The effect of metal ions on the activity and thermostability of the extracellular proteinase from a thermophilic *Bacillus*, strain EA.1

Tim COOLBEAR,* Jan M. WHITTAKER and Roy M. DANIEL

Thermophile Research Unit, University of Waikato, Private Bag, Hamilton, New Zealand

The proteinase from the extremely thermophilic *Bacillus* strain EA.1 exhibits maximum stability at a pH of approx. 6.5. In the presence of calcium ions the half-life at 95 °C of the enzyme at this pH was 17 min, and loss of activity followed first-order decay kinetics. The role of metal ions in the activity and stability of the enzyme was studied using the holoenzyme, the metal-depleted apoenzyme, and a zinc-enriched apoenzyme preparation. Zinc and calcium ions were the preferred bivalent cations for the active site and stabilization site(s) respectively. Stabilization by metal ions was not in itself a highly stringent process, but ions other than calcium which stabilized the enzyme generally had a concomitant inhibitory effect on activity. Inhibition and stabilization of the enzyme by cations were concentration-dependent effects and certain ions activated the apoenzyme but not the holoenzyme. Manganese(II) ions conferred some stability and also activated the enzyme, but in the latter case were not as effective as zinc ions. The results are discussed with reference to the ionic radii, co-ordination number and preferred ligand donors of the ions. Mercury(II) ions severely compromised enzyme activity and stability, and the effects of thiol-reactive agents suggest that thiol groups also have a role in enzyme integrity.

INTRODUCTION

The characteristics of proteinase production from 41 bacterial isolates shown to be extremely thermophilic bacilli have been described elsewhere (Coolbear *et al.*, 1991). Properties of the proteinases were described from two particular isolates, one of which was the isolate EA.1 from Mount Erebus, Antarctica. The initial studies established that EA.1 proteinase was a metalloproteinase similar in some respects to typical metalloproteinases from mesophilic bacilli (Matsubara & Feder, 1971). The thermostability of the enzyme was, however, much greater than that of the metalloproteinases previously described from more thermophilic *Bacillus* strains [e.g. thermolysin (Pangburn & Walsh, 1975) and the enzymes reported by Kubo *et al.* (1988)]. The specificity of the EA.1 proteinase was also much narrower; neither elastolytic activity nor activity against some small peptide substrates [e.g. *N*-(3-[2-Furyl]acryloyl)glycylleucinamide (Keay, 1972)] was detectable. In the present paper the relationships of metal ions to activity and thermostability were investigated, as was the possible involvement of thiol residues.

EXPERIMENTAL

Growth of *Bacillus* EA.1 and enzyme preparation

Culture of the *Bacillus* isolate EA.1 and the preparation of proteinase from culture supernatant are described elsewhere (Coolbear *et al.*, 1991). The proteinase gave a single active band during PAGE.

Preparation of apoenzyme (EA.1 apoproteinase)

EA.1 proteinase was dialysed against 0.1 M-Hepes, pH 7.1, (at 4 °C) containing 5 mM-EDTA for 20 h with one change of buffer. This was followed by further dialysis against metal-ion-free 0.01 M-Hepes buffer, pH 7.1, made up in Milli-Q water, for 24 h with one change of buffer. The dialysed material, comprising apoenzyme (EA.1 apoproteinase), was freeze dried and stored under

vacuum desiccation. All dialysis steps were performed at 4 °C using 3500 Da cut-off, Spectrapor (Spectrum Medical Industries, Inc., Los Angeles, CA, U.S.A.) dialysis tubing. Sample volume/dialysis buffer ratio was 1:1000 at each step.

Preparation of Zn²⁺-apoproteinase

Zn²⁺-apoproteinase was prepared from EA.1 apoproteinase by dialysing for 24 h, as above, against two changes of 0.01 M-Hepes (pH 7.1, made up in Milli-Q water) containing 0.5 mM-ZnCl₂. This was followed by a similar dialysis protocol with ZnCl₂ omitted. The dialysed Zn²⁺-apoproteinase was freeze dried and stored under vacuum desiccation.

Assay of proteolytic activity

Enzyme preparation (0.1 mg) was incubated in 0.2 M-Hepes buffer (pH 6.8 at the incubation temperature) containing 0.3 % azocasein in a final volume of 1.0 ml. Unless otherwise stated, the incubation mixture also contained 1 mM-CaCl₂. Other additions were as described in the text. Assays were for either 30 min at 75 °C or 60 min at 50 °C. Assays were terminated by the addition of 0.5 ml of 15 % (w/v) trichloroacetic acid, cooled, centrifuged (5 min at 6000 g) and the absorbance of the supernatant was determined at 440 nm using a Varian DMS 80 spectrophotometer (Varian Techtron Pty. Ltd., Mulgrave, Australia). Control incubations were those in which the appropriate amount of enzyme was added after precipitation by trichloroacetic acid of substrate and non-enzyme additives which had been incubated under the relevant conditions.

Determination of thermostability

For thermostability studies, enzyme preparations (apoenzyme or holoenzyme) were made up at 1 mg/ml (unless otherwise stated in the text) in 0.2 M-Hepes buffer (pH 6.8 at the temperature used), except in the case of the determination of the effect of pH on thermostability when 0.2 M-Bis-tris propane (BTP) was used. The preparations were pre-incubated at room temperature for

Abbreviations used: DTT, dithiothreitol; BTP, Bis-tris propane.

* To whom correspondence should be addressed, at present address: New Zealand Dairy Research Institute, Private Bag, Palmerston North, New Zealand.

30 min with the appropriate metal ion (or distilled water where no metal ion was incorporated). The text gives details of the inclusion and concentrations of cations and other additives in the incubation mix. The enzyme solutions were incubated at the required temperatures in sealed tubes. At specified intervals aliquots were removed and either cooled on ice, if being immediately assayed for proteolytic activity, or frozen in liquid nitrogen. When cool (or thawed from liquid nitrogen), 0.1 ml aliquots were removed from the treated samples and assayed for proteolytic activity as described above. Experiments showed that freezing in liquid nitrogen did not alter enzyme activity compared with cooling on ice, and it was therefore a convenient method for holding samples until all enzyme assays could be performed simultaneously.

RESULTS AND DISCUSSION

Thermostability of EA.1 proteinase as a function of pH and temperature

To determine appropriate basic conditions for the study, the thermostability of EA.1 proteinase was determined over the pH range 5.5–8.0 [i.e. approx. 1.2 pH units either side of the previously established activity optimum (Coolbear *et al.*, 1991)] and over the temperature range 70–90 °C. Previous work (Coolbear *et al.*, 1991) had also established that CaCl_2 stabilized the enzyme; thus these experiments were performed in the presence and absence of 5.5 μmol of CaCl_2 per mg of enzyme preparation. For determination of thermostability over a range of pH values the EA.1 proteinase was incubated over the given pH range at 75 °C for up to 5.5 h in the presence of CaCl_2 or 100 min in its absence. In the former case aliquots were removed at 30 min intervals for activity determinations; in the latter case 20 min intervals were used. Proteolytic activity was assessed using azocasein as substrate as described in the Experimental section except that 0.2 mg of enzyme was used for each assay. In all cases the pH was adjusted to 6.8. The enzyme was stable for the full incubation period in the presence of CaCl_2 over the pH range 5.5–7.0 (results not shown). At higher pH values the enzyme was subject to inactivation and exhibited half-lives of 9.6 h at pH 7.5 and 4.2 h at pH 8.0. In both instances plots of log percentage activity remaining versus time were linear, indicating that loss of activity was caused solely by thermal denaturation.

In the absence of CaCl_2 the enzyme was subject to inactivation at all pH values tested. Between pH 5.5 and 7.5 decay in enzyme activity followed first-order kinetics, but at pH 8.0 the plot of log percentage activity remaining versus time was non-linear, indicating that autoproteolysis was a factor. The half-lives of enzyme activity versus pH are shown in Fig. 1. The enzyme was clearly most stable between pH 6.0 and 7.0, with rapid decline in stability at pH values outside this range.

With no exogenous CaCl_2 the enzyme was stable over the treatment period only at 70 °C (results not presented). At higher temperatures loss of activity was apparent (Fig. 2). First-order decay kinetics were exhibited at 75 °C and 80 °C, half-lives being 112 min and 34 min respectively. At 85 °C and 90 °C loss of activity was extremely rapid with the initial half-life being less than 4 min at both temperatures. Non-linearity of plots of log percentage activity remaining versus time suggested that autoproteolysis was occurring. The presence of CaCl_2 stabilized the enzyme at all temperatures tested. No significant loss of activity over the 75 min period was observed at up to 80 °C, with some activation being noted (up to 20%). At 85 °C and 95 °C the enzyme decay followed first-order kinetics and half-lives were 85 min and 17 min at the two temperature respectively.

Enzyme thermostability was determined by periodic assay of

residual activity of enzyme samples during incubation at the temperatures of interest, kinetic analysis of these results providing unambiguous information, not only on stability but also on autolysis. 'Stability' is thus defined here as the ability of the enzyme to retain its catalytic activity under the conditions concerned, and may in some circumstances lead to the enzyme being defined as unstable in the absence of any conformational change other than that required, for example, for retention of a catalytic metal ion.

Measurements of proteolytic enzyme stability which rely on physical rather than on activity determinations are unsatisfactory for other reasons. First, irreversible inactivation by autolysis (to which the enzymes are prone, especially at higher temperatures)

Fig. 1. Variation of EA.1 proteinase half-life with pH at 75 °C

EA.1 proteinase was treated at 75 °C for up to 100 min in the absence of exogenous CaCl_2 using BTP as buffer. Half-lives were calculated from plots of log percentage activity remaining versus time except for that at pH 8 which was calculated from the initial rate of activity loss. Azocasein was used as substrate for assessment of remaining activity.

Fig. 2. Variation of thermostability of EA.1 proteinase with temperature

EA.1 proteinase was treated for up to 75 min in the presence (closed symbols) or absence (open symbols) of CaCl_2 at 75 °C (○, ●), 80 °C (□, ■), 85 °C (△, ▲) and 90 °C (▽, ▼). Remaining proteinase activity was determined using azocasein as substrate.

Fig. 3. Activity of EA.1 proteinase in the presence of bivalent metal ions with azocasein as substrate

EA.1 proteinase was assayed for 60 min at 50 °C (a) and 30 min at 75 °C (b) with azocasein in the presence of CaCl_2 (○), CoCl_2 (■), CuCl_2 (□), MgCl_2 (●), MnCl_2 (▽), SrCl_2 (△) and ZnCl_2 (▲).

complicates thermodynamic analysis of unfolding [see Takahashi & Sturtevant (1981)] and the procedures used to prevent autolysis during such determinations are generally deleterious to the interpretation of the results obtained (Mitchinson & Wells, 1989). Secondly, since the physiological function of an enzyme is catalysis, it is the consequence of altered stability on catalysis itself which is of fundamental importance and physical methods do not necessarily give this information.

Effect of bivalent metal ions on the activity of EA.1 proteinase

From the stability of EA.1 proteinase at 70 °C in the absence of exogenous CaCl_2 it can be reasonably concluded that any change in enzyme activity resulting from the inclusion of metal ions in an incubation at that temperature would be due to the effects of those ions on activity *per se*, rather than stability. It is possible, however, that certain metal ions could destabilize the

enzyme. To minimize this possibility and to facilitate the study of metal-ion effects on activity the temperature of incubation was reduced still further to 50 °C.

The results obtained on incubating EA.1 proteinase with various metal ions in the concentration range 0–11 mM at 50 °C were then compared with those obtained on repeating the experiment at 75 °C. At this latter temperature the enzyme would not be expected to lose more than approx. 10% of its initial activity in the absence of CaCl_2 during the incubation and would be fully stable in its presence. The results obtained at 50 °C and 75 °C are shown in Figs. 3(a) and 3(b) respectively.

It can be seen that the ions fall into two groups in terms of their effects on the enzyme. The first group (group A) comprises the bivalent magnesium, calcium and strontium ions which all had little effect on the enzyme at 50 °C, but activated the enzyme to a greater or lesser extent at 75 °C. The second group of ions (group B) were inhibitory to activity at both temperatures and comprised cobalt(II), copper(II), manganese(II) and zinc ions. The results can be rationalized in terms of the ionic radii, ligand binding preference and co-ordination numbers of the ions.

For group A ions the ionic radii are 0.066 nm (0.66 Å), 0.099 nm (0.99 Å), and 0.112 nm (1.12 Å) for magnesium, calcium and strontium ions respectively. These ions show a general preference for oxygen ligands, but while the two larger ions have a tendency towards eight co-ordination, the magnesium ion prefers six co-ordination because of its smaller size. The group B ions are all smaller than the calcium ion with ionic radii of 0.072 nm (0.72 Å) for Co^{2+} and Cu^{2+} , 0.074 nm (0.74 Å) for Zn^{2+} , and 0.08 nm (0.80 Å) for Mn^{2+} . Manganese(II) ions have some preference for octahedral co-ordination; due to Jahn–Teller distortion copper(II) ions tend to form tetragonal or square-planar complexes. Both zinc and cobalt(II) ions prefer lower co-ordination numbers (tetrahedral co-ordination), although cobalt(II) ions do form a number of octahedral complexes. With regard to ligand preferences for the group B ions, the manganese(II) ion accepts ligands with either oxygen or nitrogen donor atoms, but the bivalent ions to the right of the first transition series show an increasing preference for ligands with nitrogen donor atoms. In comparison with the group A ions, therefore, the group B ions have a higher preference for nitrogen or sulphur ligands. This suggests that inhibition of EA.1 proteinase by metal ions is dependent on the preferred ligand types and the co-ordination number of the ion, the former perhaps being the dominant factor. Thus the lower activation of the enzyme by magnesium ions compared with the other group A ions may be a consequence of its six co-ordination preference, while the levels of inhibition by manganese(II) and cobalt(II) ions may reflect their tendency towards nitrogen-ligand preference.

The apparent activation of the enzyme by the group A ions at 75 °C may reflect stabilization of the enzyme, since in the absence of metal ions the enzyme is subject to denaturation at this temperature. Increasing concentrations of the ions would favour increased binding levels and would be reflected in the higher activities seen. The relatively poor increase in activity observed with magnesium ions may be caused by less effective binding of the smaller ion as a result of the longer, weaker bonds, or may be a result of a reduction in the number of bonding interactions. Similarly the greater efficacy of strontium ions could reflect stronger interactions of the larger ion. The effect of the various ions on thermostability was therefore investigated.

Thermostability of EA.1 proteinase in the presence of added bivalent metal ions

The initial studies on proteinase thermostability in the presence and absence of CaCl_2 over a range of temperatures (see above)

Table 1. Thermostability of EA.1 proteinase in the presence of bivalent metal ions

Thermostability was assessed at 80 °C over a 75 min period in the presence of metal ions. Copper(II) ions were included at 2.75 mM and zinc ions at 5.5 mM. All other ions (including zinc in a second series of incubations) were included at 11 mM. Results are expressed as the ratio of half-life of enzyme in the presence of ion to that in the absence of ion. s denotes that the enzyme was stable over the period of incubation. i denotes that an initial half-life was estimated from non-linear decay kinetics.

Ion	Thermostability
Ca ²⁺ or Mn ²⁺	s
Zn ²⁺ (low)	9.1
Sr ²⁺	3.7
Zn ²⁺ (high)	1.4
Co ²⁺	1.2
Mg ²⁺	1.0
Cu ²⁺	0.5i

showed that the enzyme was stable in the absence of the ions at 70 °C and in the presence of the ions at 80 °C. These two temperatures were used to study the effect on thermostability of the metal ions as described above. All ions except the copper(II) ion (but including the zinc ion) were added at a concentration of 11 mM. Because of the extreme effect of copper(II) ions on activity the concentration of this ion used was reduced to 2.75 mM and, for the same reason, a second series of incubations were performed with ZnCl₂ at a concentration of 5.5 mM. In both cases this ensured that at least 50% of the uninhibited levels of enzyme activity would be retained at the start of the incubations.

At 70 °C the inherent stability of the enzyme was not affected by any of the ions except copper(II), which destabilized the enzyme to the extent that it exhibited a half-life of approx. 45 h (results not shown). The results for the effect of the ions on thermostability at 80 °C are given in Table 1 and are expressed as the ratio of the half-life of the enzyme in the presence of an ion to that in its absence. Calcium and manganese(II) ions conferred full stability over the time period assayed, while strontium ions and the low concentration of zinc ions had a significant stabilizing effect. The higher concentration of zinc ions as well as cobalt(II) and magnesium ions were only marginally effective. Copper(II) ions destabilized the enzyme by a factor of approx. 2, the decay kinetics being non-linear. These results can again be rationalized mainly on the basis of ion size, co-ordination number and ligand preferences. The most effective ions, calcium, manganese(II) and the somewhat larger strontium ions, co-ordinate octahedrally and accept oxygen ligands. Magnesium ions are slightly smaller and prefer six co-ordination, while the zinc, cobalt(II) and copper(II) ions tend towards lower co-ordination numbers and a preference for nitrogen ligands. The concentration effect found with zinc ions, however, suggests that other factors may be involved in stabilization. Zinc ions may be able to occupy sites not specific to stabilization *per se* but nevertheless leading to improved enzyme stability. At high concentrations of the ion other antagonistic binding would presumably negate this stabilizing effect.

Properties of EA.1 apoproteinase

A variety of techniques were investigated to derive apoproteinase preparations (results not shown). While some of these techniques led to a more complete removal of metal ions from

Table 2. Activity and stability of EA.1 apoproteinase preparation

Activity was assessed at 75 °C for 30 min before and after pre-incubation of the various preparations at 80 °C for 90 min. In the latter case the value given is the amount of activity remaining expressed as a percentage of that given by the same preparation before pre-incubation and the letter S denotes that no loss of activity was observed. The preparations of the holoenzyme, apoenzyme and Zn²⁺-apoenzyme were as described in the Experimental section.

Preparation	Initial activity (% holoenzyme)	Activity remaining after 90 min at 80 °C (%)
Holoenzyme	100	S
Apoenzyme	11	11
Apoenzyme + 1 mM-Ca ²⁺	25	98
Apoenzyme + 1 mM-Zn ²⁺	70	23
Zn ²⁺ -apoenzyme + 1 mM-Ca ²⁺	81	S

the holoenzyme, they did not subsequently permit restoration of activity upon re-introduction of metal ions. The technique described here (see the Experimental section) resulted in a preparation with low residual activity and reduced stability, but with a good response to added metal ions in terms of restoration of activity and stability towards those demonstrated by the holoenzyme (Table 2). The manner in which the metal ions were removed could be interpreted in terms of the production of a heterogeneous enzyme population containing (on the basis of residual activity of the preparation) approx. 10% holoenzyme molecules and 90% apoenzyme molecules. In this case the results presented below pertain only to the population of apoenzyme molecules. Alternatively, the preparation could be considered to be comprised of enzyme molecules partially depleted of metal ions. While the former interpretation is easier to conceptualize, the apparently irreversible loss of all activity with only slightly harsher conditions indicates that the complete removal of metal ions from the holoenzyme tends to induce an irreversible conformational change which either does not allow replacement of metal ions *per se* or does not permit catalytic events after replacement of the ions. The second interpretation given above conceives the possibility of a conformational state of the enzyme molecule which allows dynamic interaction with metal ions remaining in a depleted pool. In this case the term 'metal-ion-depleted proteinase' would be a more accurate description of the preparation than 'apoenzyme'. Although the term 'apoenzyme' is used throughout the description and discussion of the results in this paper, it is not intended to exclude this second interpretation.

Activity and thermostability of EA.1 apoproteinase in the presence of cations

The activity of the apoenzyme was determined over a range of concentrations (0–14 mM) of various cations from the previously designated groups A and B. Two patterns of apparent activation were found (Fig. 4). The group A cations calcium and strontium caused an increase in activity proportional to the ion concentration. In contrast the group B ions gave a maximum level of activation at low concentration; at higher concentrations the level of activation was reduced, the degree of reduction being different for each ion. At the higher concentrations certain of the ions were inhibitory to the apoenzyme.

For a wider range of ions two concentrations only were used to assess the effect of the ions on activity. The lower concentration, 0.41 mM, was the concentration at which most of the

Fig. 4. Activity of EA.1 apoproteinase in the presence of metal ions with azocasein as substrate

EA.1 apoproteinase was assayed for 30 min at 75 °C with azocasein in the presence of CaCl_2 (●), CoCl_2 (○), CuCl_2 (■), $\text{La}(\text{NO}_3)_3$ (□), MnCl_2 (▲), SrCl_2 (△), and ZnCl_2 (▼). Activity is expressed as a percentage of that activity given by EA.1 apoproteinase in the absence of metal ions.

Table 3. Activity of apoenzyme in the presence of exogenous cations

Activity was assessed at 75 °C for 30 min using either 0.41 mM or 5.76 mM of the various cations. The ions were added as the chlorides except for lanthanum(III) where the nitrate was used. Activity is expressed as the ratio of activity of the apoenzyme in the presence of the ion to that of the apoenzyme alone.

Ion	Cation concn. ...	Apoenzyme activity	
		0.41 mM	5.76 mM
None		1.0	1.0
Ca^{2+}		2.1	5.3
Cd^{2+}		1.3	0.7
Co^{2+}		5.5	2.4
Cu^{2+}		3.4	0.4
Fe^{3+}		1.2	1.0
Hg^{2+}		0.05	0.0
K^+		1.1	1.0
La^{3+}		2.3	0.4
Mg^{2+}		1.1	2.1
Mn^{2+}		5.1	3.8
Na^+		1.1	1.0
Ni^{2+}		1.8	0.9
Sr^{2+}		1.1	1.9
VO^{2+}		3.1	3.2
Zn^{2+}		5.5	0.3

group B ions gave maximum activation. The higher concentration, 5.76 mM, was the concentration estimated from Fig. 4 to give the maximum difference in activities between the various ions. The results, shown in Table 3, suggest that the majority of the ions gave one or other of the two established patterns. The univalent ions and iron(III) ions had little effect on the apoenzyme while the vanadyl ion stimulated activity equally at both concentrations and mercury(II) ions were almost totally inhibitory.

With regard to the effect of these ions on stability of the apoenzyme (results not shown), calcium, cadmium, lanthanum(III) and manganese(II) ions at 1 mM all stabilized the apoenzyme 2-fold at either 75 °C or 85 °C in 30 min incubations. The other ions generally had no effect at 75 °C with the exception of nickel(II) and vanadyl ions which caused some destabilization, whereas at 85 °C the majority of these ions reduced thermostability. Inclusion of the ions at 14 mM gave generally similar results; calcium, cadmium and manganese(II) ions being especially effective stabilizers at 80 °C. Additionally, although copper(II) and iron(III) ions severely inhibited activity, that activity which remained was stabilized by the presence of these ions. Of all the ions tested mercury(II) ions proved to be the most detrimental to apoenzyme stability. Only at 1 mM and 75 °C was the apoenzyme relatively unaffected by this ion species; at 75 °C with 14 mM-ion and 85 °C with 1 mM-ion the apoenzyme was completely inactivated during the 30 min incubation.

The effect of the group A ions on the apoenzyme activity is essentially the same as that on the activity of the holoenzyme. The activation of the apoenzyme by low concentrations of the group B-type ions, however, was not observed with the holoenzyme. The thermostability of the apoenzyme was affected by the various ions in a similar manner to that of the holoenzyme with the exception that zinc ions did not retain stabilization properties. In general these results can be rationalized on the same basis as those for the holoenzyme in terms of preferred co-ordination number and donor ligand.

The distinct behaviour of the two groups of ions may suggest that activation can be effected through separate binding sites with oxygen ligands for the group A ions and nitrogen and/or sulphur ligands for the group B ions. The efficacy of nitrogen-ligand binding is clearly greater than oxygen-ligand binding, demonstrated by the low levels of activation of the apoenzyme by the group A ions. Equally clearly, however, the group B ions at a higher concentration have a secondary antagonistic effect on the enzyme. Stabilization of the proteinase would appear to be dependent upon ions which bind to oxygen donor ligands; other ions which stabilize have a concomitant inhibitory effect on the enzyme. The effect of lanthanum(III) ions is interesting in view of the observations by Matthews & Weaver (1974) that this ion could substitute for calcium in thermolysin stabilization and by Khoo *et al.* (1984) that this ion was actually more effective than calcium in stabilizing caldolyisin. For the EA.1 enzyme stabilization by lanthanum(III) ions is concentration dependent.

Activity and thermostability of Zn^{2+} -apoproteinase in the presence of cations

The lack of stabilization of the apoenzyme by zinc ions was interesting in view of its effect on the holoenzyme. This might indicate that although it is unable to stabilize *per se*, it can facilitate stabilization of the holoenzyme indirectly. In view of this and the activation of the apoenzyme by zinc ions, further studies were undertaken on the Zn^{2+} -apoenzyme prepared as described in the Experimental section.

The activity and thermostability of the Zn^{2+} -apoproteinase was determined at 75 °C in the presence of various cations at concentrations of 1 mM and 14 mM. The results are presented in Table 4 in terms of the ratio of activity of the Zn^{2+} -apoenzyme in the presence of exogenous ions to that in the absence of ions. The absolute activity of the Zn^{2+} -apoproteinase was higher than that of the apoenzyme itself and the increase is attributable to a direct effect of the ion on activity rather than stability; zinc ions did not stabilize the apoenzyme (see previous section). Addition of cations at either 1 mM or 14 mM showed that only calcium ions increased the activity of the Zn^{2+} -apoenzyme. Manganese(II) ions had no effect, while all other ions tended to inhibit activity,

Table 4. Activity and thermostability of Zn²⁺-apoproteinase

Activity of the Zn²⁺-apoenzyme was determined before and after pre-incubation at 75 °C for 30 and 90 min with either 1 mM or 14 mM of the various cations. The ions were included as the chlorides except for lanthanum(III) where the nitrate was used. Activities are expressed as the ratio of activity of the Zn²⁺-apoenzyme in the presence of the various ions to the activity in the absence of the ion and without pre-incubation.

Ion added	Zn ²⁺ -apoproteinase activity					
	Pre-incubation period (min)...		0 min		30 min	
	Cation concn....		1 mM	14 mM	1 mM	14 mM
None			1.00	1.00	0.16	0.21
Ca ²⁺			1.17	1.37	1.09	1.30
Co ²⁺			0.88	0.25	0.29	0.11
Cu ²⁺			0.87	0.09	0.09	0.07
La ³⁺			0.89	0.11	0.78	0.11
Mn ²⁺			1.05	0.96	0.93	0.94
Sr ²⁺			0.95	0.82	0.26	0.25
VO ²⁺			0.96	0.88	0.12	0.16
Zn ²⁺			0.86	0.09	0.16	0.10

especially when included at the higher concentration. Of these latter ions, vanadyl ions were the least inhibitory.

With regard to stabilization (Table 4), calcium ions at 1 mM imparted full stability on the Zn²⁺-apoenzyme over the 90 min incubation period at 75 °C, as did manganese(II) ions at 14 mM. Manganese(II) ions at 1 mM conferred only partial stability, as was the case at 14 mM for lanthanum(III), cobalt(II), copper(II) and zinc ions (albeit only on that activity remaining as a consequence of the concomitant inhibitory effect of the ions).

The results with manganese(II) ions on the Zn²⁺-apoenzyme differ from those on the apoenzyme itself (see previous section) in that for the former the ion did not give higher activity than calcium ions and this may reflect competition with the more suitable zinc ions at the active site. The results with the Zn²⁺-apoenzyme are fully compatible with those of the apo- and holo-enzyme on the basis of the physicochemical properties of the ions.

Activity and thermostability of EA.1 proteinase in the presence of thiol reagents

The thiol-reactive agents mercaptoethanol and dithiothreitol (DTT) were both inhibitory to EA.1 proteinase at either 50 °C or 75 °C at virtually all concentrations tested (Fig 5). The higher initial activity at 75 °C was reflected in the greater amounts of reagents which were required to obtain 50% inhibition (11 mM and 21 mM for mercaptoethanol and 0.7 mM and 1.3 mM for DTT at 50 °C and 75 °C respectively).

The effects of the thiol-reactive agents on enzyme thermostability are presented in Table 5. Mercaptoethanol at a concentration of 5 mM had no effect on stability at 50 °C. Higher concentrations destabilized the enzyme and only at 10 mM-mercaptoethanol was CaCl₂ effective in reducing the degree of destabilization. At 75 °C all mercaptoethanol concentrations used caused loss of stability and again the effectiveness of CaCl₂ in reducing the loss was diminished at the higher concentrations of the thiol reagent. In the case of DTT, destabilization of the enzyme occurred at concentrations of the reagent at least an order of magnitude lower than those required to produce a

similar effect with mercaptoethanol. Again, inclusion of CaCl₂ was effective only at the lower concentrations of DTT used. Furthermore, at 75 °C the kinetics of decay were non-linear with all concentrations of DTT tested.

Fig. 5. Activity of EA.1 proteinase in the presence of thiol reagents

EA.1 proteinase was incubated for 60 min at 50 °C (open symbols) or 30 min at 75 °C (closed symbols) with azocasein and CaCl₂ in the presence of mercaptoethanol (○,●) and DTT (□,■).

Table 5. Half-lives of EA.1 proteinase at 50 °C and 75 °C in the presence of thiol-reactive agents

Half-lives of EA.1 proteinase were determined at 50 °C and 75 °C in the presence and absence of 11 mM-CaCl₂ at various concentrations of mercaptoethanol or DTT. Half-lives are given in hours. s denotes that the enzyme was stable over the time period of incubation (up to 2.5 h). i denotes that the value given is an initial half-life estimated from non-linear decay kinetics. In the absence of thiol-reactive agents the enzyme is stable at 50 °C in the presence or absence of CaCl₂ and at 75 °C in the presence of the salt. At 75 °C in the absence of CaCl₂ the enzyme has half-life of 1.87 h.

Agent	Half-lives in presence of thiol-reactive agent (h)			
	50 °C		75 °C	
	+ CaCl ₂	- CaCl ₂	+ CaCl ₂	- CaCl ₂
Mercaptoethanol				
5.0 mM	s	s	9.37	0.95
10.0 mM	12.5	9.37	2.13	0.77
17.5 mM	3.75	3.95	1.15	0.50
25.0 mM	2.08	2.08	0.53	0.30
DTT				
0.50 mM	0.37	5.35	0.5i	0.37i
1.00 mM	3.75	2.35	0.27i	0.22i
1.75 mM	1.87	1.70	0.50i	0.33i
2.50 mM	1.33	1.43	0.30i	0.25i

Since the enzyme is stable in the presence of 5 mM-mercaptoethanol at 50 °C the loss of activity caused by low levels of this reagent (see Fig. 5) is due to inhibition. Both thiol-reactive agents are good complexing agents for thiophilic metal ions such as Zn^{2+} and their inhibitory effect is probably caused by leaching of this ion from the active site. DTT is the more effective complexing agent, this being reflected in the lower concentration required to effect inhibition.

Destabilization of the enzyme possibly plays a more significant role in the losses of activity at the higher levels of mercaptoethanol and in the presence of DTT. The destabilization observed at 50 °C by the thiol-reactive agents, the severity of the destabilization, and the fact that the destabilization is not reduced by addition of CaCl_2 (with the exception of the partial reduction at the lower concentrations of reagent used) tend to suggest that the effect of the thiol-reactive agents is separate from that given by the various metal ions. This may indicate that the integrity of the enzyme is enhanced by thiol group interactions in the enzyme molecule, which is consistent with the extreme effect of mercury(II) ions on stability.

Conclusions

Certain features of the interaction of the various ions with EA.1 proteinase are similar to those found in other studies. The inhibition of the enzyme by higher concentrations of zinc ions is similar to that reported by Holmquist & Vallee (1974) for thermolysin, in which concentrations above 10^{-5} mM inhibited by binding to histidine at the active site. Also, the ability of cobalt(II) ions to substitute for zinc ions has been seen in other studies [i.e. with thermolysin (Holmquist & Vallee, 1974); with carboxypeptidase (Coleman & Vallee, 1960, 1961)] and the positional similarities of zinc, cobalt(II), manganese(II) and copper(II) ions in carbonic anhydrase have been reported (Liljas *et al.*, 1972).

The characteristics displayed by zinc in this study are compatible with involvement at the active site. Zinc ions (d^{10}) do not experience significant ligand-field stabilization effects: they therefore have few constraints on the co-ordination geometries they adopt and can easily occupy sites of low symmetry. Also, their complexes are labile, allowing an easy exchange of ligands.

Ionic radius partially determines an ion's ability to occupy a metal-binding site at the active centre (whether to promote or inhibit enzyme activity). The importance of ionic radii and stereochemical requirements in determining the suitability of a metal ion for substitution was demonstrated by Arnone *et al.* (1971). Ions exhibiting good activation characteristics on EA.1 proteinase have ionic radii close to that of zinc(II), while those ions which stabilize the enzyme, without inhibiting activity, have radii similar to that of the calcium ion (see Fig. 6). Neither Co^{2+} nor Zn^{2+} showed any stabilization of the apoenzyme; rather they tended to have a destabilizing effect.

Fe^{3+} Mg^{2+} Ni^{2+} Cu^{2+} Co^{2+} Zn^{2+} Mn^{2+} Cd^{2+} Ca^{2+} La^{3+} Sr^{2+}
0.64 0.66 0.69 0.72 0.72 0.74 0.80 0.97 0.99 1.06 1.12

Cu^{2+} — Activity — Mn^{2+}
 Mn^{2+} — Stability — Sr^{2+}

* La^{3+} stabilized at low concentrations only.

Fig. 6. Ionic radii of relevant cations

Ionic radii of cations used in the experiments are given in Angstroms ($1 \text{ \AA} = 0.1 \text{ nm}$).

Stabilization of EA.1 proteinase does not appear to be a very specific process, since ions with a range of characteristics [e.g. calcium, manganese(II), iron(III) and cadmium ions] stabilized the apoenzyme. Those ions which prefer softer ligands, however [cadmium ions, sulphur; copper(II) and iron(III) ions, nitrogen], were also associated with inhibition of enzyme activity. Thus it appears that non-inhibitory stabilization of EA.1 proteinase is dependent on metal ions with a preference for ligands with oxygen donor atoms. In contrast, nitrogen-ligand binding is preferred by those ions which activate the enzyme.

Clearly, therefore, there are two types of metal-ion-binding sites in EA.1 proteinase, one type related to activity, the other to stability. The zinc/calcium ion combination is most effective with no significant inter-site competition and this indicates that they are the native ions for the active site and stabilization sites respectively. The possibility of multiple stabilization sites is mooted on the basis of the known structure of thermolysin (Matthews *et al.*, 1972a,b) and is perhaps reflected by the concentration dependence of calcium ion stabilization. The ability of manganese(II) ions (and to a lesser extent vanadyl) to substitute partially in the active and stabilization sites probably reflects the intermediate ionic radius of the ion and the binding of both oxygen and nitrogen donor ligands.

Calcium ions have characteristics ideally suited to stabilization of protein structure. Usually associated with high co-ordination numbers, the ion has the ability to occupy irregular co-ordination environments. In studies on other calcium-binding proteins [staphylococcal nuclease (Arnone *et al.*, 1971); carp-muscle albumin (Nockolds *et al.*, 1972); thermolysin (Matthews *et al.*, 1974)] calcium was found to adopt distorted octahedral co-ordination with a wide variety of bond lengths and bond angles.

The stability of the EA.1 proteinase under optimum conditions of pH and calcium ion concentration is greater than any other reported metalloproteinase. Whether its mode of action is similar to that of other *Bacillus* metalloproteinases, e.g. thermolysin, remains to be seen. Certainly the mechanisms proposed for thermolysin by Hangauer *et al.* (1984) and for carboxypeptidase A by Monzingo & Matthews (1984) have features which are compatible with properties of the EA.1 proteinase. Thermolysin and carboxypeptidase both bind zinc ions at the active site [with an irregular tetrahedral-like geometry employing the ligands Gly, His, His and H_2O (Lipscomb *et al.*, 1969; Matthews *et al.*, 1974)] and both are proteolytically active as the manganese and cobalt derivatives (Coleman & Vallee, 1960, 1961; Holmquist & Vallee, 1974).

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