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Effect of Zinc Ion on the Inhibition of Carboxypeptidase A by Imidazole-Bearing Substrate Analogues

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Abstract—Competitive inhibitors of carboxypeptidase A, 2-(4-imidazoyl)hydrocinnamic acid (**1**) and its congeners (**2**–**4**) that bear an imidazole ring as the zinc-ligating functionality have been evaluated for their CPA inhibitory activity in the presence of zinc ion to find that the zinc ion augments the inhibitory potency by up to 212-fold.

Introduction

Carboxypeptidase A (CPA) is one of the most studied zinc-containing proteolytic enzymes and serves as a prototypical enzyme for metalloproteases that play important roles in the biological system.[1] The enzyme shows specificity for cleavage of C-terminal amino acid residue having an aromatic side chain. The catalytically essential zinc ion is held by His-69, Glu-72, His-196 and a molecule of water which functions as the nucleophile that attacks on the scissile peptide bond of the substrate. The other important residues at the active site of CPA are Glu-270 and Arg-145. The former functions as a general base, activating the zinc-bound water molecule to be a strong nucleophile, and the latter forms hydrogen bonds with the C-terminal carboxylate of substrate. In addition, there is present a hydrophobic pocket at the active site, the primary function of which is to recognize substrates by accommodating the aromatic side chain in the P_1' residue of substrate.[1,2] CPA has also served as a model enzyme for developing a design strategy of inhibitors that are effective for zinc proteases of medicinal interest, and a wide variety of design protocols have been reported.[3] Most of these CPA inhibitors are designed by incorporating a zinc-ligating functionality into the structural frame of a substrate-like molecule that can be recognized by the enzyme. The commonly used zinc-ligating groups for the design of zinc protease inhibitors involve carboxylate, sulfhydryl, phosphate, phosphonamide, and hydroxamate.[4] Recently, we have reported the design of CPA inhibitors that bear an imidazole group as the zinc-ligating moiety.[5] This communication reports that inhibitory potency of CPA inhibitors **2**–**4** that carry an imidazole moiety is augmented drastically by the zinc ion.

Results and Discussion

In 1960, Vallee et al. reported that the enzymic activity of CPA is reversibly inhibited by zinc chloride.[6] The discovery aroused considerable attention and several subsequent studies suggested that the second zinc ion binds the enzyme at the active site.[7] Larsen and Auld later established that the zinc inhibition of CPA is effected by zinc monohydroxide ($ZnOH^+$), and proposed that the secondary (inhibitory) zinc ion coordinates to the carboxylate of Glu-270 and there forms a hydroxide bridge between the inhibitory zinc and the catalytic zinc ions at the active site.[8] This has been confirmed by the X-ray crystal structure of the inactivated CPA.[9] Recently, Mock and Wang reported synergistic inhibition of CPA by imidazole and zinc ion, and they attributed the synergistic effect to the formation of a ternary complex of the three species, namely, zinc ion, imidazole, and the enzyme.[10] This report prompted us to evaluate our CPA inhibitors (**1**–**4**)[5] in the presence of zinc ion. These inhibitors were designed under the promise that their carboxylate would form a bifurcated hydrogen bond with the guanidinium moiety of Arg-145

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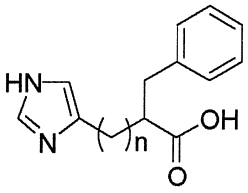
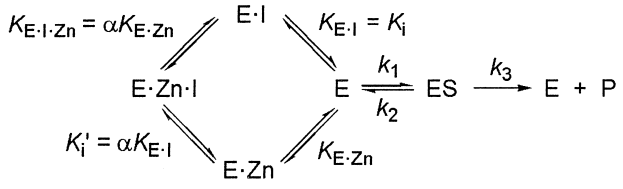
residue of the enzyme, the phenyl ring anchors in the primary substrate recognition pocket, and the imidazole ring ligates to the catalytic zinc ion at the active site of CPA. Since the imidazole heterocycle in these inhibitors is thought to ligate to the catalytic zinc ion upon the inhibitors forming complexes with CPA, it is expected that their imidazole moiety would form the ternary complexes in the presence of zinc ion to result in an improvement of the inhibitory potency. We have resynthesized[11] the inhibitors and evaluated their CPA inhibitory activity in the presence of zinc chloride at different concentrations in the range of 0–45 μM.



1 : n = 0
2 : n = 1
3 : n = 2
4 : n = 3

The inhibition of CPA by zinc ion and the imidazole-bearing inhibitors may be represented by Scheme 1, for which Yonetani and Theorell have derived a kinetic equation [eq (1)] and developed a graphical method for the kinetic analysis.[12] In the scheme and equation, α is referred to as the interaction factor, which is useful for characterizing the nature of interactions between the two inhibitors in the ternary complex of CPA·I·Zn: (i) If I_1 and I_2 interact at different sites of E, the value of α falls in $\infty > \alpha > 0$. (ii) When a positive attraction occurs between I_1 and I_2 in the E I_1 I_2 complex, α would have a value less than 1, that is, $1 > \alpha > 0$. (iii) If the two inhibitors interact repulsively in the ternary complex, the value of α would fall in the range of $\infty > \alpha > 1$.



Scheme 1.

Table 1. Kinetic parameters for the inhibition of CPA

Inhibitor	K_i' (μM)[a]	K_i (μM)[b]	K_i/K_i'[c]	α[d]
1	0.49	0.26	0.53	1.88
2	1.01	214	212	0.0047
3	0.29	15	51.7	0.0056
4	18	750	41.7	0.024

[a]Apparent inhibitory constants obtained in the presence of zinc ion.
[b]Inhibitory constants in the absence of zinc ion.
[c]The K_i/K_i' value represents the extent of enhancement of potency caused by zinc ion.
[d]The α value denotes the cooperative effect of the inhibitory zinc ion in the inhibition of CPA by the inhibitors **1**–**4**.

$$\frac{1}{v_i} = \frac{1}{V_m} + \frac{K_M}{[S]V_m}\left(1 + \frac{[Zn]}{K_{E\cdot Zn}}\right) + \frac{K_M}{[S]V_m K_i}\left(1 + \frac{[Zn]}{\alpha K_{E\cdot Zn}}\right)[I] \quad (1)$$

The values of the apparent dissociation constant (K_i') and the interaction factor for the CPA inhibition by **1**–**4** in the presence of zinc ion were obtained from the respective Yonetani–Theorell plot and are collected in Table 1 together with the inhibitory constants (K_i) determined in the absence of zinc chloride. Figure 1 exemplifies the Yonetani–Theorell plot. It can be seen from Table 1 that binding affinity of inhibitors **2**–**4** was augmented by zinc ion as much as 212-fold. In comparison, in the case of the most potent inhibitor (**1**) in the series, the zinc ion affected adversely. It may be surmised from the α values that the imidazole ring in inhibitors **2**–**4** does not bind to the catalytic zinc ion but is likely to interact with the inhibitory zinc ion. While the interaction between the inhibitory zinc ion and the imidazole in **1** is repulsive, albeit weak, there operate strong attractive interactions between the inhibitory zinc ion and the imidazole ring in the case of **2**–**4**.

From the present study, it may be expected that zinc protease inhibitors bearing an imidazole ring would have enhanced inhibitory activity in cells having high concentration of zinc ion. Comparative trace metal analyses of cancerous and noncancerous human tissue have revealed that the concentration of zinc ion in cancerous cell such as breast carcinoma is higher by 700% compared with that in normal breast cells.[13] Hence, the imidazole bearing substrate analogue inhibitors that are effective for zinc proteases associated with cancer metastasis such as matrix metalloprotease **2**[14] may potentially be explored as therapeutic agents

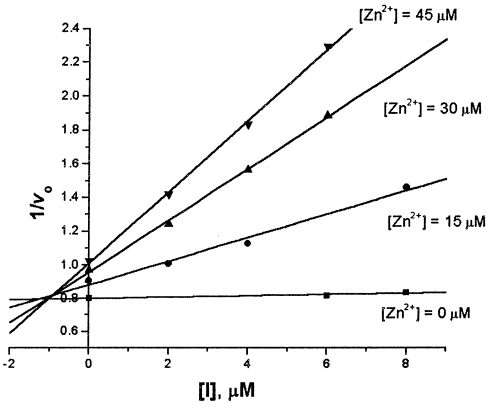


Figure 1. The Yonetani–Theorell plot for determination of the apparent dissociation constant (K_i') and the interaction factor (α) of zinc ion in the inhibition of CPA with **2** in the presence of zinc ion. In the plot, the negative concentration of **2** corresponding to the crossing point of straight lines represents the K_i' value which is equivalent to $-\alpha K_i$. Double inhibition studies for inhibition of CPA by **2** and Zn^{2+} were performed in 0.05 M Tris buffer (pH 7.5), 0.5 M NaCl, and 300 μM of hippuryl-L-phenylalanine (substrate). The concentration of **2** was varied from 0 to 8 μM at four levels of Zn^{2+} concentrations (0, 15, 30, 45 μM).

that can selectively control rapidly proliferating cancerous cells.

In conclusion, we have demonstrated that zinc ion improves the inhibitory activity of CPA inhibitors that carry an imidazole moiety as the zinc-ligating species as much as 212-fold. One can take advantage of the present result in developing therapeutic agents that can selectively inhibit pathologically important zinc metalloproteases in cells of high zinc concentrations such as breast carcinoma.

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