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Mechanistic role of each metal ion in *Streptomyces* dinuclear aminopeptidase: Peptide hydrolysis and 7×10^{10}-fold rate enhancement of phosphodiester hydrolysis

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ABSTRACT

The dinuclear aminopeptidase from *Streptomyces griseus* (*Sg*AP) and its metal derivatives catalyze the hydrolysis of the phosphoester bis(*p*-nitrophenyl) phosphate (BNPP) and the phosphonate ester *p*-nitrophenyl phenylphosphonate with extraordinary rate enhancements at pH 7.0 and 25 °C [A. Ercan, H. I. Park, L.-J. Ming, Biochemistry 45, (2006) 13779–13793.], reaching 6.7 billion-fold in terms of the first-order rate constant of the di-Co(II) derivative with respect to the autohydrolytic rates. Since phosphoesters are transition state-like inhibitors in peptide hydrolysis, their hydrolysis by *Sg*AP is quite novel. Herein, we report the investigation of this proficient alternative catalysis of *Sg*AP and the role of each metal ion in the dinuclear site toward peptide and BNPP hydrolysis. Mn(II) selectively binds to one of the dinuclear metal sites (M1), affording MnE-*Sg*AP with an empty (*E*) second site for the binding of another metal (M2), including Mn(II), Co(II), Ni(II), Zn(II), and Cd(II). Peptide hydrolysis is controlled by M2, wherein the k_{cat} values for the derivatives MnM2-*Sg*AP are different yet similar between MnCo- and CoCo-*Sg*AP and pairs of other metal derivatives. On the other hand, BNPP hydrolysis is affected by metals in both sites. Thus, the two hydrolytic catalyses must follow different mechanisms. Based on crystal structures, docking, and the results presented herein, the M1 site is close to the hydrophobic specific site and the M2 site is next to Tyr246 that is H-bonded to a coordinated nucleophilic water molecule in peptide hydrolysis; whereas a coordinated water molecule on M1 becomes available as the nucleophile in phosphodiester hydrolysis.

1. Introduction

Enzymes can effect remarkable rate accelerations by stabilizing the transition-state (TS‡) of the substrates [1]. The use of TS‡ analogues for the production of catalytic antibodies [2] and inhibition of peptidases and esterases by different phospho-centers [3–6] support the TS‡ theory. However, some phosphoesters and fluorophosphates can be hydrolyzed by serine proteases and esterases via nucleophilic attack by the active-site Ser [7], which nevertheless produces an indefinitely stable dead-end complex with the phospho-center covalently attached to the Ser. Moreover, the phosphoester bis(*p*-nitrophenyl)phosphate (BNPP) and the phosphonate ester *p*-nitrophenyl phenylphosphonate can be effectively hydrolyzed by the dinuclear aminopeptidase (AP) from *Streptomyces griseus* (*Sg*AP) with activities comparable to some native phosphoesterases [8–10]. Since phospho- and phosphono-esters are TS‡-like molecules and can inhibit peptide hydrolysis [11–13], their hydrolysis by *Sg*AP is novel and must take place according to a unique catalytic pathway of the enzyme.

*Sg*AP (30 kDa) is a Ca^{2+}-influenced extracellular enzyme of high thermal stability with a catalytic specificity toward hydrophobic substrates [14,15]. It has a di-metal center (3.65 Å apart) bound to the protein through the side chains of His85 and Asp160 in one metal site and His247 and Glu132 in another site, along with a bridging Asp97 [16,17]. *Sg*AP and the AP from *Aeromonas proteolytica* (*Ap*AP, 32 kDa) have 29.6% sequence identity, identical metal-binding ligands, and a similar three-dimensional structure [18]. However, *Ap*AP [19] along with mammalian AP-P and *Escherichia coli* Met AP [20–22] require only one metal to activate, and a second metal to modulate, its activity. Conversely, *Sg*AP requires two metal ions for catalysis on the basis of crystallographic, NMR, and kinetic studies [8–10,19,23–25].

The active site of metalloenzymes can be investigated with spectroscopic and kinetic methods by the use of various metal ions [26–29]. Different homo- and hetero-dinuclear derivatives of dinuclear APs, including Leu-specific APs from bovine lens (blLAP) [29]

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and porcine kidney (pkLAP) [30] and *Ap*AP [19], were constructed and investigated to gain further insight into the function of each metal ion in catalysis [19,31]. For example, in blLAP, the first metal-binding site shows higher effect on k_{cat} while the second metal is more influential on K_m [29]. On the other hand, the first Zn^{2+} affects K_m while the second metal affects k_{cat} in pkLAP [30] and both k_{cat} and K_m values are affected by the two metals in *Ap*AP [32]. The variations of the kinetic parameters with the different metal derivatives of these enzymes signifies the importance and the function of each metal ion in their dinuclear active center.

Different metal derivatives of *Ap*AP were prepared and their activity and inhibition by 1-butaneboronic acid investigated, from which a mononuclear mechanism was proposed [33–35]. The crystal structure of ZnZn-*Ap*AP upon binding with this inhibitor shows that the inhibitor binds one metal through two oxygen atoms, one of which may interact with the second metal [36]. Unlike *Ap*AP, *Sg*AP is inactive with only one metal and becomes fully active upon binding of the second metal [23]. In this case, selective Co^{2+} binding has been verified with NMR, showing distinct hyperfine-shifted ^{1}H NMR signals for Co,E- and Co,Co-*Sg*AP [23]. Since selective metal-binding of *Sg*AP occurs, different metal ions can be introduced to construct various hetero-dinuclear derivatives. We present herein further investigation of the role of each metal ion in the dinuclear active site in the action of *Sg*AP by the use of various hetero-dinuclear derivatives MnM2-*Sg*AP (M2 = Zn^{2+}, Co^{2+}, Ni^{2+}, or Cd^{2+}). The mechanisms of this enzyme toward the hydrolysis of peptides and the alternative hydrolysis of BNPP are proposed.

2. Experimental section

2.1. Materials and reagents

The protease mixture Pronase, the buffers, HEPES, MES, NaH_2PO_4, EDTA, 1,10-phenanthroline, phenylglyoxal monohydrate, DEAE-Sephacel and Sephadex G-50, the substrate L-Leu-*p*-nitroanilide (Leu-*p*NA), Met-*p*NA, Val-*p*NA, Ala-*p*NA, Gly-*p*NA, and BNPP were purchased from Sigma–Aldrich (St. Louis, MO) and the atomic absorption standards (>99.99%) of Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+}, and Cu^{2+} from Fisher Scientific (Pittsburg, PA). Deionized water of >18 MΩ obtained from a MiliQ system (Millipore, Bredford, MA) was used to prepare all the solutions. All the glassware and plastic ware were treated with 2 mM EDTA solution and rinsed with deionized water prior to use.

2.2. Purification, demetallization, and characterization of SgAP

*Sg*AP was purified according to the published procedures [14,23]. The fractions of the first peak from the DEAE-Sephacel column with AP activity were combined. Purified *Sg*AP (0.05–0.1 mM, 10 ml) was dialyzed for 12 h against 250 ml of 20 mM Tris/HCl buffer at pH 7.5 with 100 mM NaCl for four times, with 2.0 mM EDTA and 2.0 mM 1,10-phenanthroline for the first two times and 2.0 mM 1,10-phenanthroline for latter two, followed by dialyzing against 250 ml of 20 mM MES at pH 6.0 for four times to remove the chelators. The AP concentration was determined according to the absorption $E_{280}^{1\%} = 15$ [14,23] and by titration with atomic-absorption-grade Co^{2+} solution of known concentrations.

The formation of the various MnM-*Sg*AP derivatives was verified by their activities and after prolonged incubation to ensure the retention of the activities (otherwise, migration of metal ions in the two sites may take place). Paramagnetically shifted ^{1}H NMR features were also used as the fingerprints for identification of the Co^{2+}-containing derivatives [37–39]. The ^{1}H NMR spectra of the paramagnetic derivatives were acquired on a Varian INOVA500 spectrometer (at 500 MHz ^{1}H resonance) with a 5-mm bio-TR (triple resonance) probe by the use of the build-in polynomial 1–3-3–1 pulse sequence for samples in H_2O buffers and a presaturation pulse for samples in D_2O buffers with a 90° pulse of ~9 μs over 200 ppm spectral width and processed with a line-broadening of 40 Hz, followed by spline baseline correction.

2.3. Enzyme kinetics and inhibition

The kinetics was carried out in 0.1 M HEPES buffer at pH 8.0 containing 0.1 M NaCl and 10 mM $CaCl_2$ at 30 °C and the data were analyzed with the Michaelis–Menten model to derive the turnover k_{cat} and the Michaelis constant K_m with non-linear fitting of the rate with respect to substrate concentration. Fluoride inhibition was carried out under the same conditions with different inhibitor concentrations, but in the absence of Ca^{2+} to avoid the formation of the very insoluble CaF_2. Each inhibition study was performed at least twice and fitted to the Michaelis–Menten equation with non-linear regression and the inhibition constants K_i for different inhibition patterns are determined accordingly.

2.4. pH Profiles and thermostability

Catalytic parameters (k_{cat} and K_m) toward hydrolysis of Leu-*p*NA and BNPP and inhibitions were measured at different pHs (acetate at pH 5.0, MES at 5.5–6.5, HEPES at 7.0–8.0, TAPS at 8.5–9.5, and CAPS at 10.0). Thermostability of apo-*Sg*AP and different metal derivatives of *Sg*AP was determined on the basis of their activities toward Leu-*p*NA hydrolysis. Herein, *Sg*AP and its various metal derivatives with or without 5 mM $CaCl_2$ were incubated at various temperatures for 1.0 min followed by incubation on ice for 5 min. Then, an excess amount of corresponding metal was added and activity determined.

3. Results and discussion

3.1. Mn^{2+} binding and hetero-dinuclear active site of SgAP

The binding of Mn^{2+} to apo-*Sg*AP at pH 6.0 requires >50 equivalents to fully activate the enzyme (Fig. 1A), wherein the activity is significantly enhanced by Ca^{2+} (5 mM). At pH 8.0 without Ca^{2+}, Mn^{2+} binds to one site exclusively without showing activity (o, Fig. 1B and inset), and reaches full activation at >20 equivalents. Mn^{2+} binding becomes less selective and the enzyme is 44% less active in the presence of Ca^{2+} (●, Fig. 1B and inset). The binding of Mn^{2+} to just one metal-binding site in the active center at one equivalent (denoted the M1 site) allows the introduction of another metal ion to the second site (M2) to construct several catalytically active hetero-dinuclear derivatives in the form of Mn,M2-*Sg*AP (where M2 = Co^{2+}, Zn^{2+}, Ni^{2+}, or Cd^{2+}) (Fig. 1C). Conversely, the binding of Zn^{2+}, Cd^{2+}, or Cu^{2+} to the enzyme exhibit a non-selective pattern [23,24,40], preventing the preparation of the corresponding M1,E-*Sg*AP derivatives with an empty (*E*) M2 site. The first equivalent of Mn^{2+} must bind to the active site of *Sg*AP. Otherwise, the subsequent addition of one equivalent of Co^{2+} (▼, Fig. 1C) would not generate an active derivative since CoE-*Sg*AP is inactive [23].

The derivative MnCo-*Sg*AP shows paramagnetically shifted ^{1}H NMR features in the downfield region ~30–80 ppm (Fig. 2, bottom) different from those of CoE- [23] and CoZn-*Sg*AP [41], indicating a new Co^{2+} derivative. The spectrum is also different from that of CoCo-*Sg*AP which indicates that Co^{2+} is not bound to both metal site of the protein (by replacing the Mn^{2+}). The NMR signals of the protons around mononuclear Mn^{2+} cannot be detected due to signal broadening by the slowly relaxing unpaired electrons of Mn^{2+}. However, magnetic coupling between Mn^{2+} and a metal