate (monobasic and dibasic), and ammonium sulfate were purchased from BDH/VWR (West Chester, PA). Ethylenediaminetetraacetic acid (EDTA), citric acid, glucose, glucose oxidase, sodium formate-*d*, sodium citrate, and lysozyme were purchased from Sigma (St. Louis, MO). Isopropyl-β-D-thiogalactoside (IPTG) and ampicillin were purchased from Gold Biotechnology (St. Louis, MO). Glycerol was obtained from Fisher Biotech (Pittsburgh, PA). Sodium acetate was purchased from Amresco (Solon, OH). Luria broth (LB) was purchased from U.S. Biological (Swampscott, MA). Acetic acid was purchased from EMD Chemicals (Gibbstown, N.J.) Coomassie protein assay reagent, bovine serum albumin standard, and HisPur™ Ni-NTA resin were purchased from Thermo Scientific (Rockford, IL).

Cloning, expression, and purification of His-tagged FOX. The DNA encoding FOX was synthesized by GeneArt (Life Technologies, Grand Island, NY). During synthesis, codons were optimized for expression in *Escherichia coli*. The optimized DNA encoding FOX was inserted into the *Nde*I-*Not*I restriction sites of the pET21c(+) expression vector (EMD Bioscience, Darmstadt, Germany) and transformed into *E. coli* Novablue cells (EMD Bioscience). After isolating the plasmid DNA from the *E. coli* Novablue and sequencing to verify the presence of the recombinant FOX_{AO} gene, *E. coli* BL21 (DE3) expression host strain (EMD Bioscience) was transformed with the resulting pET21-FOX_{AO} vector. Expression and purification of His-tagged FOX_{AO} was then performed as previously described with the following modifications: harvested cells were suspended in 25 mM potassium phosphate buffer, pH 7.5, supplemented with 20 mM imidazole, 100 mM NaCl, and 10% glycerol prior to sonication [7]. This homogenate was centrifuged at 16,000 g for 20 min and the resulting supernatant was loaded on a column of HisPur™ Ni-NTA resin equilibrated with the aforementioned suspension buffer. After the column was washed and eluted, the resulting enzymes was dialyzed and stored as previously described but modified so that all steps were performed in the absence of light [7].

Determination of FOX concentrations. The total protein concentration of the purified FOX enzyme stock was determined by Bradford assay using Coomassie protein assay reagent with bovine serum albumin as the standard [24]. The molar ratio of 8-fFAD to FOX was determined by extracting 8-fFAD from FOX through heat denaturation at 100 °C for 10 min, removing precipitated protein by centrifugation, and estimating the total 8-fFAD concentration in the supernatant using the molar extinction coefficient of 9000 $M^{-1}\,cm^{-1}$ at 450 nm as previously described [25,26]. From these measurements, the molar extinction coefficient of active, flavin bound FOX was determined to be 10,200 $M^{-1}\,cm^{-1}$ at 472 nm [27].

FOX activity assays. FOX activity assays were conducted using a Hansatech Oxygraph equipped with the DW1 electrode chamber and S1 electrode to determine the initial rate of O_2 consumption in solutions employing 0.2 μM flavin-bound FOX, dissolved oxygen (0.012–0.440 mM) and sodium formate or sodium formate-*d* (0.00025–3.0 M) in either 50 mM citrate (pH 2.8–5.8), 50 mM acetate (pH 3.6–5.5), or potassium phosphate (pH 5.8–6.8). All assays were performed at 25 °C and in triplicate. Dissolved oxygen concentrations were achieved by bubbling the appropriate mixture of N_2 and O_2 into the reaction buffer plus substrate prior to the addition of enzyme. Nitrogen and oxygen gases were mixed using a Maxtec® MaxBlend™ low flow air/oxygen mixer. The steady-state kinetic mechanism and kinetic parameters for any pH were determined by fitting the initial rate data to the Michaelis-Menten equation or the Lineweaver-Burk equation in combination with EnzFitter global fitting software (Cambridge, UK) analysis. When the initial reaction rates were determined by varying the concentration of both formate (A) and oxygen (B), the data were fit to Eq. (1), which describes a sequential mechanism, where K_a and K_b represent the Michaelis constant for formate and oxygen, respectively, K_{ia} is a constant required to obtain non-parallel lines in double reciprocal plots of the initial rate versus the concentration of substrate [28], and E_t is the total enzyme used in the reaction.

$$\frac{\nu}{E_t} = \frac{k_{cat}AB}{K_{ia}K_b + K_bA + K_aB + AB} \tag{1}$$

Plots of $k_{cat}/K_{formate}$ and k_{cat}/K_{oxygen} values as a function of pH were best fit to the single ionization models defined by Eq. (2) and Eq. (3), respectively, where H is $[H^+]$, y is $k_{cat}/K_{formate}$ or k_{cat}/K_{oxygen}, C is the pH independent value of y, and K_1 represents the dissociation constant for a group on the free enzyme or substrate.

$$logy = log\left[C / \left(1 + \frac{K_1}{H}\right)\right] \tag{2}$$

$$logy = log\left[C / \left(1 + \frac{H}{K_1}\right)\right] \tag{3}$$

Solvent viscosity effects on FOX kinetic parameters. FOX activity was assayed as previously described in the presence of 0.2 μM flavin-bound FOX, dissolved oxygen (0.440 mM), and sodium formate (0.25–200 mM) in an acetate buffer solution (pH 3.6) supplemented with varying concentrations of glycerol as the viscosigen [7]. Assays were performed in triplicate at 30 °C. The solvent viscosity effects on the k_{cat} and k_{cat}/K_{oxygen} values were best fit to Eq. (4), while the solvent viscosity effects on $k_{cat}/K_{formate}$ was best fit to Eq. (5), where $(k)_o$ and $(k)_\eta$ are the kinetic parameters k_{cat} and $k_{cat}/K_{formate}$ in the absence and presence of glycerol, respectively, S is the degree of viscosity dependence, and η_{rel} is the relative viscosity at 30 °C calculated from previously reported values at 20 °C [29].

$$\frac{(k)_o}{(k)_\eta} = S\eta_{rel} + 1 \tag{4}$$

$$\frac{(k)_o}{(k)_\eta} = S[(\eta_{rel})^{-1} - 1] + 1 \tag{5}$$

Rapid reaction kinetic analyses. Rapid-reaction mixing studies were performed using an Olis RSM1000 spectrometer equipped with a temperature controlled stopped-flow handling unit. The entire sample handling unit was made anaerobic by flushing the system with an oxygen scrubbing solution of 20 mM glucose and 10 units of glucose