Catalysis of Zinc Transfer by D-Penicillamine to Secondary Chelators

Curtis R. Chong[†,‡] and David S. Auld*[,§]

Department of Pharmacology and Molecular Sciences, Medical Scientist Training Program, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, Department of Biology, Boston College, 140 Commonwealth Avenue, Chestnut Hill, Massachusetts 02467

Received July 4, 2007

The antiarthritis drug D-penicillamine (D-PEN) catalyzes zinc(II) transfer from carboxypeptidase A to chelators such as thionein and EDTA at a rate constant up to 400-fold faster than the uncatalyzed release. Once D-PEN releases zinc(II) from enzyme stronger chelators can tightly bind zinc(II) leading to complete and essentially irreversible inhibition. D-PEN is the first drug to inhibit a zinc protease by catalyzing metal removal, and the name "catalytic chelation" is proposed for this mechanism.

Introduction

Zinc metalloenzymes are involved in biological processes that control development, growth, and transmission of the genetic message in a wide range of phyla and species.[1] Chelating agents have been widely used to identify, study, and inhibit metalloenzymes.[2] Chelators that form stable ternary complexes with the enzyme bound metal are potent inhibitors. 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 (ZnCPD[a]), led to the design of specific inhibitors of angiotensin converting enzyme nearly three decades before its three-dimensional structure was determined.[3−5] However, alternative inhibition modes are possible when the target is a metalloenzyme. Recent studies of ZnCPD show the chelating drug D-penicillamine (D-PEN, β,β-dimethyl cysteine) increases the dissociation rate constant of the enzyme bound catalytic zinc ion.[6] Because D-PEN is a chelator, it can also act as the receptor of the released metal leading to inhibition of the enzyme. We have proposed the name "catalytic chelation" to account for this mechanism of inhibition.

D-PEN is used clinically to treat Wilson's disease, an inherited disorder of copper metabolism, heavy metal poisoning, and rheumatoid arthritis. Although the mechanism of D-PEN in heavy metal poisoning and Wilson's disease is attributed to its ability to bind metal, the mechanism of D-PEN in arthritis remains unknown.[7] D-PEN weakly inhibits several zinc proteases, including members of the matrix metalloprotease family,[8,9] which are implicated in the pathogenesis of rheumatoid arthritis and cancer metastasis.[10]

Although D-PEN catalyzes release of the active site zinc(II) from metalloproteases, the apoenzyme is a strong chelator that rebinds zinc(II). Consequently, the potency of D-PEN is limited by the relative affinities of it and the apoenzyme for zinc(II). In principle, the potency of drugs that catalyze metal removal like D-PEN could be increased if the released metal rapidly diffuses away from apoenzyme or is captured by a second chelator. The second chelator does not need to directly inhibit the metalloenzyme. As long as it has a higher affinity for zinc(II) than the apoenzyme it should contribute synergistically to inhibition.

We tested this hypothesis by studying the effect of EDTA and thionein (T) on the D-PEN inhibition of ZnCPD, a model zinc protease. EDTA has frequently been used for heavy metal detoxification due to its high affinity for zinc(II) (10^{16} M^{-1}).[11] 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.[12] MT has been hypothesized to participate in zinc homeostasis and heavy metal detoxification.[13] Because T is present in the body, it could serve as a physiological secondary or scavenging chelator. Neither T nor EDTA are effective inhibitors of ZnCPD even though both have a strong affinity for zinc(II). However, we show when combined with D-PEN these chelators have a synergistic effect, increasing the potency of inhibition.

Materials and Methods

Materials. Bovine ZnCPD (Cox), obtained from Sigma, was purified on a CABS-sepharose affinity column and recrystallized as previously described.[14] T from liver MT-2 was the generous gift of Dr. Klaus Jacob and was prepared as previously described.[15] The fluorescent carboxypeptidase substrate dansylglycylglycyl-L-tryptophan (DnsGGW) was characterized as previously described.[16] D-PEN was prepared fresh each day. The preparation of buffers and precautions used to avoid metal contamination are detailed elsewhere.[6]

Kinetics. Enzyme assays were performed with an Applied Photophysics Biosequential SX-18MV stopped-flow reaction analyzer. Hydrolysis of the energy-transfer based substrate DnsGGW was monitored by following the increase in tryptophan fluorescence upon cleavage of the scissile bond.[6] Initial velocity values were obtained by a linear regression of no more than the first 10% of the substrate hydrolysis. When D-PEN and ZnCPD are preincubated, the initial rate for substrate hydrolysis decreases exponentially with time. The first-order rate constant, k, for this time-dependent ZnCPD inhibition is determined by using eq 1.

$$V_t = (V_i - V_f)\, e^{-kt} + V_f \quad (1)$$

where V_i is the initial velocity in the presence of inhibitor at zero time of incubation, V_f is the initial velocity after full equilibration of the enzyme and inhibitor, and V_t is the velocity at any time t. In the presence of a second chelator V_f is essentially zero. The analysis

* To whom correspondence should be addressed. Tel.: 617-552-0814. Fax: 617-552-2011. E-mail: auldda@bc.edu.

† Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine.

‡ Medical Scientist Training Program, The Johns Hopkins University School of Medicine.

§ Boston College.

[a] Abbreviations: CABS-sepharose, [*N*-(ϵ-aminocaproyl)-*p*-aminobenzyl]-succinyl-sepharose 4B; dansyl, 5-dimethylaminonapthalene-1-sulfonyl-; D-Cys, D-cysteine; D-PEN, D-penicillamine; EDTA, ethylenediaminetetraacetic acid; MT, metallothionein; MT-2, metallothionein isoform 2; PAR, 4-(2-pyridylazo)resorcinol; SC, secondary chelator; T, thionein; ZnCPD, carboxypeptidase A.

10.1021/jm070803y CCC: $37.00
Published on Web 10/06/2007

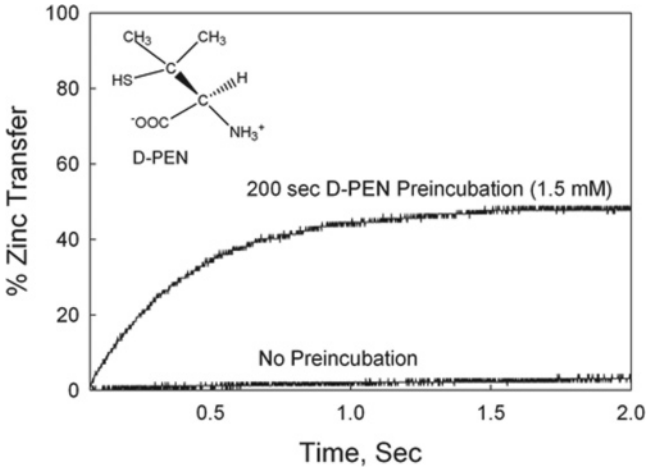

Figure 1. D-PEN catalyzes zinc(II) removal from ZnCPD. D-PEN, 1.5 mM, and ZnCPD, 5 μM, are preincubated for 200 s and mixed with 3.6 mM PAR and 1.5 mM D-PEN in the same buffer in the stopped-flow (top line). Zinc(II) transfer to PAR is monitored by the increase in absorbance at 500 nm. Essentially no transfer of zinc(II) occurs when ZnCPD, 5 μM, is mixed with 3.6 mM PAR and 3.0 mM D-PEN without preincubation (bottom line).

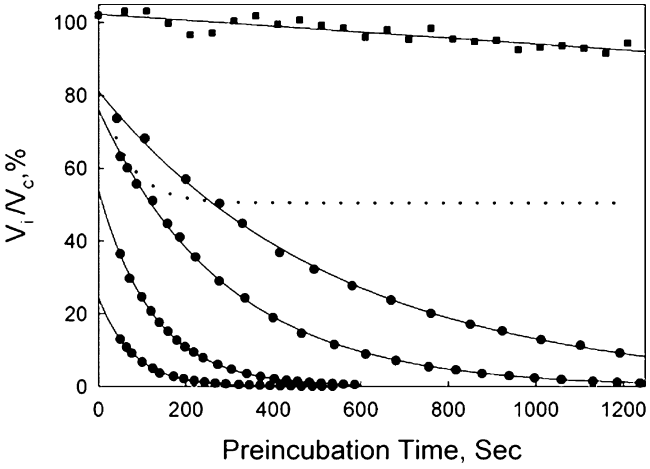

Figure 2. EDTA increases the potency of D-PEN inhibition. D-PEN, 0.25 mM (dotted line), is preincubated in the drive syringe with 2 μM ZnCPD and mixed with 0.25 mM D-PEN and 20 μM DnsGGW substrate at the indicated times. Preincubation of ZnCPD and 1 mM EDTA results in minimal inhibition even at the longest time studied (■). In contrast, when ZnCPD is preincubated with 1 mM EDTA and varying concentrations of D-PEN (●) and mixed with substrate, 1 mM EDTA, and an equivalent amount of D-PEN, enzyme inhibition is greater. The instantaneous velocity, V_i, is expressed as the percentage of the uninhibited ZnCPD activity, V_c. The concentrations of D-PEN from top to bottom are (●): 0, 0.1, 0.25, 1, and 5 mM. The solid lines are obtained using eq 1.

of such curves was performed using the Sigma Plot 4.0 program for a two-parameter single-exponential decay. The concentration dependence of the first-order rate constant on D-PEN concentration was fitted to a two-parameter single rectangular hyperbola.

PAR Experiments. The chromophoric chelator 4-(2-pyridylazo)-resorcinol (PAR) was used to measure zinc(II) transfer from ZnCPD to D-PEN through the increase in absorbance at 500 nm upon formation of the $Zn(PAR)_2$ complex. Data were collected at a PAR concentration of 1.8 mM and the 0.2 mm path length of the stopped-flow reaction cell. The transmittance was set to 100% with buffer solution. The extinction coefficient at 500 nm was 61 500 M^{-1} cm^{-1} for the $Zn(PAR)_2$ complex.

Results

D-PEN Catalyzes Zinc(II) Removal from ZnCPD. D-Cys and D-PEN, which differ structurally by only two methyl groups on the β-carbon, inhibit ZnCPD by different mechanisms. D-Cys forms a stable ternary complex by binding to the active site metal.[17] D-PEN on the other hand destabilizes zinc(II) binding leading to dissociation of zinc(II) with a rate constant 400-fold faster than the uncatalyzed rate constant.[6] Preincubation of the enzyme with 1.5 mM D-PEN for 200 s followed by the addition of the chromophoric metal chelator PAR indicates D-PEN releases 45% of zinc(II) in the preincubation period (Figure 1, eq 2). Essentially no zinc(II) is released when the enzyme is mixed with PAR and D-PEN without preincubation.

$$\underset{(100\%)}{\text{ZnCPD}} \xrightarrow[\text{200 s preincubation}]{\text{+1.5 mM D-PEN}} \underset{(45\%)}{\text{Zn(D-PEN)}_2} + \underset{(55\%)}{\text{ZnCPD}} \xrightarrow[\text{rapid reaction}]{\text{+1.8 mM PAR}} \underset{(45\%)}{\text{Zn(PAR)}_2} + \underset{(55\%)}{\text{ZnCPD}} \quad (2)$$

The extent of zinc(II) removal in the preincubation phase is dependent on the concentration of D-PEN.

Increasing the Potency of D-PEN Inhibition. The ability of D-PEN to catalyze zinc(II) transfer to a metal-binding agent of greater affinity like PAR suggests the potency of D-PEN could be increased if a strong chelator is included in the preincubation mixture. EDTA is a powerful zinc(II) chelator that inhibits many metalloenzymes over long time periods by scavenging zinc(II) that dissociates from the protein in an S_N1 mechanism. Due to the bulky nature of its chelating ligands, EDTA frequently cannot access the enzyme active site, and, as a result, dissociation of zinc(II) from enzyme is the rate-determining step. Once released, zinc(II) rapidly binds EDTA to form a stable complex that precludes zinc(II) return to the apoenzyme. There is only about a 5% decrease in activity over a 20 min incubation of EDTA and ZnCPD under the chosen experimental conditions (Figure 2). This time-dependent decrease in activity reflects the dissociation of zinc(II) from the protein and the subsequent scavenging of the metal by EDTA.

When D-PEN, 0.25 mM, and ZnCPD, 2 μM, are preincubated in one syringe of the stopped-flow and mixed with substrate and 0.25 mM D-PEN, the instantaneous velocity decreases to approximately 51% of the uninhibited velocity with a first-order rate constant of 0.016 s^{-1} (Figure 2). After 200 s, no further time-dependent inhibition occurs, suggesting $Zn(D\text{-}PEN)_2$ and ZnCPD reach an equilibrium governed by the affinities of apoenzyme and D-PEN for zinc(II).

The addition of 1 mM EDTA to the 0.25 mM D-PEN and ZnCPD preincubation mixture decreases enzyme activity to 1% within 20 min (Figure 2). EDTA displaces the equilibrium between apoenzyme and D-PEN for zinc(II) toward D-PEN by continually transferring the zinc(II) bound by D-PEN to EDTA. Increasing the concentration of D-PEN progressively decreases the time that it takes for complete inhibition. The combination of 1 mM D-PEN and 1 mM EDTA leads to less than 1% activity within 8 min (Figure 2). The time-dependent increase in the concentration of apoenzyme leads to a concomitant decrease in enzyme activity. Inactivation is a first-order process with a rate constant of 0.013 s^{-1} at 5 mM D-PEN that is 260 times faster than the rate constant for the noncatalytic dissociation of zinc(II) from the enzyme. The enzyme activity is now decreased to 0.1% (1000-fold) within 7 min.

The dependence of the rate constant for inactivation on the D-PEN concentration is hyperbolic (Figure 3). This type of kinetic behavior is indicative of a mechanism in which D-PEN inhibition occurs in two steps. The first step is rapid formation of an enzyme·D-PEN complex with an apparent dissociation

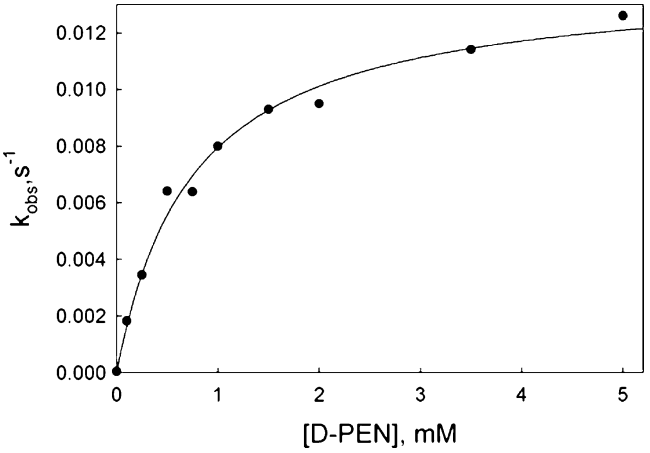

Figure 3. Dependence of the first-order inactivation rate constant, *k*, on D-PEN concentration in the presence of EDTA. The rate constants are obtained as shown in Figure 2. The solid line is derived from the function: $k = 0.0139\ s^{-1}$ [D-PEN]/(0.75 mM +[D-PEN]).

constant of 0.75 mM. This is followed by a limiting first-order decrease in activity of 0.014 s^{-1}. These results are consistent with our previous studies in the absence of EDTA, indicating that D-PEN very rapidly binds to the enzyme and then releases zinc(II) in a rate-determining step with a 0.016 s^{-1} rate constant.[6] The initial apparent binding constant of 0.75 mM compares favorably with the constant of 1.1 mM obtained from the preincubation studies with D-PEN alone.

In this mechanism, D-PEN mediates zinc(II) transfer to EDTA. The zinc(II) stability constant for EDTA is estimated to be 10^{16} M^{-1} based on the binding of the tetra-anionic species of EDTA to Zn(II) at pH 7.5.[11] The zinc(II) affinity constant for apo-ZnCPD is estimated to be 10^9 at pH 7 (D. S. Auld, unpublished). Because the zinc(II) stability constant for EDTA is about 10^7-fold greater than that of apo-carboxypeptidase, zinc(II) transfer to EDTA is essentially irreversible. As a result, the zinc(II) equilibrium in the presence of D-PEN and EDTA is governed by the affinity of EDTA for zinc(II), which in turn drives apoenzyme formation.

T, a Physiological Chelator, Increases the Potency of D-PEN. To investigate whether D-PEN could transfer zinc(II) to a physiological chelator, we preincubated T with D-PEN and ZnCPD. T, the apo-form of MT, is a protein of 60 amino acids, 20 of which are cysteine that can bind seven zinc ions.[13] This protein can also bind several other first transition and group 12 elements in place of the zinc(II). The high affinity of T for zinc(II) suggests it could augment the potency of D-PEN in a manner analogous to that observed for EDTA.

T at 2.5 μM does not inhibit ZnCPD instantaneously and displays only a slight time-dependent decrease in activity upon preincubation alone with enzyme (Figure 4), similar to that observed for EDTA (Figure 2). Preincubation of 2.5 μM T with 2 μM ZnCPD in the presence of 0.25 mM D-PEN reduces enzyme activity to 1.5% of the uninhibited velocity in 17 min. The first-order rate constant for this process, 0.0039 s^{-1}, is quite similar to that when the same concentration of D-PEN and ZnCPD are preincubated with 1 mM EDTA (0.0034^{-1}; Figure 2). Although the T concentration used in this experiment is 400-fold lower than the EDTA concentration, the rate constant of the observed inhibition is similar because it is related mechanistically to the concentration of D-PEN rather than to the concentration of EDTA or T.

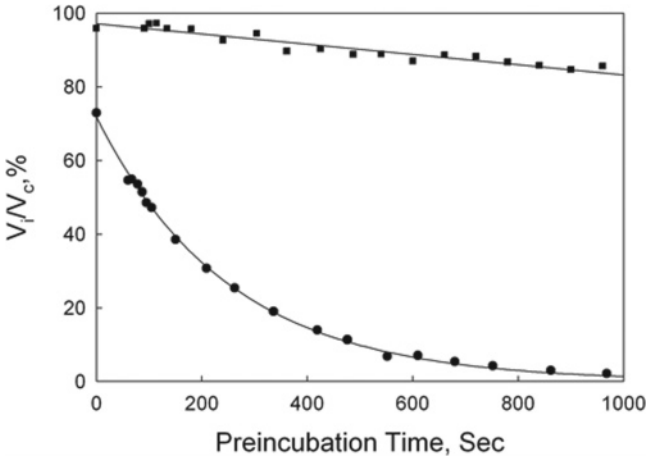

Figure 4. D-PEN catalyzes zinc(II) transfer to thionein. Thionein, 2.5 μM, and ZnCPD, 2 μM, are preincubated as described in Figure 2 caption with (●) or without 0.25 mM D-PEN (■) and mixed with 20 μM DnsGGW to obtain an instantaneous velocity, V_i, expressed as the percentage of the uninhibited ZnCPD activity, V_c. The solid line is obtained using eq 1, using a first-order rate constant for inactivation of 0.0039 s^{-1} and an initial V_i/V_c of 71.4%.

Discussion

The potency of the antiarthritis drug D-PEN as a ZnCPD inhibitor increases in the presence of a secondary chelator (SC) that binds zinc(II) with high affinity, such as T or EDTA. Although D-PEN catalyzes zinc(II) removal from ZnCPD, it can only compete effectively with apoenzyme for released zinc(II) when the concentration of D-PEN favors the $Zn(D\text{-}PEN)_2$ complex. When D-PEN is preincubated with ZnCPD, enzyme activity decreases to reach a constant level, which reflects formation of this equilibrium (Figure 2). Strong metal binding SCs such as EDTA and T shift this equilibrium by binding zinc(II) released from the enzyme

$$\text{D-PEN} + \text{ZnCPD} \leftrightarrow \text{Zn(D-PEN)}_2 + \text{CPD} \xrightleftharpoons{+\text{SC}} \text{CPD} + \text{Zn(D-PEN)}_2 + \text{Zn(SC)} \quad (4)$$

Because T and EDTA bind zinc(II) with greater affinity than apoenzyme, zinc(II) return to apoenzyme proceeds at a very slow rate, making inhibition according to this mechanism essentially irreversible, because the estimated amounts of *free* zinc(II) present in the body is in the pM to nM range.[18,19]

Experiments show zinc(II) chelating agents such as Tris buffer, citrate, and glutathione mediate zinc(II) transfer from the zinc enzyme alkaline phosphatase to T.[20] Zinc(II) transfer in these experiments is not as efficient as that observed here for D-PEN, however, as a 2 h preincubation is required to cause modest amounts of inhibition. In the absence of D-PEN, ZnCPD is essentially unresponsive to the presence of T due to the slow rate of zinc(II) dissociation from enzyme. By catalyzing zinc(II) dissociation, D-PEN renders ZnCPD susceptible to T inhibition. The rapid rates of zinc(II) removal observed for D-PEN also suggest a mechanism for regulating zinc proteins that are normally insensitive to inhibition by physiological chelators. Catalytic chelators that transfer zinc(II) from proteins to T could be part of a zinc(II) recovery pathway for homeostatic regulation.

Complications limit the number of arthritis patients who benefit from D-PEN without adverse reactions: 55−65% of patients respond to D-PEN, but side effects reduce the number who benefit without complications to 41%.[21] Long-term D-PEN

serum levels of 0.1 mM are reached in patients being treated for rheumatoid arthritis.[22] Although this level may be insufficient to cause strong instantaneous inhibition of ZnCPD, our work suggests more potent time-dependent inhibition can occur in the presence of physiologic SCs like T. Remarkably, many of the common side effects reported for D-PEN are similar to those observed for zinc deficiency in humans. These side effects include abnormalities in taste and the immune system, skin disorders, and problems with wound healing.[7,23] Further studies on other zinc metalloproteins will give insight on whether the ability of D-PEN to catalyze zinc(II) removal can account for many of its drug-related side effects.

Acknowledgment. This research was supported by NIH Grant GM-53265 to D.S.A. and by grants from the Harvard College Undergraduate Research Program, the Dean's Summer Research Award, and the Head Tutor's fund in the Department of Biochemical Sciences, Harvard University to C.R.C.

References

(1) Auld, D. S. Zinc Enzymes. *Encyclopedia of Inorganic Chemistry*, 2nd ed.; John Wiley & Sons, Ltd.: Chichester, 2005; pp 5885−5927.
(2) Auld, D. S. Use of chelating agents to inhibit enzymes. *Methods Enzymol.* **1988**, *158*, 110−114.
(3) Ondetti, M. A.; Rubin, B.; Cushman, D. W. Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. *Science* **1977**, *196*, 441−444.
(4) Natesh, R.; Schwager, S. L.; Sturrock, E. D.; Acharya, K. R. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature* **2003**, *421*, 551−554.
(5) Towler, P.; Staker, B.; Prasad, S. G.; Menon, S.; Tang, J.; et al. ACE2 structures reveal a large hinge-bending motion important for inhibitor binding and catalysis. *J. Biol. Chem.* **2004**, *279*, 17996−18007.
(6) Chong, C. R.; Auld, D. S. Inhibition of carboxypeptidase A by D-pencillamine: Mechanism and implications for drug design. *Biochemistry* **2000**, *39*, 7580−7588.
(7) Munro, R.; Capell, H. A. Penicillamine. *Br. J. Rheumatol.* **1997**, *36*, 104−109.
(8) Sapolsky, A. I.; Keiser, H.; Howell, D. S.; Woessner, J. F., Jr. Metalloproteases of human articular cartilage that digest cartilage proteoglycan at neutral and acid pH. *J. Clin. Invest.* **1976**, *58*, 1030−1041.
(9) Norga, K.; Grillet, B.; Masure, S.; Paemen, L.; Opdenakker, G. Human gelatinase B, a marker enzyme in rheumatoid arthritis, is inhibited by D-penicillamine: Antirheumatic activity by protease inhibition. *Clin. Rheumatol.* **1996**, *15*, 31−34.
(10) Nagase, H.; Visse, R.; Murphy, G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc. Res.* **2006**, *69*, 562−573.
(11) Auld, D. S. Removal and replacement of metal ions in metallopeptidases. *Methods Enzymol.* **1995**, *248*, 228−242.
(12) Vasak, M.; Kagi, J. H. R. *Metal Ions in Biological Systems*; Marcel Dekker, Inc.: New York, 1983; pp 213−273.
(13) Kagi, J. H.; Schaffer, A. Biochemistry of metallothionein. *Biochemistry* **1988**, *27*, 8509−8515.
(14) Bicknell, R.; Schaeffer, A.; Auld, D. S.; Riordan, J. F.; Monnanni, R.; et al. Protease susceptibility of zinc- and apo-carboxypeptidase A. *Biochem. Biophys. Res. Commun.* **1985**, *133*, 787−793.
(15) Maret, W. Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 237−241.
(16) Latt, S. A.; Auld, D. S.; Vallee, B. L. Fluorescence determination of carboxypeptidase A activity based on electronic energy transfer. *Anal. Biochem.* **1972**, *50*, 56−62.
(17) van Aalten, D. M.; Chong, C. R.; Joshua-Tor, L. Crystal structure of carboxypeptidase A complexed with D-cysteine at 1.75 A−Inhibitor-induced conformational changes. *Biochemistry* **2000**, *39*, 10082−10089.
(18) Outten, C. E.; O'Halloran, T. V. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **2001**, *292*, 2488−2492.
(19) Krezel, A.; Maret, W. Zinc-buffering capacity of a eukaryotic cell at physiological pZn. *J. Biol. Inorg. Chem.* **2006**, *11*, 1049−1062.
(20) Jacob, C.; Maret, W.; Vallee, B. L. Control of zinc transfer between thionein, metallothionein, and zinc proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3489−3494.
(21) Joyce, D. A. D-Penicillamine. *Baillieres Clin. Rheumatol.* **1990**, *4*, 553−574.
(22) Muijsers, A. O.; van de Stadt, R. J.; Henrichs, A. M.; Ament, H. J.; van der Korst, J. K. D-Penicillamine in patients with rheumatoid arthritis. Serum levels, pharmacokinetic aspects, and correlation with clinical course and side effects. *Arthritis Rheum.* **1984**, *27*, 1362−1369.
(23) Crawhall, J. C.; Lecavalier, D.; Ryan, P. Penicillamine, its metabolism and therapeutic applications: A review. *Biopharm. Drug Dispos.* **1979**, *1*, 73−95.

JM070803Y