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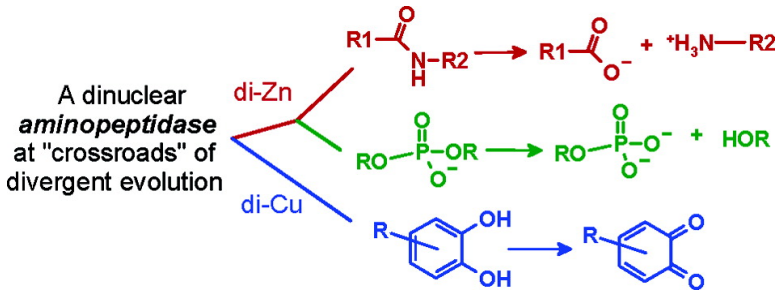

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Catechol Oxidase Activity of Di-Cu^{2+}-Substituted Aminopeptidase from *Streptomyces griseus*

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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 ($TS^{‡}$),[1] such as the tetrahedral $TS^{‡}$ during peptide hydrolysis by metallohydrolases. However, several examples of enzyme catalytic promiscuity,[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.[2] Nevertheless, these results still reflect that if the $TS^{‡}$ 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.[3] Metal substitution has thus been widely used to shed light on the structure and mechanism of metalloproteins.[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 metalloprotein systems. However, altering catalytic specificity based on simple metal substitution of metalloenzymes was rarely reported in the literature.[5] Recently, the dinuclear aminopeptidase from *Streptomyces griseus* (*Sg*AP) was found to exhibit a catalytic promiscuity toward phosphoester hydrolysis with a catalytic proficiency >40 billion under physiological conditions.[6] Since phosphoesters are structurally and mechanistically different from peptides during hydrolysis, this catalytic promiscuity is quite unusual. Herein we present that di-Cu^{2+}-substituted *Sg*AP (CuCu-*Sg*AP) exhibits a remarkable oxidative activity, approaching that of native catechol oxidase. The multifunctional character of *Sg*AP 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 *Sg*AP[7,8] and preparation of its metal derivatives[8] followed published procedures. The oxidation of the prototypical catechol oxidase substrate, 3,5-di-*tert*-butylcatechol (DTC), by CuCu-*Sg*AP follows Michaelis−Menten kinetics (○, Figure 1A),[9] affording $k_{cat} = 1.45\ s^{-1}$ and $K_m = 0.44$ mM. The catalytic efficiency of this catalysis ($k_{cat}/K_m = 3295\ M^{-1}\ s^{-1}$) under mild conditions at 25 °C and pH 7.0 in 50.0 mM HEPES and 5.0 mM Ca^{2+} is much better than that of a number of synthetic metal complexes[10] and is only ∼10 times smaller than that of catechol oxidase from gypsywort ($32\ mM^{-1}\ s^{-1}$).[11]

To demonstrate that the high oxidative activity observed herein is indeed attributed to *Sg*AP, activity profiles were obtained in stoichiometric Cu^{2+} titration of apo-*Sg*AP (Figure 1B) and thermodeactivation.[12] The results show that the activities toward the oxidation of DTC and the hydrolysis of Leu-*p*-nitroanilide[8,13] (Leu-*p*NA) are parallel. We have previously shown that metal ions bind sequentially to the two metal-binding sites of apo-*Sg*AP, with the hydrolytic activity controlled by the relative magnitude of the two metal-binding constants.[8,13] The oxidation reaction reaches full activity with the addition of 2 equiv of Cu^{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.

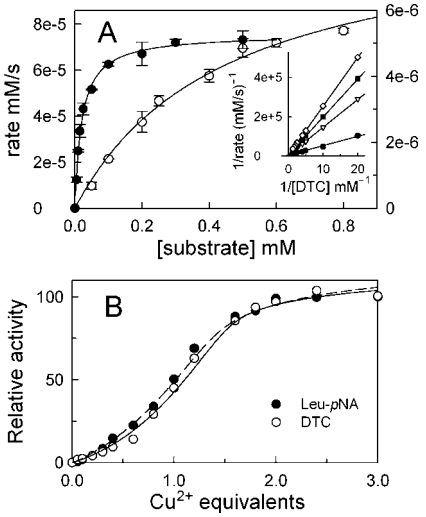

Figure 1. (A) Aerobic oxidation of DTC (○, pH 7.0, left scale) and catechol (●, pH 8.0, right scale) by CuCu-*Sg*AP.[9] The inset shows competitive inhibition of DTC oxidation by dinuclear AP-specific bestatin at 0.0, 5.0, 10.0, and 20.0 μM (from bottom), similar to the inhibition toward peptide hydrolysis. (B) Metal titration to apo-*Sg*AP in 100.0 mM HEPES at pH 7.0 and monitored with Leu-*p*NA hydrolysis and DTC oxidation, and fitted to Cd^{2+}-binding pattern previously published.[13]

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 μM for Leu-*p*NA hydrolysis and 8.8 μM 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. *Sg*AP is specific toward hydrophobic amino acids based on kinetic[7] and crystallography[14] studies. The hydrophobic pocket for specific recognition in *Sg*AP 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^{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,[9] which gives $k_{cat} = 0.066\ s^{-1}$, $K_m = 0.021$ mM, and $k_{cat}/K_m = 3220\ M^{-1}\ s^{-1}$ at pH 8.0 (●, 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_{cat} since $K_m = (k_{cat} + k_{-1})/k_1$ in Michaelis−Menten kinetics. CuCu-*Sg*AP can also oxidize a biorelevant catechol, dopamine, with $k_{cat} = 0.097\ s^{-1}$ and $K_m =$

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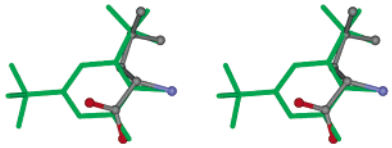

Figure 2. DTC superimposed onto Leu to reveal their structural similarity (BioMedCAChe 6.1.10). In Leu, the carboxylate and the amino groups are for metal binding, and the isobutyl group is for hydrophobic recognition.

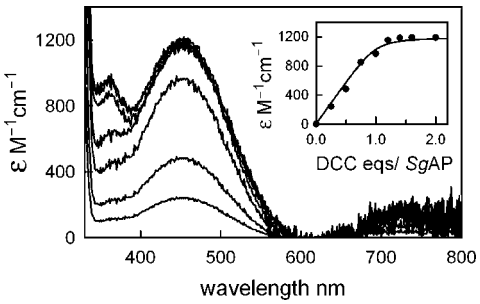

Figure 3. 4,5-Dichlorocatechol (DCC) titration to 0.1 mM CuCu-*Sg*AP in 50.0 mM HEPES at pH 7.0. The inset is the best fit to a quadratic ligand binding pattern showing 1:1 DCC:CuCu-*Sg*AP stoichiometry.

0.60 mM and 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_2O_2]-dependent increase in activity. In the presence of 10.0 mM (0.034%) H_2O_2, the k_{cat} value of CuCu-*Sg*AP 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 $Cu^{2+}{}_2$-μ-η^2-η^2-peroxo species that is isoelectronic to $Cu^{+}{}_2O_2$ and Cu^{3+}-bis-μ-oxo, which are all capable of performing the $2e^-$ oxidation of catechol to yield *o*-quinone.[15] Herein, H_2O_2 may facilitate the formation of an electrophilic $Cu^{2+}{}_2$-μ-peroxo intermediate in CuCu-*Sg*AP.

To gain further insight into substrate binding, a slow substrate, 4,5-dichlorocatechol (DCC which is only oxidized slowly at pH 8.0 with k_{cat} = 0.0045 s^{-1} and K_m = 0.15 mM), was titrated into 0.1 mM CuCu-*Sg*AP at 25 °C and pH 7.0, and the electronic spectra were collected (Figure 3). The ligand-to-metal charge transfer band at 437 nm increases upon addition of DCC and reaches saturation when more than 1 equiv of DCC is added, which can be nicely fitted to a single-substrate binding mode (Figure 3, inset) to yield a dissociation constant of 0.13 mM. The result provides direct evidence and stoichiometry (DCC:active site = 1:1) for catechol binding to the dinuclear active site of CuCu-*Sg*AP, yielding ϵ_{max} = 1200 M^{-1} cm^{-1}, that is consistent with observations in DCC or trichlorocatechol binding to a dinuclear Cu^{2+} center.[16]

Taken together, the results reported herein suggest that *Sg*AP can serve as a template for the design of potential catalysts capable of performing various chemical transformations, such as hydrolyses[6] of peptide, phosphodiester, and phosphonatoester and catechol oxidation, due to its easy purification, high thermal stability,[7] and easy preparation of metal derivatives.[8,13] The existence of a "*met*-like" di-Cu site in CuCu-*Sg*AP similar to that observed in the crystal structure of catechol oxidase[16] with a bridging OH between the two Cu^{2+} centers likely plays a role in the catalysis. Nevertheless, since the active sites of these two enzymes are quite different (an all-His vs a mixed His/carboxylato environment), the catalytic promiscuity observed here reflects that the active site does not need to be restricted to one structural pattern, which thus affords a new direction for rational design of ligands and proteins for oxidation catalysis. *Sg*AP and its metal derivatives are the only protein system to date that shows sharply different multiple catalytic activities. Thus, this enzyme and its variants prepared in the future can serve as unique dinuclear systems to provide further insight into the correlation of structure and mechanism of metal-centered hydrolytic and oxidation/oxygenation chemistry and may also serve as a "living fossil" to hint for divergent enzyme evolution.

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