Different phosphate binding modes of *Streptomyces griseus* aminopeptidase between crystal and solution states and the status of zinc-bound water

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Abstract Phosphate shows a non-competitive inhibition toward a *Streptomyces* aminopeptidase (sAP) between pH 5.85 (*K*_i = 0.48 mM) and 9.0 (110 mM), with a *p*K_a of 7.1 likely due to ionization of *H*₂PO₄⁻. This non-competitive inhibition pattern indicates that phosphate binding to sAP in solution is different from that in the crystal structure, where phosphate is bound to the active site Zn(II) ions. Fluoride uncompetitively inhibits sAP from pH 5.5 (*K*_i = 3.72 mM) to 9.0 (43.6 mM), with a *p*K_a of ~6.2 likely due to a coordinated water. The different inhibition natures and *p*K_a values indicate that the two inhibitors bind at different locations.

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Key words: Aminopeptidase; Dinuclear; Fluoride; Inhibition; Phosphate; *Streptomyces*

1. Introduction

Aminopeptidases (APs) catalyze the hydrolysis of protein or peptide substrates from the N-terminus, and are widely distributed throughout nature in bacteria, plants, and mammalian tissue [1–3]. Several biological processes, such as protein maturation, terminal degradation of proteins, and hormone level regulation require aminopeptidases to function properly [1–3]. Altered aminopeptidase activity has been associated with several pathological disorders and diseases, which involve cancer [4], AIDS [5], and eye lens cataracts [6,7].

The AP from *Aeromonas proteolytica* (aAP) has two zinc ions in its active site 3.5 Å apart, bridged by an aspartate and a water molecule [8]. A crystal structure of an inhibitor-bound (o-iodophenylalanine hydroxamate) complex of this enzyme [9] has allowed a mechanism similar to that of thermolysin and carboxypeptidase A to be proposed [10,11], in which a water molecule [8] and a hydroxide [12] bind at different locations.

To obtain a better understanding of the mechanism of sAP and how phosphate interacts with sAP, phosphate inhibition was examined at different pH values. In addition, fluoride inhibition was performed to provide insight into the role of the coordinated water molecule that was not revealed in the crystal structure.

2. Materials and methods

The crude enzyme mixture Pronase, the buffers TAPS (N-tris(hydroxymethyl) methyl-3-aminopropane sulfonic acid), HEPES (N-[2-hydroxyethyl] pipеразин-N'‐2-етанесульфоновая кислота) and MES (2-[N-morpholino]ethanesulfonic acid), NaH₂PO₄·H₂O, EDTA (ethylenediamine tetracetic acid), 1,10-phenanthroline, and the substrate *L*-leucine-*p*-nitroanilide (Leu-pNA) were purchased from Sigma Chemical Co. (St. Louis, MO), Diethylaminoethyl (DEAE) Sephacel and Sephaxide G-50 were used for purification of the protein and were also purchased from Sigma. Zinc(II) stock solutions were prepared from atomic absorption standard purchased from Fisher (Pittsburgh, PA). All the solutions were prepared from deionized water of >18 MΩ from a MilliQ system (Millipore, Bedford, MA). The glassware and plastic ware were treated with EDTA and rinsed with deionized water prior to use.

*S. griseus* aminopeptidase was prepared according to the literature procedure [19,20]. The enzyme concentration was determined according to the absorption value *E*₂₈₀ = 15 [19,20]. Kinetic measurements were performed on a Cary 3E spectrophotometer using the substrate Leu-pNA from pH 5.5 to 9.0 at 30°C. Assays were performed in 50 mM buffer in the presence of 60 mM NaNO₃ and 0.1 mM Zn²⁺. Both NaNO₃ and NaCl below 0.5 M were found not to affect the enzyme activity. The initial rate of the hydrolysis of the substrate was obtained by monitoring the release of the chromophore pNA at 405 nm (ε = 10 600 M⁻¹ cm⁻¹ which is found constant in the pH range of 5.5–9.0) [19,21]. The kinetic parameters *k*ₘ and *K*_i were obtained.
by non-linear fitting of data to the hyperbolic Michaelis-Menten equation. The substrate was first dissolved in a minimum amount of dimethyl sulfoxide (DMSO), then dissolved in a buffer at the desired pH. The amount of DMSO in the solutions showed no inhibitory effect on sAP under the experimental conditions. The enzyme concentration was 7.0 nM for the entire pH study and the phosphate concentration varied to afford the inhibitor constant. To test our methods, assays were compared to the literature values [19–21]. The results showed a $K_m$ of 0.54 mM and a $k_{cat}$ of 396 s$^{-1}$ in the presence of 1.0 mM Ca$^{2+}$, in good agreement with the previous results.

3. Results and discussion

3.1. Phosphate inhibition

Phosphate has been used extensively as a probe to study active sites of metalloproteins and has been shown to bind positively charged residues such as lysine and arginine [22–24]. Crystalllographic study of sAP also suggested that phosphate is bound to this enzyme [18]. In order to elucidate if mechanistic information could be obtained from phosphate binding to sAP, the inhibitory nature of phosphate was investigated in the pH range of 5.85–9.0. Non-competitive inhibition was observed at every pH tested in this study. The inhibitor constant ($K_i$) increased from 0.48 ± 0.06 mM at pH 5.85 to 110 ± 1.7 mM at pH 9.0. Only very weak inhibition was observed at pH 10. The AP activity can be completely recovered when phosphate is removed by ultrafiltration, indicating the enzyme is intact during the inhibition. A representative result is shown in Fig. 2 as plots of 1/rate versus 1/[S] at pH 7.5.

Purely non-competitive inhibition and partially non-competitive inhibition can be distinguished by plotting rate versus [I] (inset, Fig. 2) [25]. For purely non-competitive inhibition the rate approaches zero at high inhibitor concentrations, whereas the curve for partial non-competitive inhibition reaches a limiting velocity. In other words, the substrate-inhibitor complex cannot convert to products in purely non-competitive inhibition. In addition, the plots of 1/V$\text{max}$ versus the concentration of phosphate yielded a linear correlation at every pH in this study (data not shown), indicating that the enzyme has only one phosphate binding site [25].

The nature of the phosphate inhibition and the crystal structure assignment of the zinc-bound phosphate present a controversial situation. The non-competitive kinetics in this study indicate that phosphate and the substrate bind at different sites, thus tetrahedral phosphate cannot bind to the metal center as a transition-state analog. However, the crystal structure of sAP reveals that phosphate bridges both Zn ions by one oxygen, with another oxygen bound to Zn1 (Fig. 1). This phosphate binding mode is similar to that of transition-state analogues bound to blAP with one bridging and one terminal oxygen bound to the dinuclear active site [14–17], and the aAP-inhibitor complex with one bridging oxygen [9]. If phosphate did bind to sAP in solution as it is shown in the crystal structure, at least a mixed type of inhibition would be expected in which the enzyme-inhibitor complex has a lower affinity than the free enzyme for the substrate [25]. This was not observed in our study. The justification

![Fig. 1. Structure of the di-zinc active site in S. griseus aminopeptidase with the bound phosphate [18]. Protein Data Bank code 1xjo.](image1)

![Fig. 2. The plot of 1/V (µmol/s)$^{-1}$ versus 1/[S] mM$^{-1}$ at pH 7.5 for phosphate inhibition, in which the lines intersect on the x-axis characteristic of non-competitive inhibition. Phosphate concentrations are 0.0 (●), 5.0 (■), 20 (▲), and 50 (▼) mM. The inset is a plot of $V$ (µmol/s) versus [phosphate] at pH 5.85, at which phosphate exhibits the highest inhibition in this study. The solid line is a fitting toward the equilibrium with only one phosphate bound. The rate at > 140 mM phosphate is beyond detection limits under the experimental conditions within 20 min.](image2)

![Fig. 3. The plot of 1/V (µmol/s)$^{-1}$ versus 1/[S] mM$^{-1}$ at pH 7.0 for fluoride inhibition, in which the parallel lines are indicative of non-competitive inhibition. Fluoride concentrations are 0.0 (●), 20 (■), 40 (▲), and 80 (▼) mM. The inset is a plot of 1/$K_{mapp}$ versus [fluoride] (mM) at pH 7.0, in which the linear relationship indicates that sAP binds only one fluoride ion.](image3)
of a bound dibasic phosphate (rather than a sulfate) in the crystal structure is based on phosphate's acidity relative to sulfate, in which a hydroxy1 group is present in phosphate that can interact with Glu-131 and Arg-202 [18]. However, a region near the active site (Glu-196 to Arg-202) could not be traced in the crystal structure due to poor electron density, which could have posed a problem in the assignment of the phosphate interactions.

Phosphate has been shown to bind to arginine in enzymes such as copper-zinc superoxide dismutase [24,26], ribonuclease A [27], and several others [28]. Moreover, arginine has been implicated as a residue essential to the function of a number of enzymes [24-29], such as the above enzymes, carboxypeptidase A [30], and glutamine synthetase [31], which become inactive or significantly decrease in activity once their arginine residues are chemically modified. The crystallographic study of sAP suggests that an arginine residue (Arg-202) is located near the active site [18]. Based on our study phosphate does not bind directly to the zinc ions in sAP, therefore it is suspected that the phosphate binding site is Arg-202. This arginine may play a critical role in the action of sAP, likely to be involved in hydrogen bonding and/or acting as a general acid during the hydrolytic process.

3.2. Fluoride inhibition

Fluoride has been utilized to probe metal-bound water or hydroxide in several metalloproteases [32-38], thus has been chosen as a probe to study the status of the coordinated water in sAP. The kinetic data clearly show that F$^-$ uncompetitively inhibits the hydrolysis in the pH range of 5.5-9.0 (Fig. 3). The apparent $1/K_{in}$ versus $[F^-]$ plot shows a linear relationship, which indicates that $F^-$ is a pure uncompetitive inhibitor and the enzyme binds only one $F^-$ [25] (inset, Fig. 3). The inhibitor constant ($K_a$) is $3.72 \pm 0.25$ mM at pH 5.5 and increases to $43.6 \pm 0.8$ mM at pH 9.0. This uncompetitive inhibition nature reflects that the inhibitor can bind to the enzyme only after the substrate is bound, suggesting that a water/hydroxide becomes available for $F^-$ substitution after substrate binding. One possibility is a change from bridging to terminal bound hydroxide that becomes replaceable. Fluoride was shown to be an uncompetitive inhibitor towards Leu-pNA hydrolysis by aAP [12]. It was proposed that a zinc bound hydroxide was substituted by $F^-$ and that this hydroxide was supposedly the attacking nucleophile. This is also likely the case in sAP.

3.3. pH dependence of phosphate and fluoride inhibitions

The pH dependence of phosphate and fluoride inhibitions is shown in Fig. 4. A $pK_a$ of 7.1 is obtained for phosphate inhibition from non-linear regression fitting of the data. This $pK_a$ is consistent with that of ionization of $H_2PO_4^-$, reported at 7.12 at 25°C [39]. The pH dependence of $F^-$ inhibition reveals a $pK_a$ of 6.2. Fluoride is expected to bind hard acids such as $Zn^{2+}$ based on the hard/soft concept of acids and bases [40]. A pH dependence study of sAP activity also reveals a similar $pK_a$ of $\sim 6.0$ in the $k_{cat}$ versus pH plot, suggested to be due to ionization of a coordinated water (H.I. Park and L.-J. Ming, unpublished observation). On the basis of the uncompetitive pattern of $F^-$ inhibition, the pH dependence of this inhibition, and pH-activity profile, we propose that the binding of $F^-$ is associated with the displacement of a catalytically significant coordinated water/hydroxide that has a $pK_a$ of 6.2.

4. Concluding remarks

Crystallographic studies of transition state analogs with bAP and aAP suggest that the zinc ions function in the binding and polarization of the substrate [9,14-17]. The phosphate inhibition study clearly shows that phosphate and the substrate bind to sAP at different locations. Therefore, the binding of phosphate to sAP in solution cannot be the same as that proposed from the crystal structure. This conclusion is supported by our preliminary $31P$ NMR studies of phosphate binding to CoZn-sAP, which do not suggest direct phosphate-metal interaction. A detailed investigation of phosphate binding to the Co$^{2+}$ derivative of sAP by the use of NMR is in progress. Fluoride inhibition has provided insight into a zinc-bound coordinated water of sAP. The uncompetitive inhibition pattern of $F^-$ towards sAP is similar to that observed in aAP and can suggest possible mechanistic similarities between the two enzymes despite their low sequence homology (24% in aligned regions) [8,18] and very different functional groups in the proximity of the active site zinc ions.

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