Catechol Oxidase Activity of Di-Cu\textsuperscript{2+}-Substituted Aminopeptidase from *Streptomyces griseus*

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The predominant theory concerning enzymatic catalysis is that enzymes have evolved to perform specific chemical transformations on their respective substrates by stabilizing the transition state (TS\textsuperscript{A}),\textsuperscript{1} such as the tetrahedral TS\textsuperscript{D} during peptide hydrolysis by metallohydrolases. However, several examples of enzyme catalytic promiscuity,\textsuperscript{2} that is, the catalyses of more than one type of reactions by a single enzyme, have been observed, particularly within enzyme superfamilies which can be mostly attributed to divergent evolution.\textsuperscript{2} Nevertheless, these results still reflect that if the TS\textsuperscript{A} of an alternative reaction can be stabilized by an enzyme the reaction may take place effectively. While many metal-substituted derivatives of metalloenzymes are active, some are inert, which nevertheless can provide structural information regarding the enzyme—substrate complexes that may not be readily available from active derivatives.\textsuperscript{3} Metal substitution has thus been widely used to shed light on the structure and mechanism of metalloproteins.\textsuperscript{4} Using apo metalloprotein molecules as natural ligands and tuning their structures/activities with the active-site metals are unique approaches toward exploration and discovery of new metalprotein systems. However, altering catalytic specificity based on simple metal substitution of metalloenzymes was rarely reported in the literature.\textsuperscript{5} Recently, the dinuclear aminopeptidase from *Streptomyces griseus* (SgAP) was found to exhibit a catalytic promiscuity toward phosphoester hydrolysis with a catalytic proficiency >40 billion under physiological conditions.\textsuperscript{5} Since phosphoesters are structurally and mechanistically different from peptides during hydrolysis, this catalytic promiscuity is quite unusual. Herein we present that di-Cu\textsuperscript{2+}-substituted SgAP (CuCu-SgAP) exhibits a remarkable oxidative activity, approaching that of native catechol oxidase. The multifunctional character of SgAP and its metal derivatives makes this enzyme an exceptional candidate for further exploration of protein structure and function in combination with molecular biology techniques.

The purification of SgAP\textsuperscript{7,8} and preparation of its metal derivatives\textsuperscript{8} followed published procedures. The oxidation of the prototypical catechol oxidase substrate, 3,5-di-tert-butylcatechol (DTC), by CuCu-SgAP follows Michaelis–Menten kinetics (C, Figure 1A),\textsuperscript{9} affording $k_{\text{cat}} = 1.45 \, \text{s}^{-1}$ and $K_m = 0.44 \, \text{mM}$. The catalytic efficiency of this catalysis ($k_{\text{cat}}/K_m = 3295 \, \text{M}^{-1} \, \text{s}^{-1}$) under mild conditions at 25 °C and pH 7.0 in 50.0 mM HEPES and 5.0 mM Ca\textsuperscript{2+} is much better than that of a number of synthetic metal complexes\textsuperscript{10} and is only ~10 times smaller than that of catechol oxidase from *gypsywort* (32 mM\textsuperscript{–1} s\textsuperscript{–1}).\textsuperscript{11}

To demonstrate that the high oxidative activity observed herein is indeed attributed to SgAP, activity profiles were obtained in stoichiometric Cu\textsuperscript{2+} titration of apo-SgAP (Figure 1B) and thermodeactivation.\textsuperscript{12} The results show that the activities toward the oxidation of DTC and the hydrolysis of Leu-p-nitroanilide\textsuperscript{8,13} (Leu-pNA) are parallel. We have previously shown that metal ions bind sequentially to the two metal-binding sites of apo-SgAP, with the

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) Aerobic oxidation of DTC (○, pH 7.0, left scale) and catechol (●, pH 8.0, right scale) by CuCu-SgAP.\textsuperscript{2} The inset shows competitive inhibition of DTC oxidation by dinuclear AP-specific bestatin at 0.0, 5.0, 10.0, and 20.0 \muM (from bottom), similar to the inhibition toward peptide hydrolysis. (B) Metal titration to apo-SgAP in 100.0 mM HEPES at pH 7.0 and monitored with Leu-pNA hydrolysis and DTC oxidation, and fitted to Cd\textsuperscript{2+}-binding pattern previously published.\textsuperscript{13}

hydrolytic activity controlled by the relative magnitude of the two metal-binding constants.\textsuperscript{8,13} The oxidation reaction reaches full activity with the addition of 2 equiv of Cu\textsuperscript{2+} and can be well fitted as in the hydrolytic catalysis (Figure 1B). The data indicate that the two completely different reactions, hydrolysis and oxidation, are carried out by a single enzyme with a dimetal active center.

Both hydrolytic and oxidative activities are found to be competitively inhibited by the dinuclear AP-specific inhibitor bestatin (inset, Figure 1A) with similar $K_i$ values, 11.0 \muM for Leu-pNA hydrolysis and 8.8 \muM for DTC oxidation. This observation indicates that the inhibitor and the two substrates bind to the active site in a similar way in these two reactions. SgAP is specific toward hydrophobic amino acids based on kinetic\textsuperscript{2} and crystallography\textsuperscript{14} studies. The hydrophobic pocket for specific recognition in SgAP may facilitate the binding of DTC to the active site via its tert-butyl groups. DTC binding can also be promoted via deprotonation of the OH groups in DTC facilitated by the strong Lewis acidic Cu\textsuperscript{2+}. The structural similarity between these two substrates can be clearly shown when they are superimposed to each other (Figure 2). To further demonstrate this point, the rate of catechol oxidation is measured,\textsuperscript{9} which gives $k_{\text{cat}} = 0.066 \, \text{s}^{-1}$, $K_m = 0.021 \, \text{mM}$, and $k_{\text{cat}}/K_m = 3220 \, \text{M}^{-1} \, \text{s}^{-1}$ at pH 7.0 (●, Figure 1A); however, it is not noticeable at pH 7.0. The decrease in $K_m$ compared to that at pH 7.0 is probably due to the decrease in $k_{\text{cat}}$ since $K_m = (k_{\text{cat}} + k_{-\text{cat}})/k_i$ in Michaelis–Menten kinetics. CuCu-SgAP can also oxidize a biorelevant catechol, dopamine, with $k_{\text{cat}} = 0.097 \, \text{s}^{-1}$ and $K_m =...
oxidation, due to its easy purification, high thermal stability, and the iso-butyl group is for hydrophobic recognition. A small catalytic efficiency of 162 M$^{-1}$ s$^{-1}$ at pH 7.0, which might be attributed to its different metal-binding and recognition from Leu (cf. Figure 2).

The oxidation of DTC showed a [H$_2$O$_2$]-dependent increase in activity. In the presence of 10.0 mM (0.034%) H$_2$O$_2$, the $k_{cat}$ value of CuCu-SgAP is further increased (2.03 s$^{-1}$) with only a small change in $K_m$ (0.32 mM), which affords a second-order rate constant nearly doubled to 6344 M$^{-1}$ s$^{-1}$. The proposed mechanism of catechol oxidase includes an active Cu$^{2+}$-η$_2$-µ$_2$-η$_2$-peroxo species that is isoelectronic to Cu$^{2+}$-O$_2$ and Cu$^{3+}$-bis-µ-oxo, which are all capable of performing the 2e$^-$ oxidation of catechol to yield o-quinone. Herein, H$_2$O$_2$ may facilitate the formation of an electrophilic Cu$^{2+}$-η$_2$-peroxo intermediate in CuCu-SgAP.

To gain further insight into substrate binding, a slow substrate, 4,5-dichlorocatechol (DCC) which is only oxidized slowly at pH 4.5, was used as a model substrate. The ligand-to-metal charge transfer band at 437 nm increases upon addition of DCC and reaches saturation were collected (Figure 3). The ligand-to-metal charge transfer band was fitted to a quadratic ligand stoichiometry. SgAP showed nearly doubled to 6344 M$^{-1}$ s$^{-1}$ at pH 7.0. The inset is the best fit to a quadratic ligand binding pattern showing 1:1 DCC:active site stoichiometry.

Table 1: Reaction mechanism of CuCu-SgAP.

References:


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