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Astacus Protease, a Zinc Metalloenzyme†

Walter Stöcker, Russell L. Wolz, and Robert Zwilling*
Department of Physiology, Institute of Zoology, University of Heidelberg, Im Neuenheimer Feld 230, D-6900 Heidelberg, FRG
Daniel J. Strydom and David S. Auld
Center for Biochemical and Biophysical Sciences and Medicine and Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, 250 Longwood Avenue, Boston, Massachusetts 02115
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ABSTRACT: *Astacus* protease, an endoprotease of molecular weight 22 614, from the freshwater crayfish *Astacus fluviatilis* contains 0.97 ± 0.03 mol of zinc/mol of protein, as measured by atomic absorption spectroscopy. The metal chelating agents ethylenediaminetetraacetate (EDTA), 1,10-phenanthroline, dipicolinic acid, 8-hydroxyquinoline-5-sulfonic acid, and 2,2′-bipyridyl inactivate the enzyme reversibly in a time-dependent manner. Inactivation by 1,10-phenanthroline occurs within a few minutes whereas EDTA requires days. The inactivation data are consistent with a proposed model in which a transient ternary enzyme–metal–chelator complex is formed that subsequently dissociates to yield apoenzyme plus a chelator–metal complex. The half-life for metal dissociation in the absence of chelator is estimated to be 40 days, much slower than for carboxypeptidase A (28.3 min) or angiotensin converting enzyme (92.4 s) though much faster than carbonic anhydrase (5.4 years). Dialysis against 1,10-phenanthroline results in inactive apoenzyme which can be reactivated by the addition of stoichiometric amounts of zinc, copper, or cobalt to 100%, 70%, or 50% of native activity, respectively, indicating that the metal is required for catalysis. Cobalt-*Astacus* protease exhibits an absorption spectrum with a maximum at 514 nm (ϵ_{514} = 76.5 M^{-1} cm^{-1}) and shoulders at 505 and 550 nm, indicative of a distorted tetrahedral-like geometry about the cobalt ion. This spectrum is similar to that seen for metalloneutral proteases such as thermolysin. On the basis of similarities of sequences for thermolysin about residues 142–148 (HELTHAV) and residues 92–98 for *Astacus* protease (HELMHAI), histidyl residues 92 and 96 may be ligands to the zinc, and Glu-93 may play a role in catalysis.

Proteolytic enzymes have become the focus of much attention because of their importance in many diverse physiological systems such as complement activation, hormone production, blood coagulation, and digestion (Neurath & Walsh, 1976). The freshwater crayfish *Astacus fluviatilis*, an invertebrate species that represents a taxonomic point distant from both microorganisms and chordates, has been useful in examining the evolutionary aspects of proteases (Zwilling & Neurath, 1981).

As was first described by Pfleiderer et al. (1967), the stomachlike cardia of this crayfish contains an endoprotease, *Astacus* protease, which has been considered unique by virtue of its cleavage specificity. It use as an aid in the structural analysis of tubulin revealed that *Astacus* protease prefers a

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* Address correspondence to this author.

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small aliphatic residue in the P_1' position (i.e., the N-terminal position after cleavage) (Krauhs et al., 1982). Aside from *Astacus* protease, two other proteases from the digestive tract of this animal have been characterized through elucidation of their complete primary structures. Of these, *Astacus* trypsin and *Astacus* carboxypeptidase proved to be homologous (43.6% and 44.7%, respectively) with their mammalian (bovine) counterparts (Titani et al., 1983, 1984). *Astacus* protease is a single polypeptide composed of 200 amino acids with a molecular weight of 22 614. It, however, shows no homology with any other protease or protein sequenced to date (Titani et al., 1987).

Early investigations failed to detect any reversible or irreversible inhibitors of synthetic or natural origin and thus shed no light on its catalytic mechanism (Zwilling et al., 1981; Zwilling & Neurath, 1981). The present work demonstrates that *Astacus* protease is a zinc metalloenzyme. Preliminary reports have been made (Wolz et al., 1987a,b).

Materials and Methods

Materials. Solutions of metal ions were prepared from the spectrophotometrically pure sulfate or chloride salts ("SpecPure" grade, Johnson–Matthey, Royston, U.K.). 2,2′-Bipyridyl (BP),[1] 1,10-phenanthroline (OP), and 8-hydroxyquinoline-5-sulfonic acid (HQSA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1,7-Phenanthroline (mP) was from G. F. Smith Chemical Co. (Columbus, OH). Dipicolinic acid (DPA) was from Eastern Organic Chemicals. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), succinyl-Ala-Ala-Ala-*p*-nitroanilide (STANA), and diphenylthiocarbazone (dithizone) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and purchased from Fisher Chemical Co., Merck (Darmstadt), or Eastman Kodak Co. (Rochester, NY).

Methods. Astacus protease was isolated from the digestive juice of the European freshwater crayfish *Astacus fluviatilis* Fabr. as previously described (Zwilling & Neurath, 1981).

For each batch, protein concentration was determined by amino acid analysis on a Waters amino acid analyzer employing the picotag method (Bidlingmeyer et al., 1984). The concentration was calculated on the basis of the known molecular weight of 22 614 (Titani et al., 1987). A Gilford Model 200 spectrophotometer was used to measure the optical density at 280 nm, and a molar absorptivity constant, ϵ, of 42 800 M^{-1} cm^{-1} was determined. Subsequent protein concentrations were based on the absorbance at 280 nm.

Enzyme activity was measured colorimetrically using STANA as a substrate (Bieth et al., 1974; Geiger, 1984). Routine assays were performed at 25 °C with 1 mM substrate and 0.4–1.5 μM enzyme buffer in either 20 mM HEPES or 200 mM TEA at pH 7.8. Increasing absorption at 405 nm due to the release of *p*-nitroaniline was monitored with a Gilford Model 200 spectrophotometer or with an LKB-Ultrospec 4050 spectrophotometer interfaced with ann Apple IIe computer.

All experiments requiring metal-free conditions were carried out in plasticware or glassware soaked in 30% nitric acid and then rinsed carefully with metal-free water. Teflon beakers and disposable metal-free polypropylene pipet tips, vials, and plastic cuvettes were used for all assays of the apoenzyme. All buffers and solutions were extracted with 0.01% dithizone in CCl_4 to remove adventitious metal ions (Thiers, 1957). Metal-free dialysis tubing was prepared by extensive washing with metal-free water at 80 °C (Auld, 1988a).

Metal analyses were performed by flameless atomic absorption spectroscopy using a Perkin-Elmer Model 5000 absorption spectrometer equipped with a graphite furnace.

Apo-*Astacus* protease was prepared by the procedure developed for carboxypeptidase A (Auld 1988b). A 3-mL solution of enzyme, 56 μM, in 50 mM HEPES buffer, pH 7.5, was dialyzed for 4 days at 4 °C against four changes of 100 mL of the same buffer containing 10 mM 1,10-phenanthroline. Subsequently, excess chelator was removed by dialysis versus three changes of 50 mL of metal-free buffer. The apozenzyme was stored in solution at 4 °C.

The absorption spectrum of cobalt-*Astacus* protease was recorded with a Cary 219 spectrophotometer interfaced with an Apple computer. First, the spectrum of a solution of 56 μM apo-*Astacus* protease in 20 mM HEPES, pH 7.5, was recorded. Then, substoichiometric amounts of $CoSO_4$ were added in a stepwise fashion. At each step, a difference spectrum was recorded using the initial solution of metal-free enzyme as the reference. Molar absorptivity constants were calculated for the observed absorption bands and are expressed in units of M^{-1} cm^{-1}.

Table I: Metal Content of Purified *Astacus* Protease[a]

metal	mol of metal/mol of *Astacus* protease	
	prepn 1	prepn 2
Zn	0.94	1.01
Mg		0.035
Ni	0.015	0.013
Fe	0.036	0.029
Mn	0.00047	<0.0004
Cd	0.035	0.024
Cu	0.0008	<0.006
Co	0.0012	<0.009

[a] The protein concentration was 4.27×10^{-5} M for preparation 1 and 5.02×10^{-5} M for preparation 2. The zinc content of the last dialysate was $< 1 \times 10^{-7}$ M.

Table II: Inhibition of *Astacus* Protease by Metal Binding Agents[a]

inhibitor	concn (mM)	incubation time	activity (%)
EDTA	5	6 days	50
OP	1	1 h	12
DPA	5	1 h	6
HQSA	2	1 h	11
BP	5	10 min	25

[a] Samples of inhibitor plus enzyme were preincubated prior to the assay under the standard conditions of 20 mM HEPES, pH 7.8, and 1 mM STANA, 25 °C.

Results

Metal Analysis. The metal content of *Astacus* protease was determined by atomic absorption spectroscopy. Enzyme samples were dialyzed extensively against metal-free 20 mM HEPES, pH 7.8, and the last dialysis buffer was used as a reference. The analyses demonstrate the presence of Zn but the absence of Cd, Co, Cu, Fe, Mg, Mn, and Ni (Table I). On the basis of a protein concentration determined by amino acid analysis and the known protein molecular weight of 22 614 (Titani et al., 1987), the zinc content is 0.97 ± 0.03 mol/mol of protein.

[1] Abbreviations: OP, 1,10-phenanthroline; mP, 1,7-phenanthroline; BP, 2,2′-bipyridyl; HQSA, 8-hydroxyquinoline-5-sulfonic acid; DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; STANA, succinyl-Ala-La-Ala-*p*-nitroanilide; TEA, triethanolamine; SDS, sodium dodecyl sulfate.

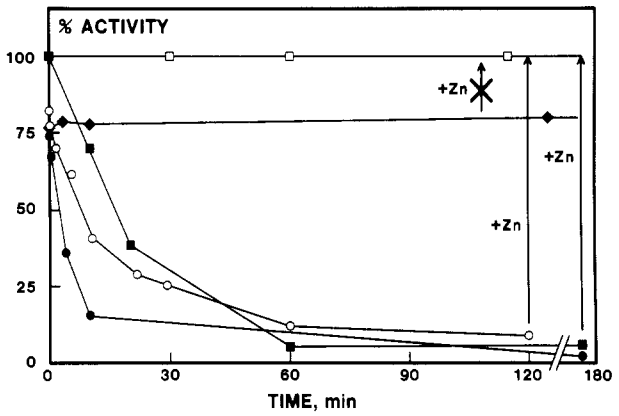

FIGURE 1: Time-dependent inactivation of *Astacus* protease by 1 mM OP (○), 2 mM OP (●), and 5 mM DPA (■). The time course for the activity in the absence of any chelator (□) and in the presence of the nonchelating isomer mP (1 mM) also is shown (♦). Assays were performed at 25 °C in 20 mM HEPES, pH 7.8, after preincubation of inhibitor and 4.1×10^{-7} M *Astacus* protease. Assays were initiated by addition of STANA to a final concentration of 1 mM. The arrows indicate addition of zinc to a final concentration of 300 μM. Activity is completely restored by addition of zinc in the case of OP and DPA, but not in the case of mP.

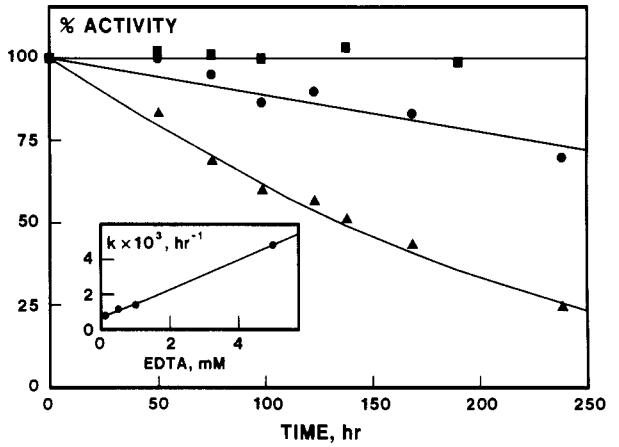

FIGURE 2: Time course of the activity of *Astacus* protease, 23 μM, by zero (■), 1 mM (●), and 5 mM EDTA (▲). Samples were incubated at the given EDTA concentrations in 200 mM TEA, pH 7.8, and assayed in the incubation buffer with 1 mM STANA. Inset: Dependence of the observed rate constant of inactivation, k, on EDTA concentration. The rate constants were calculated from the times of 50% inactivation.

Inhibition Studies. Recent studies demonstrated that *Astacus* protease is inhibited by the metal binding agents EDTA and OP (Wolz et al., 1987a,b). Table II shows that in addition to these agents, it is also inhibited by DPA, HQSA, and BP. In all cases, inhibition is reversed by dilution.

Inhibition by such chelators is markedly time dependent. Figure 1 shows the time course of inactivation of *Astacus* protease incubated in 20 mM HEPES, pH 7.8, containing millimolar concentrations of either OP or DPA. With these chelators, the times required for 50% inactivation are on the order of a few minutes. In contrast to 1,10-phenanthroline, the nonchelating isomer 1,7-phenanthroline (mP) at 1 mM exhibits only a slight instantaneous and no time-dependent inhibition. Furthermore, whereas the inactivation of *Astacus* protease by the chelating agents OP and DPA is fully reversible upon addition of zinc, addition of excess zinc does not alleviate the inhibition by the nonchelating mP (Figure 1). These observations are consistent with the hypothesis that the time-dependent inhibition by OP and DPA is related to their metal binding properties.

Table III: Inhibition Parameters for Phenanthroline Isomers

	$K_i \times 10^4$ (M)	$\bar{n}$
OP, instantaneous	30.9	1.15
OP, 1-h preincubation	5.0	2.12
mP, instantaneous[a]	29.5	1.33

[a] No change in activity upon preincubation.

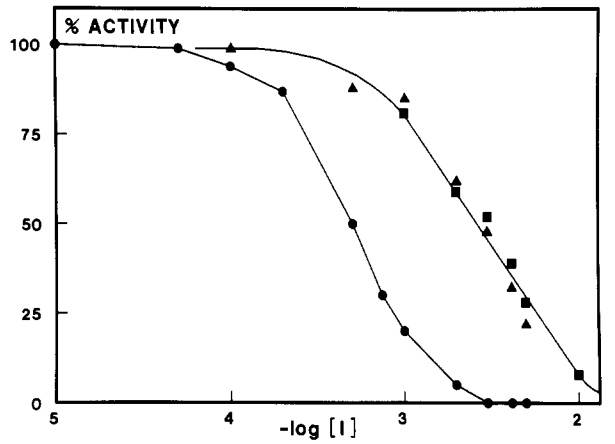

FIGURE 3: Inhibition of *Astacus* protease as a function of the concentration of the 1,10- and 1,7-phenanthroline isomers. Assays in the presence of 1,10-phenanthroline were performed after 1-h preincubation of enzyme with inhibitor (●) or without preincubation (▲). Incubation had no effect on the mP curve (■). Assay conditions were the same as in Figure 1.

The time required for 50% inactivation by EDTA is on the order of days (Figure 2). The rate constant of inactivation, k, is related to the half-live of inactivation by the equation

$$k = \ln 2/t_{1/2}$$

The rate constant for EDTA inhibition depends linearly upon the concentration of EDTA used up to 5 mM (Figure 2, inset).

In order to assess the details of the phenanthroline inhibition, the concentration dependence of inhibition was studied for both 1,10-phenanthroline and its nonchelating analogue 1,7-phenanthroline. Since the extent of inhibition by OP is time dependent (Figure 1), activities were measured "instantaneously" (i.e., enzyme and inhibitor were mixed at the start of the assay) and after a 1-h preincubation of enzyme and inhibitor (Figure 3, Table III).

Analysis of the data is accomplished by using the function:

$$\log (V_c/V_i - 1) = -\log K_i + \bar{n} \log [I]$$

where V_c and V_i are the velocities in the absence and presence, respectively, of inhibitor (I) (Coombs et al., 1962; Auld, 1988c). The resulting intercept with the log [I] axis yields the inhibitor concentration required for 50% inactivation, and the value of the slope gives $\bar{n}$, which is the order of the inhibitor in the reaction (i.e., the stoichiometry). The inhibition observed for instantaneous mP, incubated mP, and instantaneous OP all are characterized by a K_i value of approximately 3.0 mM (Table III). In marked contrast, the preincubated OP curve is characterized by a 6-fold lower K_i value of 0.5 mM. The values of $\bar{n}$ are about 1.2 for both mP and "instantaneous" OP inhibition. On the other hand, preincubated OP gives an $\bar{n}$ value of 2.1.

Preparation and Characterization of Apo-Astacus Protease. Zinc is removed from *Astacus* protease by successive dialyses of the native enzyme against 10 mM OP followed by metal-free buffer to remove excess chelator. This procedure yields preparations of apoenzyme with a zinc content of less than

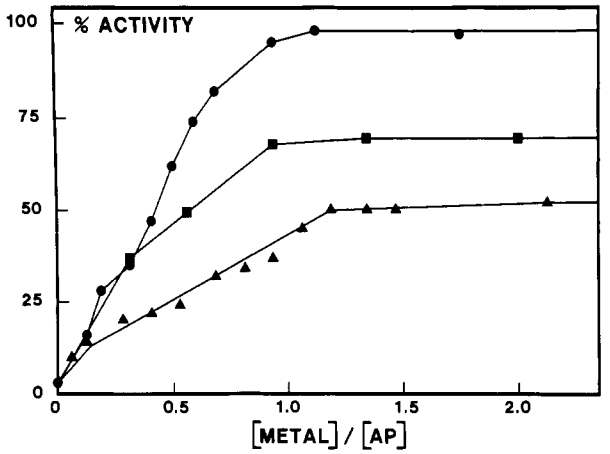

FIGURE 4: Reactivation of apo-*Astacus* protease, 7.5×10^{-7} M, by titration with Zn^{2+} (●), Cu^{2+} (■), and Co^{2+} (▲). Activities were measured immediately after metal addition under standard assay conditions at 25 °C with 1 mM STANA in 20 mM HEPES, pH 7.8. Addition of zinc to the cobalt or copper enzymes raises activity to the level of the zinc enzyme.

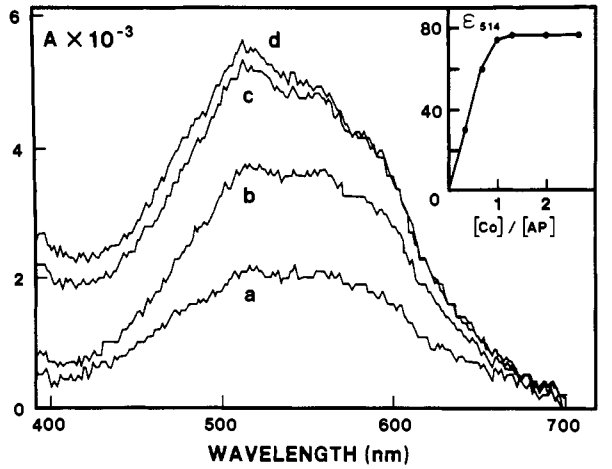

FIGURE 5: Visible absorption spectra of cobalt-*Astacus* protease, 5.6×10^{-5} M, in 20 mM HEPES, pH 7.8, recorded after addition of 0.33 (a), 0.66 (b), 0.99 (c), and 1.32 (d) mol of Co^{2+} to a mole of apo-*Astacus* protease. The ϵ_{514} increases linearly until 1 mol of Co^{2+} is incorporated (inset).

0.007 mol/mol, as determined by atomic absorption spectrometry. The apoenzyme exhibits less than 3% catalytic activity under the assay conditions of 7.5×10^{-7} M enzyme.[2]

Addition of zinc to apo-*Astacus* protease fully regenerates the native activity of the enzyme (Figure 4). Stepwise additions of substoichiometric amounts of zinc show that full activity is regained when 1 mol of zinc is incorporated per mole of protein. At this point in the titration curve, there is a sharp break, and further addition of zinc does not increase activity beyond values found for the native enzyme (Figure 4).

Apo-*Astacus* protease prepared in the manner described above is stable for at least 1 month when stored in solution at 4 °C. Thereafter, full activity can be restored by the addition of zinc ions. Cobalt or copper ions can also reactivate apo-*Astacus* protease (Figure 4). The activity titration curves show that with these two metals, maximum activity is also reached at a ratio of 1 mol of metal/mol of protein. The maximum activity levels of the copper and cobalt enzymes, however, are about 70% and 50%, respectively, of that of the zinc enzyme. Addition of excess zinc to the cobalt or copper enzyme brings the activity up to the level of the zinc enzyme, indicating that metal exchange readily occurs. Other metals tested for reactivation included Ni(II), Fe(II), Cd(II), Mn(II), Ca(II), and Mg(II), but none of these ions restores activity to more than 3% of that of the native enzyme.

Apo-*Astacus* protease was titrated with incremental amounts of Co(II) and the visible absorption spectrum measured. The absorption increases during the titration until a level of 1 mol of Co/mol of protein is reached, and then no further changes are seen (Figure 5). This confirms the 1:1 metal to protein stoichiometry measured by atomic absorption (Table I) and activity titration (Figure 4). The visible absorption spectra reveal a main peak at 514 nm and shoulders at 505 and 550 nm (Figure 5). No strong absorption bands were observed in the wavelength region 300–360 nm (data not shown).

Discussion

The results of the metal analyses and kinetic studies demonstrate that *Astacus* protease is a metalloenzyme which contains one zinc atom per mole of protein. The zinc is required for activity since the apoprotein is devoid of activity but can be reactivated by addition of metal. The apoenzyme proved to be very stable. Even after 1 month of storage in solution at 4 °C, full, native activity could be regenerated by the addition of zinc, demonstrating that the metal is not required for the structural stability of the protein.

Activity also could be restored by the addition of cobalt or copper to the apoprotein. This yielded metal-substituted enzymes with maximum activities of 50% and 70%, respectively, of that of the native enzyme-catalyzed hydrolysis of STANA. Cobalt has been substituted frequently for zinc in metalloenzymes and often results in enzymatically active species (Vallee & Galdes, 1984). In contrast, substitution of copper into a zinc enzyme rarely produces an enzymatically active species. Thus, copper-carboxypeptidase A displays only 2–3% of the native activity toward oligopeptide substrates (Schäffer & Auld, 1986) while copper-*Aeromonas* aminopeptidase is the only example with a relatively high amidase activity (Prescott et al., 1985).

The zinc is very tightly bound to the protein. One indication of this is that millimolar concentrations of EDTA are required to inactivate *Astacus* protease and even then it is an extremely slow process. Thus, the half-life of inactivation by 5 mM EDTA is about 6 days. The rate constant, k, of inactivation depends linearly on the concentration of EDTA up to 5 mM (Figure 2, inset). Extrapolation of the k values to a zero concentration of EDTA yields an inactivation half-life of about 40 days ($k = 1.25 \times 10^{-5}$ min^{-1}). This probably represents the rate constant for nonchelated metal dissociation from the enzyme. By way of comparison, the times of zinc dissociation from angiotensin converting enzyme (92.4 s) and carboxypeptidase A (28.3 min) are much faster, but that for carbonic anhydrase (5.4 years) is even slower (Kleeman et al., 1986).

The data presented here also give some insight into the mechanism of metal removal. In general, two types of mechanisms can be envisioned (Vallee & Galdes, 1984; Auld, 1988c). In an S_N1-type reaction, the metal spontaneously dissociates from the enzyme in a rate-limiting step, and then the free metal reacts rapidly with the chelator in solution. In contrast, an S_N2 mechanism includes the rapid formation of a ternary enzyme–metal–ligand complex. In most cases, this is followed by dissociation of the chelator-bound metal, but in some cases [e.g., alcohol dehydrogenase (Drum & Vallee, 1970)], the ternary complex is stable.

[2] Under these conditions, 3% activity corresponds to a zinc enzyme concentration of 2.2×10^{-8} M, which is approximately the background contamination level of the zinc ions in the assay.

Scheme I

$$E\text{-}M \underset{+L}{\overset{K_i^*,\ -L}{\rightleftharpoons}} E\bullet M\bullet L \overset{K_1}{\rightleftharpoons} E + M\bullet L \underset{+L}{\overset{-L}{\rightleftharpoons}} L\bullet M\bullet L \underset{-L}{\overset{+L}{\rightleftharpoons}} L_3\bullet M$$

K_2 K_3

One criterion for judging which of these mechanisms may be applicable is the dependence of the rate of inactivation on the concentration of chelator. For an S_N1 mechanism, the rate is independent of chelator concentration and is a measure of the rate of nonchelated metal dissociation from the protein. If a concentration dependence is seen, then a higher order mechanism is implicated (Kidani & Hirose, 1977; Billo, 1979).

EDTA inactivation and 1,10-phenanthroline inactivation of *Astacus* protease are both time and concentration dependent (Figures 1 and 2). The dependence of the inactivation rate on the chelator concentration indicates an S_N2 pathway. The linear dependence of the rate constant on EDTA concentration indicates, however, that even at the highest EDTA concentration tested (5 mM), there is no accumulation of the intermediate enzyme–metal–chelator.

Another criterion which is consistent with an S_N2 mechanism is the stoichiometry of the reaction as given by $\bar{n}$ (Coombs et al., 1962). When $\bar{n}$ is near 1, it is likely that a ternary enzyme–metal–chelator complex is formed. A value of $\bar{n}$ which is 2 or greater usually implies that the chelator is removing the metal from the protein or that there is more than one binding site on the protein (Vallee & Galdes, 1984; Auld, 1988c).

The present studies have shown that there is a reversible, "instantaneous" inhibition of *Astacus* protease by both OP and mP (Figure 3). It is unusual for the nonchelating isomer, mP, to cause inhibition, but it has been reported that it can bind to proteins through hydrophobic and/or aromatic interactions (Yielding & Tomkins, 1962). In the case of *Astacus* protease, inhibition by mP is not time dependent and is not affected by the addition of excess zinc (Figure 1). The K_i value of 2.95 mM measured for mP is nearly identical with the value of 3.09 mM measured for instantaneous inhibition by OP. Under these conditions, the $\bar{n}$ values for both phenanthroline isomers are near 1. It therefore seems reasonable to propose that such inhibition is due to fast, reversible binding which leaves the metal in place. This could occur in a hydrophobic binding pocket near the metal binding site.

Upon preincubation of the enzyme with OP for 1 h, the K_i value of OP shifts from 3 to 0.5 mM, and the $\bar{n}$ value shifts from 1.2 to 2.1 (Table III). Since OP removes the zinc from the protein, it is likely that bis- and tris(phenanthroline)–zinc complexes form sequentially in solution (Auld, 1988c).

Thus, the overall mechanism of metal removal from *Astacus* protease by OP appears to be consistent with Scheme I where E is enzyme, M is metal, L is chelating ligand, and K_i^*, K_1, K_2, and K_3 are dissociation constants. Under conditions of instantaneous inhibition, the experimental value obtained for K_i is equal to the value of K_i^* in this model. The observed K_i in the case of the preincubated system is a composite term of the values of K_i^*, K_1, K_2, and K_3.

This model has been proposed previously for the action of OP on several metalloenzymes such as carboxpeptidase A (Coombs et al., 1962; Billo, 1979), carbonic anhydrase (Kidani & Hirose, 1977), and thermolysin (Holmquist & Vallee, 1974; Voordouw et al., 1976).

Ligation of the metal by sulfur atoms of cysteines is not consistent with the observed electronic absorption spectrum of cobalt-*Astacus* protease. If this were the case, intense

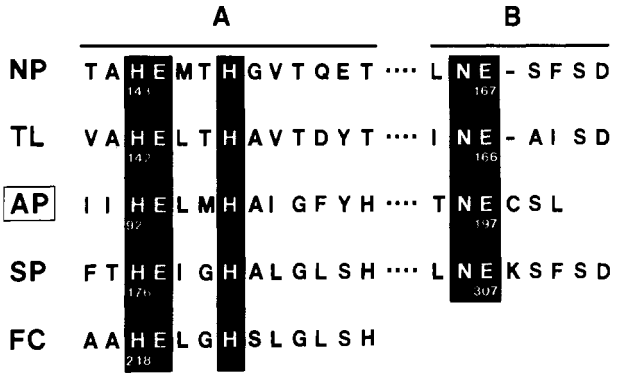

FIGURE 6: Amino acid sequences about the zinc histidyl ligands (A) and glutamyl ligand (B) of thermolysin (TL) are compared to similar sequences of *Astacus* protease (AP), *B. subtilis* neutral protease (NP), *Serratia* protease (SP), and fibroblast collagenase (FC).

absorption would be seen in the region between 220 and 360 nm (Kägi & Vallee, 1961; Vallee & Galdes, 1984). Furthermore, it is known from the primary structure that *Astacus* protease does not contain free thiols. The four cysteine residues which are present form two internal disulfide bridges (Titani et al., 1987).

On the other hand, the visible absorption spectrum of cobalt-*Astacus* protease resembles that obtained for both thermolysin (Holmquist & Vallee, 1974) and carboxypeptidase A (Latt & Vallee, 1971), indicating that the metal is probably coordinated in an irregular tetrahedral geometry. The molar absorptivity at 514 nm, 80 M^{-1} cm^{-1}, of the fully formed complex is closely similar to that observed for thermolysin (Holmquist & Vallee, 1974). Thus, histidyl and glutamyl residues are likely ligands to the zinc since it is known from X-ray structure analyses of both thermolysin and carboxypeptidase A that two histidines, one glutamic acid, and one water molecule are the ligands bound to the zinc (Lipscomb et al., 1968; Colman et al., 1972). In this regard, it should be noted that *Astacus* protease contains five histidyl residues in the linear sequence between residues 87 and 104 and three glutamyl residues in the sequence 36–39 (Titani et al., 1987). In addition, the amino acid sequence HEXXH is found at positions 92–96, a sequence observed in thermolysin where the histidines are ligands to the zinc and the glutamyl residue is a likely catalytic residue (Coleman et al., 1972, Levy et al., 1975) (Figure 6A). This sequence is also found in the zinc proteases *Bacillus subtilis* (Vasantha et al., 1984), *Serratia* neutral protease (Nakahama et al., 1986), and a metallocollagenase (Goldberg et al., 1986; McKerrow et al., 1987). A number of the amino acids adjacent to this region are also either identical or quite similar for all these metalloproteins (Figure 6A). All three zinc ligands of thermolysin occur in α-helical regions of the protein as demonstrated by X-ray diffraction studies (Matthews et al., 1974). Chou and Fasman (1978) calculations on *Astacus* protease indicate that both the glutamyl region (residues 33–39) and part of the histidyl-rich regions (residues 90–96 and 100–109) have a high probability of being in an α-helix.

Glu-166 is the third zinc ligand in thermolysin (Figure 6B). A similar sequence of amino acids around a glutamyl residue is observed in *Bacillus subtilis* (Vasantha et al., 1984) and *Serratia* neutral protease (Nakahama et al., 1986) and at the C-terminal end of *Astacus* protease (Titani et al., 1987). In the case of *Astacus* protease, the Cys-198 residue is linked to Cys-42, bringing several other glutamyl residues close to the above-postulated metal binding site. Thus, a potential glutamyl ligand could come from any of the residues Glu-36, -38, -39,

or -197. In this scenario, regardless of which glutamate is liganded to the metal, potentially three other glutamyl residues could be brought close to the metal binding site. Such a preponderance of negative charges in the active-site region may explain the high catalytic activity of the enzyme toward Arg and Lys residues in the S_1 and S_2 subsites (Stöcker and Auld, unpublished observations).

Astacus protease having an amino acid composition of 200 and a molecular weight of 22614 (Titani et al., 1984) is one of the smallest zinc proteases currently known. Several of the hemorrhagic toxins from *Crotalus atrox* (Bjarnason & Tu, 1978; Bjarnason & Fox, 1983; Pandya & Budzynski, 1984; Kruzel & Kress, 1985), *Agkistrodon acutus* (Nikai et al., 1982), and *Bitis arietans* (Strydom et al., 1986) have reported molecular weights of 20000–27000 on the basis of SDS or gel permeation chromatography. While sequence information is not generally available on this class of zinc proteases, the results on proteinase A, consisting of 213 amino acids, from *Bitis arietans* show no extensive similarity to *Astacus* protease (D. Strydom, unpublished results).

The results presented here have given new insight into the nature of *Astacus* protease. The structural and functional features which were investigated previously had given rise to the assumption that is might be a member of a new group of proteolytic enzymes found thus far only in the digestive tract of decapod crustaceans (Zwilling et al., 1981; Zwilling & Neurath, 1981). The amino acid sequence of the protein was not found to show homology with any other protein sequenced to date (Titani et al., 1987). With the new knowledge that *Astacus* protease is a metalloenzyme, comparisons can now be made with other members of this mechanistic class. Thermolysin and a number of related metalloproteases contain one catalytically essential zinc (Auld, 1987). In contrast to *Astacus* protease, these bacterial neutral endoproteases also contain four bound calcium ions which are noncatalytic but are required for fully activity (Morihara, 1974; Hofmann, 1985). As discussed above, *Astacus* protease is similar to thermolysin with regard to the visible spectrum of the cobalt enzyme (Holmquist & Vallee, 1974) and in its amino acid sequence around a proposed metal binding site (Figure 6). Thermolysin, however, does not possess further extensive similarity with *Astacus* protease. It is larger (M_r 34000) and contains no cysteines (Titani et al., 1972). Comparisons of *Astacus* protease with mammalian metalloproteases such as collagenases (Goldberg et al., 1986) or angiotensin converting enzyme (Das & Soffer, 1975; Bünning & Riordan, 1985) also reveal a variety of functional and structural differences. Thus, *Astacus* protease may yet represent a distinct group of endoproteolytic enzymes within the heterogeneous class of zinc metalloproteases.

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Characterization of a Microtubule-Stimulated Adenosinetriphosphatase Activity Associated with Microtubule Gelation–Contraction†

Baochong Gao‡ and Richard C. Weisenberg*
Department of Biology, Temple University, Philadelphia, Pennsylvania 19122
Received December 28, 1987; Revised Manuscript Received March 10, 1988

ABSTRACT: A microtubule-stimulated ATPase is associated with particles that are responsible for microtubule gelation–contraction in vitro. These particles have been proposed to be slow axonal transport, component a, particulates (SCAPs) [Weisenberg, R. C., Flynn, J. J., Gao, B., Awodi, S., Skee, F., Goodman, S., & Riederer, B. (1987) *Science (Washington, D.C.) 238*, 1119–1122]. The SCAP ATPase activity is stimulated approximately twofold by microtubules. The microtubule-stimulated ATPase activity correlates with the occurrence of microtubule gelation–contraction. Both microtubule-stimulated ATPase activity and microtubule gelation–contraction are inhibited by millimolar calcium, 0.3 M KCl plus 2 mM ethylenediaminetetraacetic acid (EDTA), 5 μM vanadate, and millimolar *N*-ethylmaleimide (NEM). Neither the ATPase activity nor microtubule gelation–contraction is affected by high magnesium concentrations (up to 8 mM) or by the anti-ATPase drugs ouabain, oligomycin, sodium azide, and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA). Magnesium is required for both ATPase activity and microtubule gelation–contraction. Microtubule-stimulated hydrolysis of GTP, CTP, ITP, and UTP is less than 50% of ATP hydrolysis, and microtubule gelation–contraction is reduced in these nucleotides. On the basis of these results we propose that the microtubule-stimulated ATPase activity associated with SCAPs is a previously undescribed enzyme that is responsible for microtubule gelation–contraction in vitro and that is the likely motor for component a of slow axonal transport.

Slow axonal transport is generally divided into two major categories according to the rate of transport and the proteins involved. Component a of slow axonal transport (SCa) has a rate of 0.2–1.1 mm/day and consists primarily of cytoskeletal proteins, including tubulin, neurofilament proteins, and brain spectrin (Tytell et al., 1981; Lasek et al., 1984). These proteins appear to be transported as an insoluble complex (Grafstein et al., 1970; Lorenz & Willard, 1978; Tashiro et al., 1984; Filliatreau & De Giamberadino, 1985). Component b of slow transport consists primarily of soluble proteins and has a rate of 2–8 mm/day (Lasek et al., 1984). The mechanism of SCa transport, the form of the transported protein, and the "motor" involved have not been clearly demonstrated.

Crude calf brain microtubule proteins can undergo gelation–contraction in the presence of ATP (Weisenberg & Cianci, 1984). During microtubule gelation–contraction, movement of particulate material along microtubules occurs at a rate of about 1 μm/min (Weisenberg et al., 1986). Recently a particulate fraction has been isolated from crude microtubule proteins which is required for gelation–contraction of microtubules (assembled from purified tubulin) in the presence of ATP (Weisenberg et al., 1987). These particulates have a protein composition consisting primarily of tubulin, neurofilament, and spectrin polypeptides, which is similar to the composition of SCa. Movement of these particulates along microtubules, at a rate of about 1 μm/min, occurs in the presence of ATP. Because of similarities in their rate of movement, protein composition, and solubility, we have proposed that the particles responsible for microtubule gelation–contraction in vitro are the transported components of SCa

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* Address correspondence to this author.
‡ Present address: National Heart, Lung and Blood Institute, National Institutes of Health, Building 3, Room B1-22, Bethesda, MD.

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