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The ins and outs of biological zinc sites

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Abstract The inner shell coordination properties of zinc proteins have led to the identification of four types of zinc binding sites: *catalytic*, *cocatalytic*, *structural*, and *protein interface*. Outer shell coordination can influence the stability of the zinc site and its function as exemplified herein by the zinc sites in carbonic anhydrase, promatrix metalloproteases and alcohol dehydrogenase. Agents that disrupt these interactions, can lead to increased off rate constants for zinc. D-penicillamine is the first drug to inhibit a zinc protease by catalyzing the removal of the metal. Since it can accept the released zinc we have referred to it as a catalytic chelator. Agents that catalyze the release of the metal in the presence of a scavenger chelator will also inhibit enzyme catalysis and are referred to as enhanced dechelation inhibitors.

Keywords Metalloenzymes · Zinc enzymes · X-ray structure · Chelators · Chelation · Zinc · Thionein · D-pencillamine · Matrix metalloproteinase · Carbonic anhydrase · Alcohol dehydrogenase · Carboxypeptidase

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Introduction

Zinc, copper, and iron are the three of the most prevalent metals in biological systems. The proof of the nutritional importance of zinc spanned nearly 100 years from the first studies in the fungus *Aspergillus niger* (Raulin 1869) to that in humans (Prasad et al. 1963). The rapid changes in technology over the last 40 years have led to establishing its importance on the molecular level. Protein bound zinc is involved in a wide variety of metabolic processes including carbohydrate, lipid, nucleic acid and protein synthesis, and degradation (Auld 2005). Recent studies estimate zinc proteins make up to 10% of the human proteome (Andreini et al. 2006a, b). The function and stability of these zinc proteins is greatly influenced by the zinc binding site.

Classification of zinc sites based on their inner shell coordination properties

Four distinct types of zinc binding sites in enzymes have thus far been identified: catalytic, structural (Vallee and Auld 1990b), cocatalytic (Vallee and Auld 1993), and protein interface (Auld 2001a, b), and the most frequent amino acid ligands are His, Glu, Asp, and Cys (Auld 2005; Patel et al. 2007). Catalytic zinc sites are generally composed of a bound water molecule and three protein ligands, two of which come from a short amino acid spacer (Vallee and Auld

1990a). Structural zinc sites contain four protein ligands and no metal bound water (Vallee and Auld 1990b). While Cys is the most frequent ligand of these sites, any combination of four Cys, His, Glu, and Asp residues in principle can form this type of zinc site (Auld 2004b). Twelve combinations of the 22 permutations of these four ligands have been observed so far. The role of the structural zinc site is to maintain the localized structure of the protein which could in turn influence protein folding or function by supplying residues involved in catalysis that arise from within the spacer arms (Auld 2004b).

Cocatalytic zinc sites are found in enzymes containing two or more zinc and/or other transition metals in close proximity to each other that operate in concert as a catalytic unit (Auld 2004a; Vallee and Auld 1993). The distance between the metals is determined by type of amino acid (Asp, Glu, His, or a carboxylated Lys) that bridges the two metals. Sometimes a water molecule forms a bridge between the metal atoms in a cocatalytic site. Asp and His are the most frequent ligands in this type of site (Auld 2004a). The ligands to these sites often come from nearly the whole length of the protein. This is the likely reason for the difficulty in classifying a number of zinc enzymes in the absence of a three-dimensional structure since the metals are important not only to catalytic function but to protein folding. Protein interface zinc sites were first identified based on the observations that zinc can have an impact on the quaternary structure of a protein (Auld 2001a). They are composed of amino acid ligands that reside in the binding surface between two protein subunits or interacting proteins and generally have the coordination properties of catalytic or structural zinc binding sites (Auld 2001a, 2005; Maret 2004).

Outer shell coordination of zinc protein sites

Catalytic zinc binding sites are often composed of His and Glu/Asp residues that coordinate the zinc by imidazole and carboxyl groups. The zinc dissociation constant for these protein sites is in the nM to pM range. Since the log of the zinc binding constants for imidazole and acetate is about 2.3 and 1.5, respectively (Sillen and Martell 1971) the theoretical value for a zinc dissociation constant for two imidazoles and one acetate is approximately in the μM range. The much lower zinc dissociation constants for protein bound zinc reflects the proximity and restricted mobility of the ligands in the protein. The first kind of stabilization comes from the secondary support structure that supplies the ligands to the zinc. In catalytic zinc sites an α-helix or a β-sheet frequently supply the ligands in the short spacer (Auld 2005). There is strong correlation between the short spacer length and the type of secondary support structure. In thermolysin the secondary support structure is an α-helix and the spacer is three. This permits the ligands to be juxtaposed in an orientation that favors a tetrahedral-like coordination sphere. In carbonic anhydrase the short spacer is one since the secondary support structure is a β-sheet forcing the ligands to come from the same side of the sheet.

The second level of restricted mobility comes from outer shell interactions. Comparative structural studies of four of the first zinc enzymes, alcohol dehydrogenase, carbonic anhydrase, carboxypeptidase, and thermolysin led to the identification of carbonyl and carboxylate "orienters" (Argos et al. 1978).

Hydrophylic orienters in carbonic anhydrase

An elegant series of structure–function studies on mutant carbonic anhydrases demonstrated the importance of these interactions to the stability of the zinc site and to an effect on the nucleophilicity of the metal bound water (Christianson and Fierke 1996; Kiefer et al. 1995; Lesburg and Christianson 1995). The zinc is coordinated by the NE2 of H94 and H96 and ND1 of H119 (pdb# 1CA2). The non-zinc binding NH in all of these imidazoles is H-bonded to another highly conserved residue in the protein. The ND1 hydrogens of H96 and H94 donate a hydrogen bond to the backbone amide carbonyl of N244 and the side chain amide carbonyl of Q92, respectively, while the NE2 hydrogen of H119 H-bonds the OE2 of the side chain of carboxylate of E117. Mutagenesis studies of the orienters indicate the zinc dissociation constant, K_D, is increased by a factor of 5- to 10-fold when the native H-bond is eliminated (Table 1) (Kiefer et al. 1995). The effects also appear to be additive. Thus the combined mutation of Q92A and E117A leads to a 40-fold increase in K_D while the individual mutations lead to 4- and 10-fold increases, respectively (Table 1). The study also demonstrated the H-bond between E117 and H119 determines slow zinc dissociation (Kiefer

Table 1 Stability and functional parameters for native and carbonic anhydrase II mutants

CA II mutant	Zinc K_D, pM	Zinc $k_{off} \times 10^3$, h^{-1}	pK_a
Native	4	5.1	6.8
Q92A	18	10	6.8
Q92E	5	0.9	7.7
E117A	40	1,500	6.9
Q92A/E117A	160	1,100	6.8

Data from Kiefer et al. 1995

et al. 1995). Disruption of this H-bond increases the zinc k_{off} rate constant by 300-fold while the elimination of the Q92 and H96 H-bond leads to only a twofold enhancement (Table 1). The studies further showed that the hydrogen bond network influences the zinc bound water pK_a by modulating the electrostatic environment of the zinc (Kiefer et al. 1995). In the case of the Q92E mutation a neutral hydrogen bond acceptor is replaced by a negatively charged acceptor. The positive charge on the zinc should therefore be reduced, making the ionization of the zinc bound water more difficult. This is indeed the result (Kiefer et al. 1995) (Table 1). The pK_a is increased by 0.9 units for this modification. The zinc off rate constant is also decreased by about 5-fold indicating a strong H-bond. Structural determination by X-ray diffraction confirms this interpretation (Lesburg and Christianson 1995). The reverse effect would be expected for a mutation that removes a negative charge since this should increase the charge on the zinc and decrease the pK_a of the zinc bound water. However, this did not occur when the H-bond interaction between the carboxylate of E117 and the ND1 hydrogen of H119 was eliminated. The pK_a of the zinc bound water does not change (Kiefer et al. 1995) (Table 1). The X-ray studies of the E117A mutant found a chloride ion bound in the region of the E117 carboxylate H119 interaction (Lesburg and Christianson 1995). However, the kinetic assays did not contain chloride ions leaving the results uninterruptable. Closer inspection of the structure of the native enzyme in this region provides another explanation for these results (Auld 2005). There are several potential H-bonds in the vicinity of the carboxylate of E117 in addition to the one with H119 (Fig. 1). Thus the OE2 of E117 is 2.57 Å and 2.93 Å from the NE2 of H119 and ND1 of H107, respectively, while the OE1 of E117 is 2.83 Å and 2.87 Å from the amide

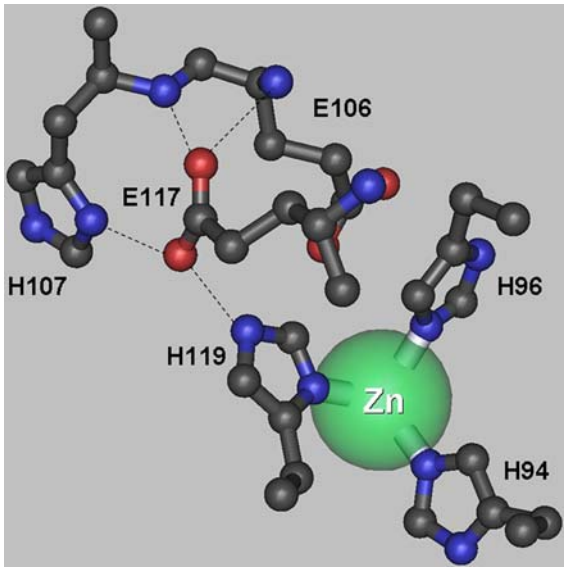

Fig. 1 The human carbonic anhydrase II (pdb# 1CA2) carboxyl oxygens of E117 hydrogen bond the imidazole NH of H107, the non-zinc binding NH of H119 and amide NH's of H106 and E106. Figure prepared using the program Cn3D of the National Center for Biotechnology Information

nitrogens of H107 and E106, respectively. This complex pattern of H-bonds would be expected to disperse the negative charge of the carboxylate onto several residues instead of concentrating it on the H119 residue. Thus removal of this H-bonding interaction should not greatly change the charge on the H119 ligand or in turn affect the pK_a of the zinc bound water in agreement with the obtained results. The intricate pattern of H-bonds may also explain why the disruption of the E117 interaction with H119 has a 150-fold higher effect on the zinc dissociation rate constant than the disruption of the Q92 interaction with H96. The latter interaction lacks this extensive H-bonding network.

Stabilization of Cys ligation to zinc in promatrix metalloproteases

Matrix metalloproteases are expressed in an inactive pro form. Their propeptides contain a cysteine residue in a highly conserved short sequence of amino acids, PRCGN(V)PD (Vallee and Auld 1990b). A number of studies on fibroblast collagenase or MMP1 showed it can be activated by surfactants such as sodium dodecyl sulfate, chaotropic agent such as SCN^-, disulfides such as glutathione, sulfhydryl alkylating agents such as

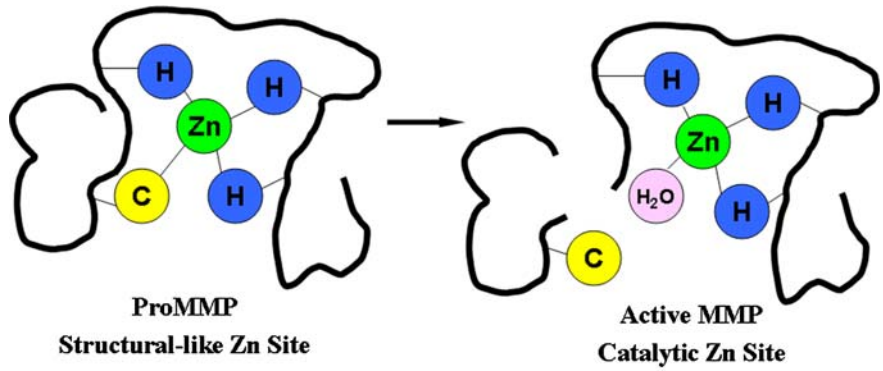

Fig. 2 Schematic of mechanism of activation of promatrix metalloproteinases. The displacement of the propeptide cysteine by water is induced by either proteolytic cleavage and/or conformational changes of the propeptide

N-ethylmalemide, and oxidants such as NaOCl in addition to the then well established activating agents for these enzymes, trypsin, and organo murcurial compounds (Springman et al. 1990) (and references therein) (Fig. 2). These results suggested it was the dissociation or displacement of the cysteine from the catalytic zinc atom in the latent form that led to its activation. They called this activation the "Cysteine Switch" mechanism since the dissociation of the cysteine from the zinc in the latent enzyme switches the role of the zinc from a non-catalytic to a catalytic one. It was further reasoned that the amino acids surrounding the propeptide cysteine acted like Velcro in stabilizing the tetracoordinate structural like zinc site in the latent form (Vallee and Auld 1990b). This represented a new zymogen activation mechanism based on zinc coordination properties. The displacement of the zinc bound cysteine by water converts a structural-like zinc site into a catalytic zinc site (Fig. 2). During the next decade the structural studies of the enzyme were focused on inhibitors bound to the active form of the enzyme. The first coordinates of a proMMP were published in 1999 (Morgunova et al. 1999) (pdb#1CK7) on type IV human collagenase, proMMP-2, followed by human proMMP-1 in 2005 (Jozic et al. 2005) (pdb#1SU3). Examination of the proMMP-2 structure shows how the conserved prolines on each side of the cysteine establish a velcro-like protection of the latent form of the enzyme (Fig. 3). They place the conserved R101 guanidinium ion and D106 carboxylate groups in an ion pair that shields the metal site. In addition the terminal carboxyl oxygen OD1of the D106 (2.88 Å) and the backbone carbonyl of Pro105 (2.98 Å) are within H-bonding distance of the non-metal binding NE2 of H413. All of these interactions should help to stabilize the latent form of the enzyme. Water molecules are excluded from the

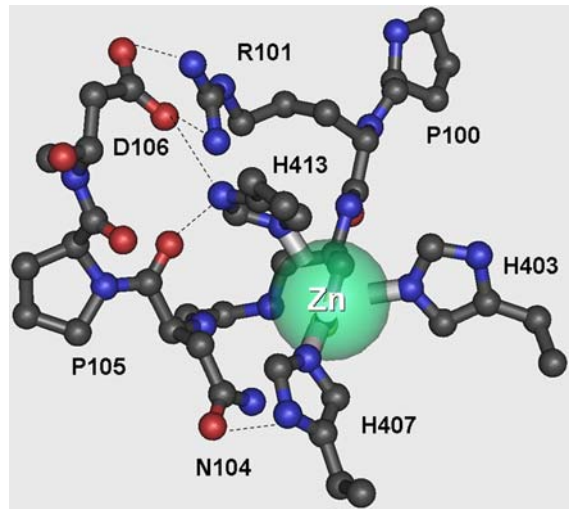

Fig. 3 Stabilization of the latent form of human proMMP-2 (pdb# 1CK7) by the interactions of the amino acids (PRCGNPD) on each side of C102 (bound to the catalytic zinc from beneath). The interaction of the N104 amide carbonyl with H407 can not occur when valine is present at this position. Figure prepared using the program Cn3D of the National Center for Biotechnology Information

catalytic site cleft of proMMP1 with the exception of the S1′ pocket (Jozic et al. 2005). The same interaction of the conserved aspartic acid and arginine residues is observed in the latent enzyme as well as the interaction of the OD1 of the aspartate carboxyl group with the histidine ligand.

Secondary structure stabilization of the structural zinc site of alcohol dehydrogenases

Alcohol dehydrogenases have one structural and one catalytic zinc site (Vallee and Auld 1990b). X-ray crystallographic studies reveal that the structural zinc is bound tetrahedrally to four sulfur atoms of Cys 97, 100, 103, and 111 in a separate loop structure near the

surface of the protein (Eklund et al. 1976). This site is highly conserved for all classes of the ADH family (Auld and Bergman 2008).

Examination of zinc binding to synthetic peptides (length 20–23 amino acids) corresponding to the structural zinc site of horse liver ADH, with and without amino acid replacements/deletions, indicate that both the number of Cys residues, the presence of His, and the spacing between Cys/His residues are important variables (Bergman et al. 2008). The metallochromic chelator 4-(2-pyridylazo) resorcinol (PAR) is effective in direct titration of peptide/zinc complexes and allows the determination of zinc binding constants. In this study Cys is replaced by Ala in each of the four cysteine positions of the replica peptide yielding peptides with only three potential sulfur ligands for binding zinc (Table 2). The study included peptides with 2,1, and no Cys residues in this binding region (Bergman et al. 2008). Depending on the number and position of ligands, the zinc binding constants range in an ordered fashion from tight to low binding (from 10^{10} to 10^{6} M^{-1}). Exclusion chromatography reveals that all four, and three Cys residues peptides generate 1–1 zinc binding (Table 2). However, X-ray absorption fine structure (XAFS) measurements of the peptide/zinc complexes indicate that the single His residue, corresponding to His105, is a zinc ligand. The presence of His in the peptides results in a three Cys/one His coordination with a zinc binding constant, K_B, for the peptide replica of 7.0×10^{9} M^{-1}.

The three Cys/one His coordination for the peptide replica leaves one Cys which does not bind. Judging from the zinc stability constants of the three Cys analogs, Cys103 is a likely candidate (peptide 4, Table 2). When this Cys residue is replaced by Ala, the peptide exhibits almost the same zinc stability as the replica (K_B 6.9×10^{9} M^{-1}). Moreover, the XAFS spectra for the peptide replica and the peptide where Ala replaces Cys103, are essentially identical. When Ala replaces His (peptide 17, Table 2), zinc binds tighter (K_B 1.3×10^{10} M^{-1}), and XAFS analysis reveals a distinct four Cys coordination (Bergman et al. 2008).

The coordination of His105 to the zinc in the peptides suggests that its replacement by Cys103 as ligand in the protein is likely to be accompanied by structural strain and higher energy which could be due to forces exerted upon Cys103 and/or residue 105 in the protein. Analysis of 52 ADH family member sequences using the PIR BLAST and Multiple Alignment programs as well as inspection of three-dimensional structures, provides insight into why this zinc coordination might occur (Bergman et al. 2008). The data reveal that position 105 is not conserved and that Asn (62%), Ser (21%), and His (10%) are the three residues most frequently found at this position. Three-dimensional structures of ADH enzymes exist for the three major variants of residue 105. They show that Cys103 is directed towards the interior of the protein to ligate the zinc while the side chain of the residue at position 105 is directed to the exterior solvent with its potential zinc binding nitrogen or oxygen of the side chain 11.5–13 Å from the structural zinc ion.

Further examination suggests why the residue at position 105 likely exists in this conformation. An α-helix of 14 residues extending from amino acid 324–337 places the ε-amino group of Lys323 within H-bonding distance of the amide carbonyl oxygens of residues 103, 105, and 108 (Fig. 4). While the residues at positions 105 and 108 are not conserved, the interaction of their amide carbonyl oxygen with the

Table 2 Structures, zinc-to-peptide stoichiometries, and zinc binding constants for peptide analogs of the replica of the zinc binding alcohol dehydrogenase segment (residues 93–115 with Cys ligands underlined) (Bergmann et al. 2008)

	Peptide	Zinc/peptide[a] (n = 3–10)	K_B Zinc (M^{-1})[a] (n = 2–5)
	Four cysteines (replica peptide)		
1	FTPQ C GK C RV C KHPEGNF C LKND	1.1	7.0×10^{9}
	Three cysteines		
2	FTPQ A GK C RV C KHPEGNF C LKND	1.2	2.7×10^{9}
3	FTPQ C GK A RV C KHPEGNF C LKND	1.2	2.6×10^{9}
4	FTPQ C GK C RV A KHPEGNF C LKND	1.2	6.9×10^{9}
5	FTPQ C GK C RV C KHPEGNF A LKND	1.1	1.1×10^{10}
	Zero histidine		
17	FTPQ C GK C RV C KAPEGNF C LKND	0.9	1.3×10^{10}

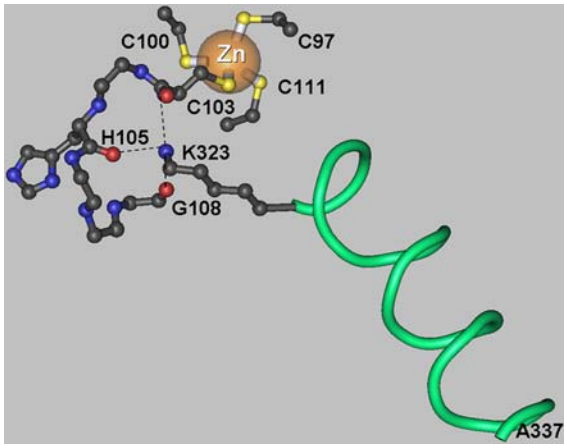

Fig. 4 Three-dimensional structure of the structural zinc site of alcohol dehydrogenase (pdb# 2JHG). The helix ending with Ala337 positions the ε-amino group of Lys323 within H-bonding distance of the backbone carbonyls of amino acid residues Cys103, His105, and Gly108. Figure prepared using the program Cn3D of the National Center for Biotechnology Information

ε-amino group of Lys323 is maintained throughout the structures. This interaction directs the side chain of residue 105 to the exterior solvent. Lys323 is conserved in all 52 sequences of the ADH enzymes, further indicating the importance of this interaction. The available three-dimensional structures for class I–IV ADH family members show this same type of interaction with the conserved Lys at the beginning of a 13–14 residue α-helix (Auld and Bergman 2008).

Enhanced dechelation of protein bound zinc by small molecule inhibitors

Chelating agents have been widely used to identify, study, and inhibit metalloenzymes (Auld 1988). Potent inhibitors of metalloenzymes displace the metal bound water to from stable ternary complexes (TCI). This principle forms the present inhibitor based drug design for metalloenzymes. Thus studies of the binding of sulfhydryl inhibitors to the catalytic zinc site of a model metalloprotease, carboxypeptidase A, led to the design of specific inhibitors of angiotensin converting enzyme nearly three decades before its three-dimensional structure was determined (Natesh et al. 2003; Ondetti et al. 1977; Towler et al. 2004). However, alternative inhibition modes are possible when the target is a metalloenzyme. Recent studies of carboxypeptidase A show the chelating drug D-penicillamine (D-PEN, β,β-dimethyl cysteine) increases the dissociation rate constant of the enzyme bound catalytic zinc ion by 400 fold (Chong and Auld 2000). Since D-PEN is a chelator it can also act as the receptor of the released metal leading to inhibition of the enzyme. We proposed the name "catalytic chelation" to account for this mechanism of inhibition by a chelating agent. In a more general sense any molecule that can disrupt outer shell interactions leading to an increased zinc dissociation rate could lead to enzyme inhibition if a scavenger chelator is present to accept the released metal. Such a molecule is referred to as an enhanced dechelation inhibitor, EDI (Eq. 1).

$$\mathrm{EDI} + \mathrm{ZnE} \leftrightarrows \mathrm{Zn} + \mathrm{EDIE} \overset{+\mathrm{SC}}{\leftrightarrows} \mathrm{EDIE} + \mathrm{Zn(SC)} \qquad (1)$$

We tested this hypothesis by studying the effect of EDTA and thionein on the D-PEN inhibition of carboxypeptidase A (ZnCPD), a model zinc protease (Chong and Auld 2007). EDTA has frequently been used for heavy metal detoxification due to its high affinity for zinc(II) (10^{16} M^{-1}) (Auld 1995). Thionein (T), the apo-form of metallothionein (MT) is capable of tightly binding seven zinc ions with twenty cysteine ligands with a stability constant for Zn_7T of 10^{13} M^{-1} at neutral pH (Vasak and Kagi 1983). MT has been hypothesized to participate in zinc homeostasis and heavy metal detoxification (Kagi and Schaffer 1988; Krezel and Maret 2007). Since thionein is present in the body, it could serve as a physiological scavenger chelator.

Neither EDTA nor thionein are effective inhibitors of ZnCPD even though both have a strong affinity

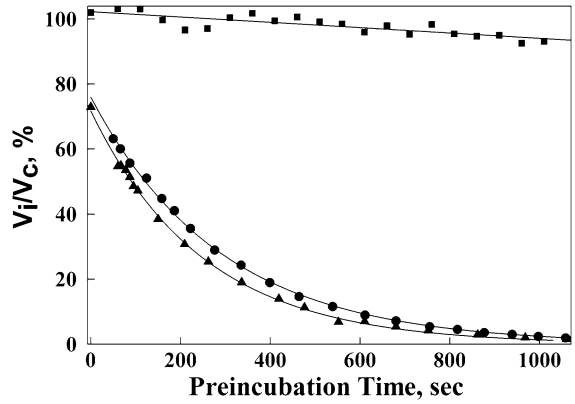

Fig. 5 D-PEN, 250 μM, catalyzes zinc transfer from carboxypeptidase A to either EDTA, 1 mM (●) or thionein, 2.5 μM (▲). In the absence of D-PEN, the activity is reduced by less than 10% over this time period in the presence of EDTA (■) or thionein. Data from (Chong and Auld 2007)

for zinc(II) (Fig. 5). However, when combined with D-PEN these chelators have a synergistic effect, increasing the potency of inhibition. Thus the addition of 1 mM EDTA or 2.5 μM thionein to an incubation mixture of 0.25 mM D-PEN and ZnCPD decreases enzyme activity to 1% within 20 min (Fig. 5) (Chong and Auld 2007). The discover of enhanced dechelation inhibitors could lead to a better understanding of zinc homeostasis and give an alternative approach to inhibiting metalloenzymes.

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