



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.

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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.

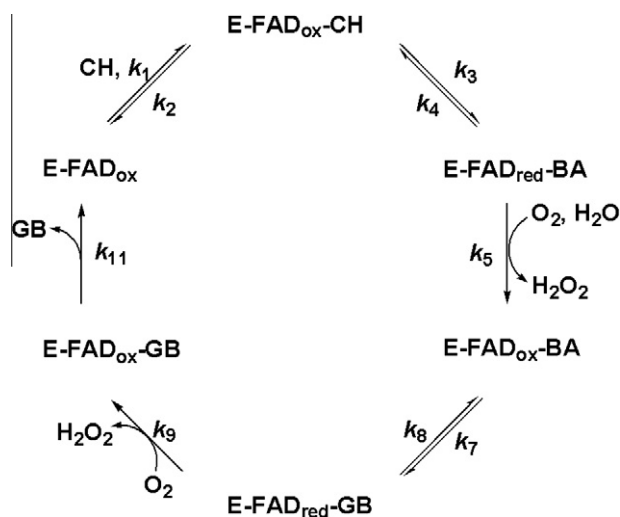
By using site-directed mutagenesis the mechanistic roles of several functional groups in the active site of the choline oxidase from *Arthrobacter globiformis* have been elucidated. His99, which is the site of covalent attachment of the flavin to the protein moiety, is important for the optimal positioning 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/codAmg plasmid harboring the wild-type gene was used as template for mutagenesis [20]. Upon mutagenesis, the entire mutant gene (pET/codAmg-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 O₂/N₂ 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 $K_{aldehyde} \ll K_{oxygen}K_{ia}$

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

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

The reductive half-reaction with betaine aldehyde was carried out by using a Hi-Tech SF-61 stopped-flow spectrophotometer at 25 °C and pH 10.0. The rate constants for flavin reduction were measured by monitoring the decrease in absorbance at 452 nm that results from the anaerobic mixing of the enzyme and betaine aldehyde, as previously described for the wild-type enzyme [6]. Glucose (5 mM) and glucose oxidase (0.5 μM) were added to the substrate and enzyme solutions to scavenge possible trace amounts of oxygen. The Ser101Ala enzyme was mixed anaerobically with an equal volume of betaine aldehyde, obtaining reaction mixtures with 10 μM enzyme and 0.2–5 mM betaine aldehyde. At each concentration of the substrate, the rate constants for flavin reduction were recorded in triplicate, with measurements usually differing by ≤5%. Stopped-flow traces were fit to Eq. (3), which describes a single exponential process where k_{obs} is the observed first-order rate constant for flavin reduction, A is the value of absorbance at 452 nm at time t , B is the amplitude of the absorbance change, and C is an offset value that accounts for the non-zero absorbance value at infinite time. Kinetic parameters for the reductive half-reaction were determined by using Eq. (4), where k_{obs} is the observed first-order rate constant for the reduction of the enzyme-bound flavin at any given concentration of substrate, k_{red} is the limiting first-order rate constant for flavin reduction at saturated substrate concentration, and ${}^{\text{app}}K_d$ is the macroscopic dissociation constant for binding of the substrate to the enzyme

$$A = B \exp(-k_{\text{obs}}t) + C \quad (3)$$

$$k_{\text{obs}} = \frac{k_{\text{red}}A}{{}^{\text{app}}K_d + A} \quad (4)$$

Results

The Ser101Ala enzyme was expressed and purified to high yields by using the same protocol previously used for the wild-type enzyme [11,20]. As in the case of wild-type choline oxidase, the enzyme-bound flavin was present as a mixture of oxidized and anionic flavosemiquinone throughout the purification procedure, with the fully oxidized enzyme obtained after extensive dialysis at pH 6.0 [9,20]. The purified Ser101Ala enzyme showed absorbance bands centered at 373 nm ($\epsilon = 8.9 \text{ mM}^{-1} \text{ cm}^{-1}$) and 452 nm

($\epsilon = 9.5 \text{ mM}^{-1} \text{ cm}^{-1}$) at pH 8.0, compared to 367 nm ($\epsilon = 10.0 \text{ mM}^{-1} \text{ cm}^{-1}$) and 455 nm ($\epsilon = 11.4 \text{ mM}^{-1} \text{ cm}^{-1}$) for the wild-type enzyme [9], consistent with an altered flavin microenvironment in the mutant enzyme. The enzymatic activity of the Ser101Ala enzyme was 3.3 s^{-1} with saturated choline (i.e., 10 mM) at pH 7.0 and 25 °C, compared to 15 s^{-1} for the wild-type [11], suggesting that Ser101 is important, but not essential, for catalysis.

The Ser101Ala enzyme crystallized in the primitive monoclinic space group $P2_1$ with eight subunits assembled as four homodimers in the asymmetric unit. X-ray data to 2.2 Å were used to refine the structure of the Ser101Ala enzyme to a final R-factor of 22.8% (Table 1). The final model consisted of residues 1–530¹, FAD, and acetate from the crystallization solution in the active site of the enzyme. The acetate ligand in the active site of the Ser101Ala enzyme had the same orientation and positioning in the eight subunits that crystallized; its oxygen atoms are 3.8 Å from both the flavin N(5) atom and the N(3) atom of His351 (Fig. 1C). When the homodimers of the Ser101Ala enzyme were superimposed on those of the wild-type enzyme the average rmsd values were 0.41 Å for 527 equivalent C α atoms in each chain, indicating no significant structural differences between the backbone atoms of the two enzymes. The active sites of the two structures are compared in Fig. 1, showing essentially no differences in the relative location of the flavin and the active site residues in the two enzymes, although there are differences in the conformation of the flavin. Interestingly, although the replacement of the side chain on residue 101 from serine to alanine resulted in loss of a hydrogen bond with His351, all of the active site residues are located in the same positions and orientations in the mutant and wild-type enzymes. Moreover, the electron density maps clearly indicate that FAD in the Ser101Ala enzyme is covalently linked to the His99 N ϵ 2 atom as seen in the wild-type [11].

A notable difference between the wild-type and the Ser101Ala enzymes is the isoalloxazine moiety of the FAD cofactor in the variant enzyme being more planar than that of the wild-type. In the latter enzyme, the C(4a) atom was shown to be in an sp^3 hybridization due to the presence of an oxygen adduct that is not observed in the Ser101Ala variant enzyme resulting in the C(4a) atom being sp^2 hybridized [11,29]. In the Ser101Ala enzyme the isoalloxazine ring system showed a slight bend between the two planes containing the benzene and pyrimidine moieties defining an 8° angle along the N(5)–N(10) axis (Fig. 1B). A similar bend was recently observed in the structure of another active site mutant form of choline oxidase where Val464 is replaced with alanine, for which the C(4a) atom of FAD was also shown to be sp^2 hybridized [14]. In summary, the major differences observable in the structures of the Ser101Ala and wild-type enzymes are the hybridization of the flavin C(4a) atom and the loss of the hydroxyl group of Ser101.

The steady state kinetic parameters with choline or betaine aldehyde as substrate for the Ser101Ala enzyme were determined at varying concentrations of oxygen, by measuring the initial rates of oxygen consumption with a Clark-type oxygen electrode at pH 10.0 and 25 °C. As for the case previously reported for the wild-type enzyme [8,9], the best fits of the data were obtained with Eq. (1) for choline and Eq. (2) for betaine aldehyde (Fig. 2). As summarized in Table 2, the Ser101Ala enzyme showed between threefold and fourfold increases in the second-order rate constants for oxygen ($k_{\text{cat}}/K_{\text{oxygen}}$) with choline and betaine aldehyde. In contrast, a 10-fold decrease in both the

Table 1
Crystallographic data collection and refinement statistics.

Space group	$P2_1$
Unit cell dimensions (Å)	
A	69.26
B	346.03
C	105.92
β	94.33
Unique reflections	150,964
R_{merge} (%) overall (final shell)	11.1(25.8) ^a
$I/\sigma(I)$ overall (final shell)	14 (3.2)
Completeness (%) overall (final shell)	90.4 (50.3)
Data range for refinement (Å)	10–2.47
R_{cryst} (%) ^b	22.8
R_{free} (%) ^c	29.2
No. of solvent atoms (total occupancies)	333 (207.3)
RMS deviation from ideality	
Bonds (Å)	0.014
Angle distance (Å)	0.021
Average B-factors (Å ²)	
Main-chain atoms	26.4
Side-chain atoms	27.2
FAD	18.5
Solvent	20.3

^a Values in parentheses are given for the highest resolution shell.

^b $R_{\text{cryst}} = \sum |F_{\text{obs}} - F_{\text{cal}}| / \sum F_{\text{obs}}$.

^c $R_{\text{free}} = \sum_{\text{test}} (|F_{\text{obs}}| - |F_{\text{cal}}|)^2 / \sum_{\text{test}} F_{\text{obs}}^2$.

¹ In the 3D structure of the Ser101Ala enzyme reported here (XXXX), the last 16–18 amino acids at the C-terminus of different subunits are not observable. Similarly, the wild-type enzyme was reported with coordinates for the first 528 residues and no ordered density for the last 18 amino acid residues at the C-terminus (2JBV). A third structure of a choline oxidase variant recently reported also has coordinates for the first 530 residues, but not for the last 16 residues at the C-terminus (3LJP). This is most likely due to the C-terminal end of the protein being highly flexible.

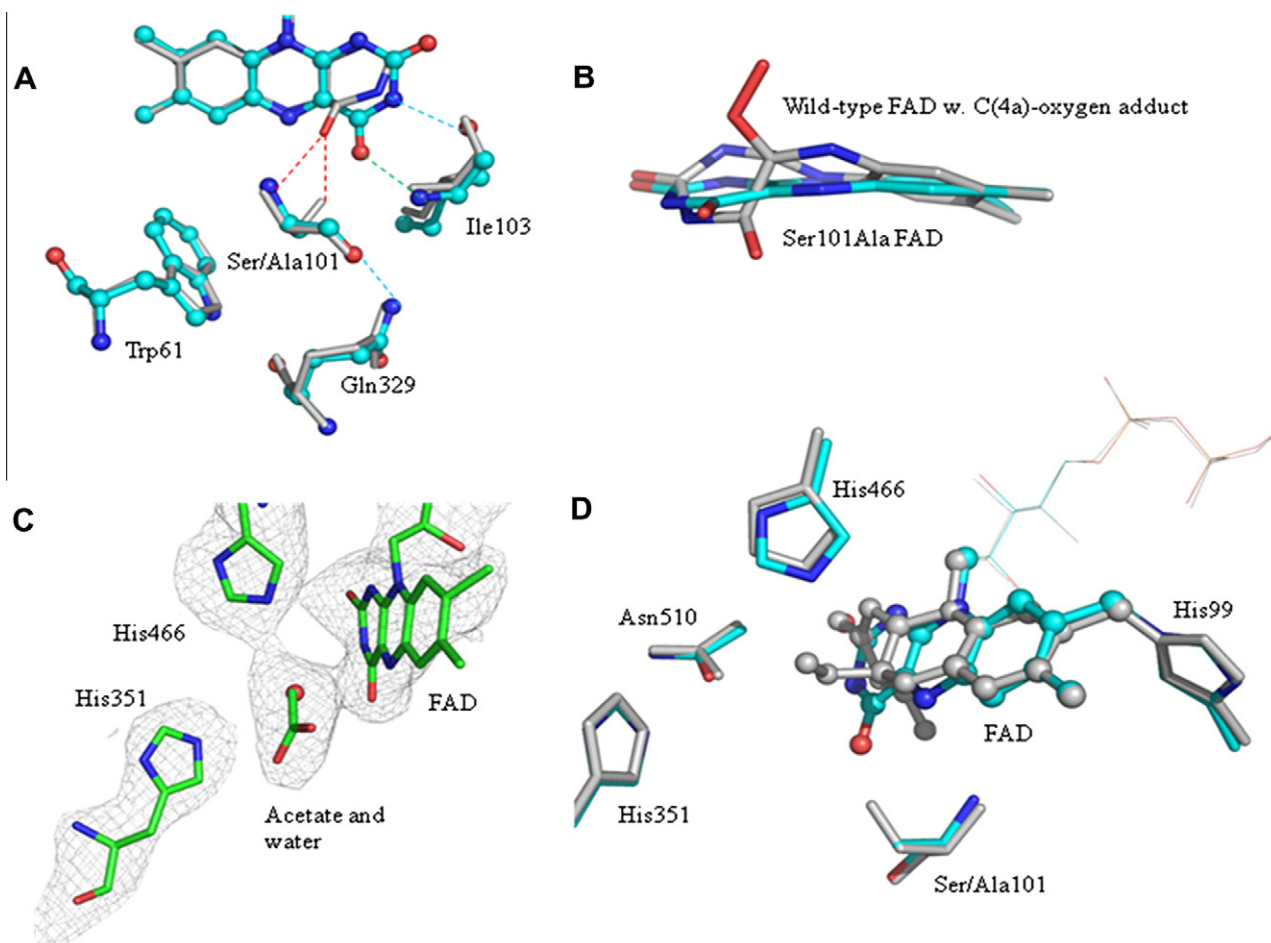


Fig. 1. Comparison of crystal structures of the Ser101Ala and wild-type enzymes of choline oxidase. The residues with carbons in cyan represent the Ser101Ala structure, whereas the carbon atoms for the wild-type enzyme are in gray. Panel A shows the interactions at the Ser101Ala mutation site. Hydrogen bonds only present in the wild-type enzyme are colored red, those only in the Ser101Ala enzyme are colored green, and those observed in both enzymes are colored cyan. Panel B illustrates the superposition of the FAD isoalloxazine ring from the wild-type (grey) with the C(4a)-oxygen adduct (red) and the Ser101Ala enzymes (colored by element type) of choline oxidase. Note that the isoalloxazine ring of the Ser101Ala enzyme is more planar than that of the wild-type structure. Panel C shows the omit-Fourier density map with contour level at 2 for the active site of the Ser101Ala structure. Panel D shows a comparison of the active sites of wild-type and Ser101Ala choline oxidase. The wild-type enzyme structure is from pdb file 2jbv [11].

second-order rate constant for the capture of choline ($k_{\text{cat}}/K_{\text{choline}}$) and the overall turnover number of the enzyme with choline as substrate (k_{cat}) were observed. Since the $k_{\text{cat}}/K_{\text{betaine aldehyde}}$ value could not be determined by using a steady state kinetic approach due to $K_{\text{aldehyde}} \ll K_{\text{oxygen}}K_{\text{ia}}$, the reductive half-reaction with betaine aldehyde was investigated by anaerobically mixing the enzyme and the substrate in a stopped-flow spectrophotometer at pH 10.0 and 25 °C. As previously reported for the wild-type enzyme [6], the absorbance at 452 nm of the Ser101Ala enzyme decreased in a single exponential manner upon mixing with betaine aldehyde (Fig. 3). A plot of the k_{obs} value for flavin reduction as a function of the concentration of betaine aldehyde followed saturation kinetics, allowing the determination of the limiting rate constant for flavin reduction at saturated substrate (k_{red}) and the macroscopic equilibrium constant for the formation of the enzyme–betaine aldehyde complex ($^{\text{app}}K_{\text{d}}$). A second-order rate constant for the capture of betaine aldehyde to yield species committed to catalysis ($k_{\text{red}}/K_{\text{d}}$) of $52,000 \text{ M}^{-1} \text{ s}^{-1}$ was calculated from the stopped-flow kinetic data (Table 2), which was sixfold lower than the value of $320,000 \text{ M}^{-1} \text{ s}^{-1}$ previously determined for the wild-type enzyme [6]. Thus, replacement of Ser101 with alanine results in lowered second-order rate constants for both the reductive half-reactions, but increased second-order rate constants for both the oxidative half-reactions catalyzed by the enzyme.

Discussion

In choline oxidase, each of the four half-reactions in which the flavin is reduced by the organic molecules and oxidized by molecular oxygen is adjusted to achieve the most efficient overall turnover of the enzyme rather than maximal efficiencies. This conclusion stems from the comparison of the steady state and rapid reaction kinetic data of the Ser101Ala and the wild-type enzymes. Indeed, in spite of increased efficiencies in the oxidative half-reactions catalyzed by the enzyme lacking the hydroxyl group on residue 101, there is a 10-fold decrease in the overall turnover of the enzyme with choline that is imputable to decreased efficiencies in the reductive half-reactions catalyzed by the enzyme. In the wild-type enzyme, Ser101 is on the *si*-face of the isoalloxazine moiety of the flavin with its hydroxyl group being 4 Å away from the flavin N(5) atom. Such a geometric arrangement of residue 101, as well as of all of the other residues in the active site of choline oxidase, is maintained in the crystallographic structure of the Ser101Ala enzyme, which clearly lacks the hydroxyl group. Thus, the differences seen in the kinetic properties of the mutant and wild-type enzymes are solely due to the lack of the hydroxyl group on residue 101, rather than originating from other geometric or steric factors.

Replacement of Ser101 with alanine results in decreased efficiencies in the reductive half-reactions with betaine aldehyde

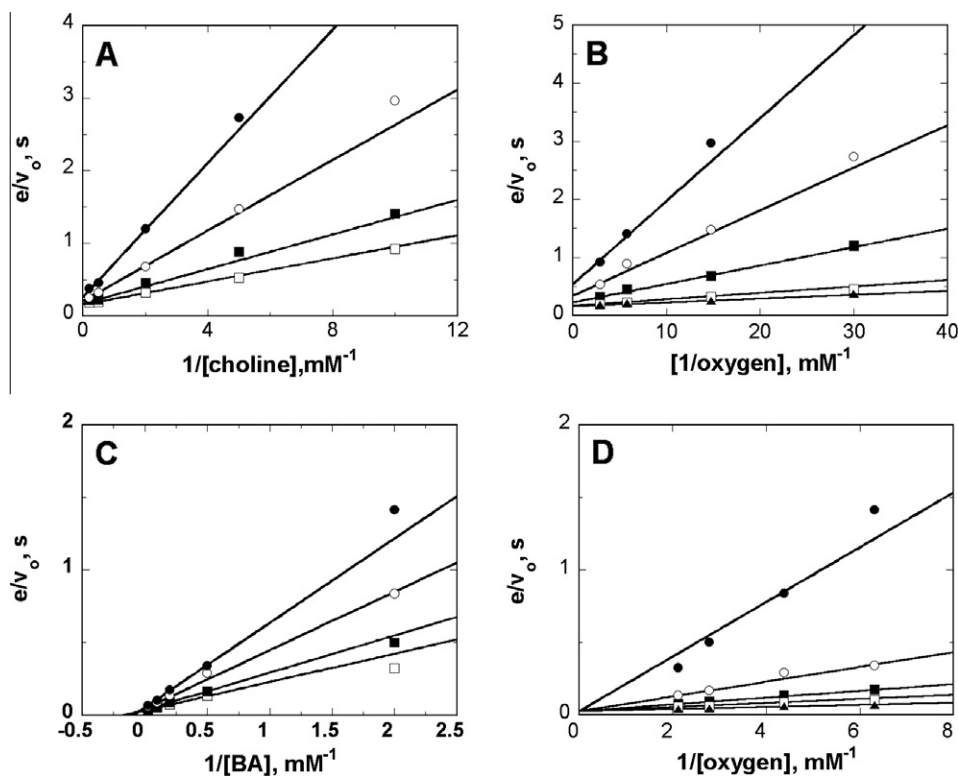


Fig. 2. Double reciprocal plots of Ser101Ala catalyzed oxidation of choline and betaine aldehyde. Choline oxidase activity was measured at varying concentrations of both choline and oxygen or betaine aldehyde and oxygen in 100 mM sodium pyrophosphate, pH 10.0, at 25 °C. Panel A, e/v_o as a function of the inverse choline concentration determined at several fixed concentrations oxygen: (●) 0.033 mM; (○) 0.068 mM; (■) 0.172 mM; (□) 0.344 mM. Panel B, e/v_o as a function of the inverse oxygen concentration determined at several fixed concentrations choline: (●) 0.1 mM; (○) 0.2 mM; (■) 0.5 mM; (□) 2 mM; (▲) 5 mM. Panel C, e/v_o as a function of the inverse betaine aldehyde concentration determined at several fixed concentrations oxygen: (●) 0.16 mM; (○) 0.23 mM; (■) 0.36 mM; (□) 0.47 mM. Panel D, e/v_o as a function of the inverse oxygen concentration determined at several fixed concentrations betaine aldehyde: (●) 0.5 mM; (○) 2 mM; (■) 5 mM; (□) 10 mM; (▲) 40 mM.

Table 2

Comparison of the kinetic parameters of Ser101Ala^a and wild-type^b choline oxidase at pH 10.0.

Substrate	Kinetic parameters	Definition ^c	Ser101Ala	Wild-type
Choline	k_{cat} (s ⁻¹)	$\left(\frac{k_3 k_7}{k_3 + k_7} \right)^d$	6.7 ± 0.1	60 ± 1
	k_{cat}/K_m (M ⁻¹ s ⁻¹)	$\frac{k_1 k_3}{k_2 + k_3}$	$25,600 \pm 2000$	$237,000 \pm 9000$
	k_{cat}/K_{oxygen} (M ⁻¹ s ⁻¹)	$\frac{k_5 k_9}{k_5 + k_9}$	$261,000 \pm 6500$	$86,400 \pm 3600$
	K_m (mM)	$\frac{k_2 (k_2 + k_3)}{k_1 (k_3 + k_7)}$	0.26 ± 0.02	0.25 ± 0.01
	K_{oxygen} (mM)	$\frac{k_3 k_7 (k_5 + k_9)}{k_5 k_9 (k_3 + k_7)}$	0.026 ± 0.001	0.69 ± 0.03
	K_{ia} (mM)	$\frac{k_2 k_4 k_9}{k_1 k_3 (k_5 + k_9)}$	3.6 ± 0.1	0.14 ± 0.01
Betaine aldehyde	k_{cat} (s ⁻¹)	k_7	43 ± 3	133 ± 4
	k_{cat}/K_{oxygen} (M ⁻¹ s ⁻¹)	k_9	$203,400 \pm 33,400$	$53,400 \pm 1600$
	K_{oxygen} (mM)	k_7/k_9	0.2 ± 0.03	2.5 ± 0.01
	k_{red} (s ⁻¹)	k_7	47 ± 2	135 ± 4
	K_d (mM)	– ^e	0.9 ± 0.1	0.45 ± 0.1

^a Conditions: 50 mM sodium pyrophosphate, pH 10.0, at 25 °C.

^b From Ref. [6].

^c Kinetic data are readily accounted for with the kinetic mechanism of Scheme 1 [6].

^d While $k_7 \gg k_3$, k_{cat} is equal to $(k_2 + k_3)/k_1$ and K_{oxygen} is equal to $k_3(k_5 + k_9)/(k_5 k_9)$, as for the case of the Ser101Ala enzyme.

^e Not applicable to the mechanism of Scheme 1.

and choline. Evidence for this conclusion comes from the comparison of the steady state and rapid reaction kinetic data determined with the Ser101Ala and the wild-type enzymes presented here. Indeed, the second-order rate constants for the capture of choline ($k_{cat}/K_{choline}$) and betaine aldehyde (k_{red}/K_d) to yield enzyme–substrate complexes that are committed to flavin reduction were 10- and sixfold lower in the enzyme lacking the hydroxyl group on residue 101. In the wild-type enzyme, the chemical steps of flavin reduction with choline and the aldehyde intermediate are fully

rate-limiting in both reductive half-reactions and are the sole contributors to the overall rate of turnover of the enzyme [6]. Moreover, the rate for the overall turnover with betaine aldehyde as substrate is limited solely by the chemical step of flavin reduction, with a rate constant of 135 s^{-1} [6]. The latter conclusion also applies to the Ser101Ala enzyme, since the rate constant for flavin reduction with betaine aldehyde is very similar to the turnover number of the enzyme (i.e., 47 s^{-1} and 43 s^{-1} for k_{red} and k_{cat} , respectively). In contrast, the overall turnover of the Ser101Ala

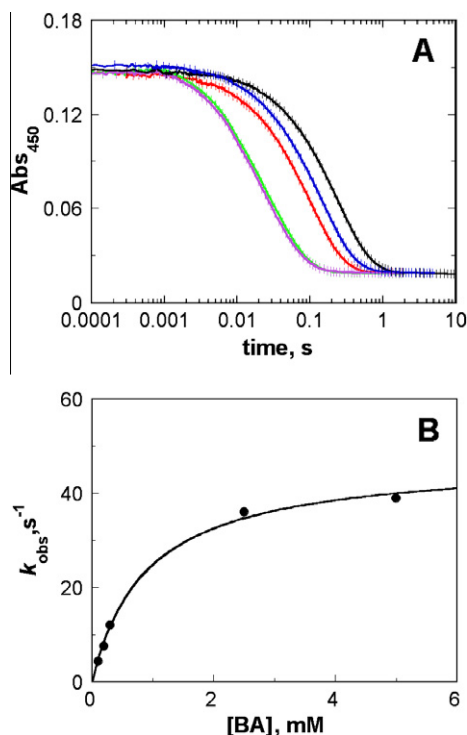


Fig. 3. Reductive half-reaction of the Ser101Ala enzyme with betaine aldehyde. Panel A shows the reduction traces with 0.1 mM (black), 0.2 mM (blue), 0.3 mM (red), 2.5 mM (green) and 5 mM (fuchsia) betaine aldehyde. All traces were fit to Eq. (3). Time indicated is after the end of flow, i.e., 2.2 ms. For clarity, one experimental point every 5 is shown (vertical lines). Panel B shows the observed rate constants for flavin reduction as a function of the concentration of betaine aldehyde. Data were fit to Eq. (4). Buffer used was 50 mM sodium pyrophosphate, pH 10.0.

enzyme with choline is primarily limited by the chemical step of flavin reduction with choline (e.g., with a k_{cat} value of 6.7 s^{-1} and a k_{red} value of 6.8 s^{-1} with choline as substrate at pH 10.0 [Hongling Yuan and Giovanni Gadda; unpublished results]) without contribution of the subsequent chemical step of flavin reduction involving the aldehyde intermediate. Since the substrate for the second reaction of flavin reduction in choline oxidase is the *gem*-diol species rather than the aldehyde, it is possible that the extra hydroxyl group of the *gem*-diol substrate compensates for the lack of the hydroxyl group on residue 101 in the mutant enzyme [7], thereby making the chemical step of flavin reduction significantly faster with betaine aldehyde than choline. The alternative possibility of betaine aldehyde being released from the active site of the Ser101-Ala enzyme turning over with choline rather than proceeding through the second step of flavin reduction seems less likely based on the similarity of the three dimensional structures of the mutant and wild-type enzymes and the fact that the active site is completely secluded from the bulk solvent in choline oxidase [11,14,29].

Lack of a hydroxyl group on residue 101 in the active site of choline oxidase results in increased efficiencies in the oxidative half-reactions with either choline or betaine aldehyde as substrate. Evidence for this conclusion comes from the comparison of the steady state kinetic data, showing that the second-order rate constants for reaction of the reduced Ser101Ala enzyme with oxygen ($k_{\text{cat}}/K_{\text{oxygen}}$) were three- to fourfold larger than those observed with the wild-type enzyme. The microenvironment in proximity of the reactive C(4a) atom of the reduced flavin is considerably less hydrophilic in the Ser101Ala enzyme than in the wild-type enzyme, suggesting that this may be the cause of the increased reactivity with oxygen of the reduced flavin

in the mutant enzyme. The importance of a hydrophobic microenvironment in proximity of the C(4a) atom of the flavin was recently demonstrated in choline oxidase with variant enzymes where Val464 was replaced with either alanine or threonine [14]. In that case, the less hydrophobic character of the mutant enzymes at position 464, which is proximal to the C(4a) atom of the flavin on the opposite face of the flavin than Ser101, resulted in 50-fold decreases efficiencies in the $k_{\text{cat}}/K_{\text{oxygen}}$ value with choline as substrate for the mutant enzyme [14]. Alternatively, the increased efficiencies in the oxidative half-reactions may be due to the absence of the hydrogen-bonding interaction between the O(4) atom of the flavin and the side chain of the residue 101. Interestingly, the Ser101Ala variant is the first case of increased efficiency in the oxidative half-reaction among many mutant variants of choline oxidase where no changes were observed with respect to the wild-type enzyme (His99Asn, Glu312Asp, His351Ala, and His466Ala) or where decreased efficiencies were observed (Val464Ala and Val464Thr) [10,11,13–15]. To our knowledge, the choline oxidase Ser101Ala mutant represents the first instance in which the efficiency of a flavoprotein oxidase for the reaction of the reduced flavin with oxygen has been increased by at least threefold with respect to the wild-type enzyme.

The flavin adduct between the C(4a) atom of FAD and oxygen that was previously observed in the crystal structure of the wild-type form of choline oxidase [11] was not observed in the structure of the Ser101Ala enzyme reported in this study. In the case of the wild-type enzyme many interactions were shown to stabilize the flavin adduct within the active site of the enzyme, among which a hydrogen bond involving the O(4) atom of the flavin and the hydroxyl group of Ser101 [11,29]. Thus, it is possible that the absence of the flavin adduct in the Ser101Ala enzyme is due to the inability to form a hydrogen bond between the O(4) atom of the isoalloxazine ring and the side chain of residue 101. Alternatively, the lack of a flavin adduct in the structure of the Ser101Ala enzyme may simply be due to the FAD not being reduced during the data collection at the synchrotron, thereby preventing the formation of the C(4a) adduct between the reduced enzyme-bound flavin and oxygen. A more planar flavin with respect to the wild-type enzyme was recently reported for another mutant form of choline oxidase, where Val464 was replaced with alanine [14]. In that case, a significantly lowered apparent affinity of the reduced enzyme for oxygen, which could not be saturated at concentrations of oxygen as high as 1 mM, was proposed to explain the lack of a flavin adduct in the crystal structure of the mutant enzyme [14].

In conclusion, replacement of Ser101 with alanine in the active site of choline oxidase produced a variant enzyme with increased efficiencies in the oxidative half-reactions where the reduced flavin reacts with oxygen and decreased efficiencies in the reductive half-reactions with choline and the aldehyde intermediate of reaction. Interestingly, this mutation has disclosed the importance of a protein hydroxyl group in close proximity of the flavin C(4a) atom in 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.

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