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 pK_a of 7.1 likely due to ionization of $H_2PO_4^-$. 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 pK_a of ~6.2 likely due to a coordinated water. The different inhibition natures and pK_a values indicate that the two inhibitors bind at different locations.

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 (D-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 carboxylate (Glu-151) in the active site of aAP appears to form a hydrogen bond with the bridging oxygen of the inhibitor. A fluoride inhibition study on aAP revealed that the anion inhibits uncompetitively and displaces one metal-bound water or hydroxide after substrate binding [12]. Bovine lens leucine aminopeptidase (blAP) has also been characterized by means of X-ray crystallography. Its active site contains two zinc ions 2.9 Å apart, bridged by a bidentate glutamate, a monodentate aspartate, and a hydroxide [13]. Crystal structures of blAP-inhibitor complexes allowed a reaction mechanism to be proposed for this enzyme [14–17]. In this mechanism, the N-terminal amino group of the substrate binds to one of the zinc ions while the scissile carbonyl group binds to the other zinc ion. The bridging hydroxide then attacks the carbonyl carbon of the peptide substrate to generate the *gem*-diolate intermediate.

The aminopeptidase from *Streptomyces griseus* (sAP) belongs to the same family as aAP and blAP and is the fourth AP to have its crystal structure resolved [18]. This AP shows maximum activity at pH 8.0 and is specific towards large hydrophobic amino acids, preferably leucine. It requires two zinc ions for activity [19–21], and its activity is significantly enhanced by calcium [19,20]. The crystal structure reveals that the zinc ions in the active site are 3.6 Å apart [18], with one zinc ion (Zn1) bound to His-85 and Asp-160 and the other (Zn2) bound to Glu-132 and His-247 (Fig. 1). Ligands bridging the two zinc ions are Asp-97 and a non-protein ligand, identified as dibasic phosphate. This phosphate was shown to bridge the two metal ions by one oxygen with a second oxygen bound to Zn1 (Fig. 1). The hydroxo group of this phosphate was suggested to interact with Glu-131 and Arg-202 near the active site.

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] piperazine-*N*′-2-ethanesulfonic acid) and MES (2-[*N*-morpholino]ethanesulfonic acid), $NaH_2PO_4{\cdot}H_2O$, EDTA (ethylenediamine tetraacetic 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 Sephadex 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 $E^{1\%}_{280}$ = 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 $NaNO_3$ and 0.1 mM Zn^{2+}. Both $NaNO_3$ 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_{cat} and K_m were obtained

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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]. Crystallographic 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 $1/V_{maxi}$ 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

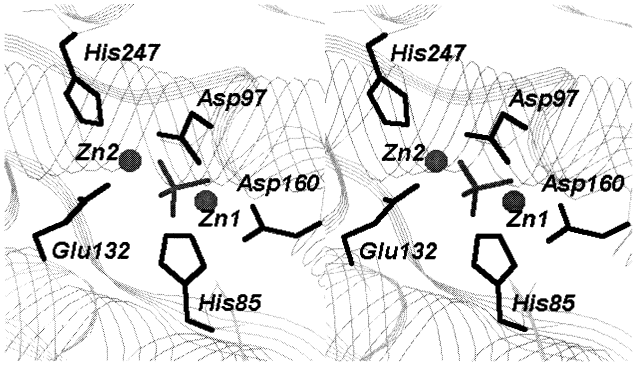

Fig. 1. Structure of the di-zinc active site in *S. griseus* aminopeptidase with the bound phosphate [18], Protein Data Bank code 1xjo.

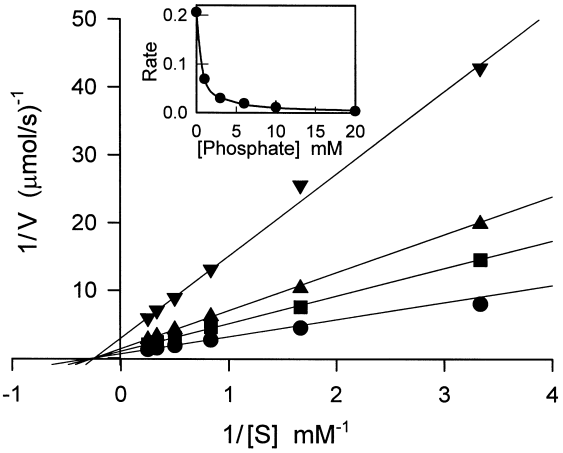

Fig. 2. The plot of $1/V$ (μmol/s)$^{-1}$ versus 1/[S] mM^{-1} at pH 7.5 for phosphate inhibition, in which the lines intersect on the *x*-axis characteristic of non-competitive inhibition. Phosphate concentrations are 0.0 (●), 5.0 (■), 20 (▲), and 50 (▼) mM. The inset is a plot of V (μmol/s) versus [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.

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 only 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

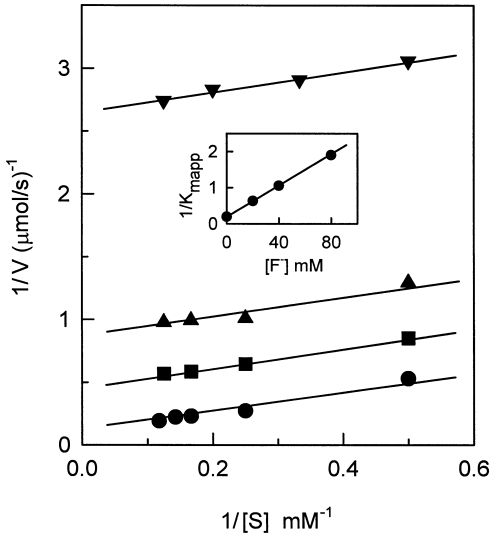

Fig. 3. The plot of $1/V$ (μmol/s)$^{-1}$ versus 1/[S] mM^{-1} at pH 7.0 for fluoride inhibition, in which the parallel lines are indicative of uncompetitive inhibition. Fluoride concentrations are 0.0 (●), 20 (■), 40 (▲), and 80 (▼) mM. The inset is a plot of $1/K_{mapp}$ versus [fluoride] (mM) at pH 7.0, in which the linear relationship indicates that sAP binds only one fluoride ion.

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 hydroxyl 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 $1/K_m$ 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_i) is 3.72 ± 0.25 mM at pH 5.5 and increases to 43.6 ± 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 terminally 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.

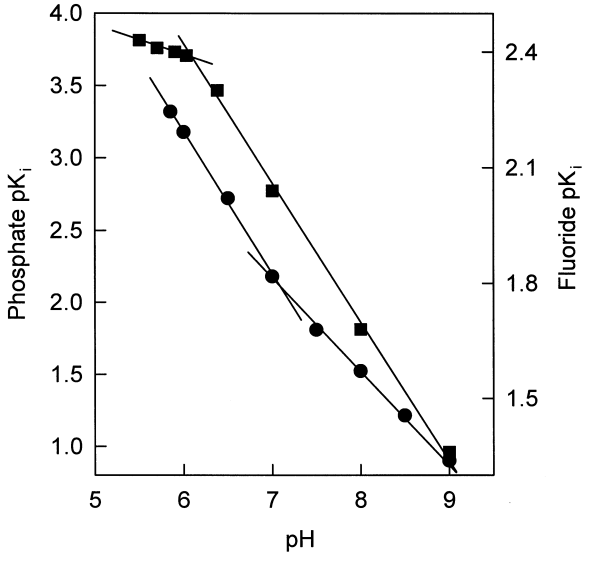

Fig. 4. The pH dependence of fluoride (■) and phosphate (●) inhibitions. The Dixon plot for phosphate, and plots from ($1/K_{mapp}$) versus [I] for fluoride determined the inhibitor constants. The pK_a values of 7.05 and 6.2 for the pH dependence of phosphate and fluoride were obtained by non-linear fitting, but displayed here in linear fashion.

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 blAP 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 ^{31}P 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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References

[1] Taylor, A. (1993) FASEB J. 7, 290–298.
[2] Taylor, A. (1993) Trends Biochem. Sci. 18, 167–172.
[3] Taylor, A., Brown, M.J., Daims, M.A. and Cohen, J. (1983) Invest. Ophthalmol. Vis. Sci. 24, 1172–1181.

[4] Frost, P. and Greig, R. (1990) The Role of Cellular Proteases and Inhibitors in Invasion and Metastasis, Kluwer, New York.
[5] Kempt, D.J. (1994) Methods Enzymol. 241, 334–370.
[6] Umezawa, H. (1980) Recent Results Cancer Res. 75, 115–125.
[7] Taylor, A., Daims, M.A., Lee, J. and Surgenor, T. (1982) Curr. Eye Res. 2, 47–56.
[8] Chevrier, B., Schalk, C., D'Orchymont, H., Rondeau, J.M., Moras, D. and Tarnus, C. (1994) Structure 2, 283–291.
[9] Chevrier, B., D'Orchymont, H., Schalk, C., Tarnus, C. and Moras, D. (1996) Eur. J. Biochem. 237, 393–398.
[10] Christianson, D.W. and Lipscomb, W.N. (1989) Acc. Chem. Res. 22, 62.
[11] Matthews, B.W. (1988) Acc. Chem. Res. 21, 333.
[12] Chen, G., Edwards, T., D'souza, V.M. and Holz, R.C. (1997) Biochemistry 36, 4278–4286.
[13] Burley, S.K., David, P.R., Taylor, A. and Lipscomb, W.N. (1990) Proc. Natl. Acad. Sci. USA 87, 6878–6882.
[14] Strater, N. and Lipscomb, W.N. (1995) Biochemistry 34, 9200–9210.
[15] Strater, N. and Lipscomb, W.N. (1995) Biochemistry 34, 14792–14800.
[16] Burley, S.K., David, P.R. and Lipscomb, W.N. (1991) Proc. Natl. Acad. Sci. USA 88, 6916–6920.
[17] Burley, S.K., David, P.R., Sweet, R.M., Taylor, A. and Lipscomb, W.N. (1992) J. Mol. Biol. 224, 113–140.
[18] Greenblatt, H.M., Almog, O., Maras, B., Spungin-Bialik, A., Barra, D., Blumberg, S. and Shoham, G. (1997) J. Mol. Biol. 265, 620–636.
[19] Ben-Meir, D., Spungin, A., Ashkenazi, R. and Blumberg, S. (1993) Eur. J. Biochem. 212, 107–112.
[20] Spungin, A. and Blumberg, S. (1989) Eur. J. Biochem. 183, 471–477.
[21] Lin, L.-Y., Park, H.-I. and Ming, L.-J. (1997) J. Biol. Inorg. Chem. 2, 744–749.
[22] Bertini, I., Donaire, A., Messori, L. and Moratel, J.M. (1990) Inorg. Chem. 29, 202–205.
[23] Todd, M.J. and Hausinger, R.P. (1989) J. Biol. Chem. 264, 15835–15842.
[24] Mota de Freitas, D. and Valentine, J.S. (1984) Biochemistry 23, 2079–2082.
[25] Segel, I.H. (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, Wiley, New York.
[26] Mota de Freitas, D., Luchinat, C., Banci, L., Bertini, I. and Valentine, J.S. (1987) Inorg. Chem. 26, 2788–2791.
[27] Takahashi, K. (1968) J. Biol. Chem. 243, 6171–6179.
[28] Riordan, J.F. (1979) Mol. Cell. Biochem. 26, 71–92.
[29] Borders, C.L. and Riordan, J.F. (1975) Biochemistry 14, 4699–4704.
[30] Riordan, J.F. (1973) Biochemistry 12, 3915–3923.
[31] Powers, S.G. and Riordan, J.F. (1975) Proc. Natl. Acad. Sci. USA 72, 2616–2620.
[32] Dixon, N.E., Blakely, R.L. and Zerner, B. (1980) Can. J. Biochem. 58, 481–488.
[33] Vallee, B.L. and Galdes, A. (1984) Adv. Enzymol. 56, 283–430.
[34] Bertini, I. and Luchinat, C. (1984) Adv. Inorg. Biochem. 6, 71–111.
[35] Williams, A.C. and Auld, D.S. (1986) Biochemistry 25, 94–100.
[36] Ganzhorn, A.J. and Chanal, M.C. (1990) Biochemistry 29, 6065–6071.
[37] Vincent, J.B., Crowder, M.W. and Averill, B.A. (1991) Biochemistry 30, 3025–3034.
[38] Yang, J.J., Artis, D.R. and van Wart, H.E. (1994) Biochemistry 33, 6516–6523.
[39] Meites, L. (1963) Handbook of Analytical Chemistry, McGraw-Hill, New York.
[40] Martell, A.E. and Hancock, R.D. (1996) Metal Complexes in Aqueous Solutions, pp. 199–202, Plenum Press, New York.