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Structural and kinetic studies on the Ser101Ala variant of choline oxidase: Catalysis by compromise ☆,☆☆

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ABSTRACT

The oxidation of choline catalyzed by choline oxidase includes two reductive half-reactions where FAD is reduced by the alcohol substrate and by an aldehyde intermediate transiently formed in the reaction. Each reductive half-reaction is followed by an oxidative half-reaction where the reduced flavin is oxidized by oxygen. Here, we have used mutagenesis to prepare the Ser101Ala mutant of choline oxidase and have investigated the impact of this mutation on the structural and kinetic properties of the enzyme. The crystallographic structure of the Ser101Ala enzyme indicates that the only differences between the mutant and wild-type enzymes are the lack of a hydroxyl group on residue 101 and a more planar configuration of the flavin in the mutant enzyme. Kinetics established that replacement of Ser101 with alanine yields a mutant enzyme with increased efficiencies in the oxidative half-reactions and decreased efficiencies in the reductive half-reactions. This is accompanied by a significant decrease in the overall rate of turnover with choline. Thus, this mutation has revealed the importance of a specific residue for the optimization of the overall turnover of choline oxidase, which requires fine-tuning of four consecutive half-reactions for the conversion of an alcohol to a carboxylic acid.

Introduction

The reaction of choline oxidation catalyzed by choline oxidase (E.C. 1.1.3.17; choline-oxygen 1-oxidoreductase) has been extensively characterized (see [1] for a recent review) due to its relevance for genetically engineering crops with increased tolerance to environmental stress and the potential for designing therapeutic agents against pathogenic bacteria [2–5]. In brief, the reaction includes two reductive half-reactions where the FAD cofactor is reduced by the alcohol substrate and by an enzyme-associated aldehyde intermediate (Scheme 1) [6]. Each reductive half-reaction is followed by an oxidative half-reaction where the reduced FAD cofactor is oxidized by molecular oxygen with formation of hydrogen peroxide [6]. In the wild-type enzyme, the first reductive half-reaction is initiated by a kinetically fast abstraction of the hydroxyl proton of choline, which results in the formation of a transient alkoxide intermediate [6]. This is followed by a rate-limiting hydride ion transfer from the α-carbon of the alkoxide intermediate to the N(5) atom of the flavin resulting in the oxidation of choline to betaine aldehyde and reduction of the flavin [6]. Betaine aldehyde is subsequently hydrated in the active site of the enzyme to form *gem*-diol choline [7,8]. In the second reductive half-reaction, the *gem*-diol choline is oxidized to the product, glycine betaine. In the oxidative half-reactions, the reduced flavin reacts with oxygen by transferring two electrons to oxygen to form oxidized flavin and hydrogen peroxide [9]. These reactions occur when the organic product of the enzymatic reaction is bound at the active site, rather than after its release to the solvent [6,9]. Maximal overall enzymatic turnover is therefore attained through a fine balancing of the requirements that are necessary for each of the half-reactions to go forward efficiently without negatively impacting any of the other half-reactions.

By using site-directed mutagenesis the mechanistic roles of several functional groups in the active site of the choline oxidase from *Arthrobacter globiformis* have been elucidated. His99, which is the site of covalent attachment of the flavin to the protein moiety, is important for the optimal positioning of FAD in the enzyme–

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☆☆ The atomic coordinates and structure factors have been deposited in the Protein Data Bank as entry 3NNE.
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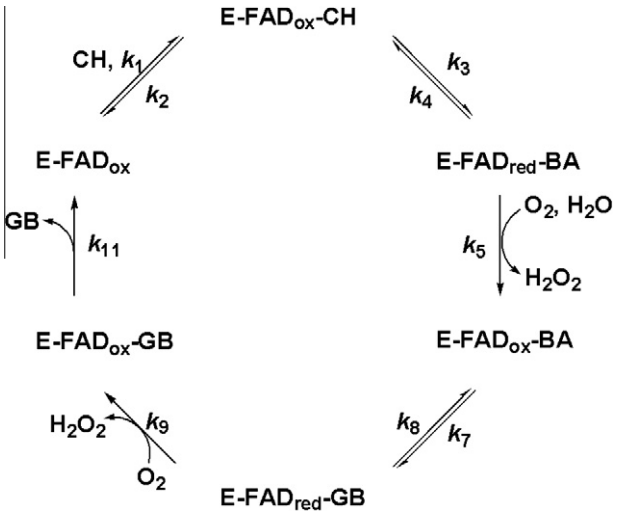

Scheme 1. The steady-state kinetic mechanism of choline oxidation catalyzed by choline oxidase. E, enzyme; FADox, oxidized flavin; CH, choline; FADred, reduced flavin; BA, betaine aldehyde; GB, glycine betaine.

alkoxide complex, thereby facilitating the environmentally assisted transfer of the hydride ion in the oxidation of choline [10]. Glu312 is the main site of substrate anchoring through electrostatic interaction with the trimethylammonium group of choline, playing a role for the optimal positioning of the substrate for efficient catalysis [11,12]. His351 is important for the binding and positioning of the substrate for efficient hydride ion transfer, for stabilization of the transition state developed during choline oxidation, and for fine-tuning the polarity of the active site [13]. Val464 participates primarily in the oxidative half-reaction where the reduced flavin is oxidized by oxygen, with minimal effects on the reaction of choline oxidation [14,15]. Finally, His466 modulates the electrophilicity of the enzyme-bound flavin, the polarity of the active site and contributes to the stabilization of the transition state for the oxidation of choline to betaine aldehyde [16,17]. Interestingly, none of the three active site histidines of choline oxidase (i.e., His99, His351, and His466) provides electrostatic stabilization of either the superoxide anion intermediate or the transition state that is formed in the reaction of the reduced flavin with oxygen [10,13,16]. In contrast, such an electrostatic stabilization is exerted by the positive charge of the enzyme-bound organic molecule undergoing oxidation in the reaction catalyzed by choline oxidase, as suggested by mechanistic studies with a substrate analog devoid of positive charge [18,19].

In the crystal structure of wild-type choline oxidase, the side chain of Ser101 is less than 4 Å from the N(5) atom of FAD and within hydrogen-bonding distance of the oxygen atom of DMSO, a ligand that was used in the crystallization of the enzyme [11]. This suggests that Ser101 may be actively involved in the oxidation of choline catalyzed by the enzyme. Here, we report the expression, purification, as well as the crystallographic and kinetic characterizations of the Ser101Ala variant of the enzyme. The results showed that replacement of Ser101 with alanine increases the apparent rates for the oxidative half-reactions by threefold, while decreasing those for the reductive half-reactions and the overall turnover of the enzyme by 10-fold. Thus, while the hydroxyl side chain of Ser101 is not essential for catalysis, it is important for the optimization of the enzymatic turnover of choline oxidase.

Experimental procedures

The mutant gene for the Ser101Ala enzyme was prepared using the QuikChange™ Site-Directed Mutagenesis kit following the manufacturer's instructions in the presence of 2% DMSO, as previously described [17,20]. The pET/*cod*Amg plasmid harboring the wild-type gene was used as template for mutagenesis [20]. Upon mutagenesis, the entire mutant gene (pET/*cod*Amg–Ser101Ala) was sequenced at the DNA Core Facility of Georgia State University to confirm the presence of the desired mutation. As an expression host, competent *Escherichia coli* Rosetta(DE3)pLysS cells were transformed with the mutant plasmid by electroporation. The mutant enzyme was expressed and the resulting enzyme was purified to homogeneity as previously described for wild-type choline oxidase [17,18,20].

Crystals of the Ser101Ala enzyme were grown by the hanging-drop vapor-diffusion method at room temperature. Purified Ser101-Ala (2 μL) at a concentration of 5 mg mL^{-1} was mixed with 2 μL from a 500 μL reservoir solution consisting of 80 mM sodium cacodylate, 20% v/v PEG6000, 20% v/v glycerol, 150 mM Mg–acetate at pH 6.0. Single crystals were transferred into a cryoprotectant consisting of reservoir solution containing 25% (v/v) glycerol and allowed to soak for approximately 2 min prior to flash-freezing in liquid nitrogen for data collection at Beamline 12B of the National Synchrotron Light Source at Brookhaven National Laboratory, NY. The data were integrated, scaled, and merged using the HKL2000 package [21]. The structures were solved by molecular replacement with PHASER [22] using the structure of wild-type choline oxidase (2JBV from the Protein Data Bank) as the starting model [23]. Refinement was carried out using Refmac5 [24] in CCP4 [25] and manual adjustment used the molecular graphics program COOT [26]. Structural figures were made with PyMol software [27].

Steady state kinetic parameters were measured with the method of the initial rates [28] at varying concentrations of both choline, or betaine aldehyde, and oxygen in 50 mM sodium pyrophosphate, pH 10.0, at 25 °C. Kinetic assays were performed at pH 10.0 because at this pH value the kinetic parameters k_{cat} and k_{cat}/K_m of choline oxidase are maximal and independent of pH [9]. Initial rates were determined by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen monitoring system (Hansatech Instrument Ltd.) at 25 °C. The assay reaction mixture was equilibrated at the desired concentration of oxygen by sparging the appropriate O_2/N_2 gas mixture for 10 min before the reaction was started with the addition of the enzyme. The initial rates measured with choline were fit to Eq. (1), which describes a sequential steady state kinetic mechanism in which $K_{choline}$ and K_{oxygen} are the Michaelis constants for choline and oxygen and k_{cat} is the overall turnover number of the enzyme (e) when saturated with both substrates. The initial rates measured with betaine aldehyde were fit to Eq. (2), which describes a sequential steady state kinetic mechanism of the type described by Eq. (1) where $K_{aldehyde} \ll K_{oxygen}K_{ia}$

$$\frac{v}{e} = \frac{k_{cat}[\text{choline}][\text{oxygen}]}{K_{choline}[\text{oxygen}] + K_{oxygen}[\text{choline}] + [\text{choline}][\text{oxygen}] + K_{ia}K_{oxygen}} \quad (1)$$

$$\frac{v}{e} = \frac{k_{cat}[\text{betaine} - \text{aldehyde}][\text{oxygen}]}{K_{oxygen}[\text{betaine} - \text{aldehyde}] + [\text{betaine} - \text{aldehyde}][\text{oxygen}] + K_{ia}K_{oxygen}} \quad (2)$$