The second zinc atom in the matrix metalloproteinase catalytic domain is absent in the full-length enzymes: a possible role for the C-terminal domain

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Abstract Domain deletion mutants of the matrix metalloproteinases consisting of the catalytic domain only contain two zinc atoms per molecule. One is essential for catalysis, while the other may fulfil a structural role. We have analysed the zinc contents of both the full-length and the truncated mutants of prostromelysin-1 and progelatinase A and report that the second zinc atom is not present in the full-length form of the proenzymes. Thus it seems likely that the role proposed for this zinc atom in maintaining the structure of the enzyme catalytic domain is performed by the C-terminal domain in the full-length enzyme.

Key words: Stromelysin-1; Gelatinase A; Metal analysis

1. Introduction

The matrix metalloproteinases are endopeptidases which require zinc for catalytic activity. They are members of the family of enzymes termed the 'metzincins' [1] which have as a common feature the zinc-binding motif HExxHxxGxxH in their catalytic domains. The crystal structures of the catalytic domains of human neutrophil and fibroblast collagenases [2–5] and the NMR structure of the catalytic domain of human stromelysin-1 [6] have recently been reported. In addition, the structures of several other members of the metzincin family including the crayfish enzyme astacin [7,8], adamalysin II from snake venom [9] and the 50 kDa metalloproteinase from *Serratia marcescens* [10] have been solved. Despite limited sequence identity these enzymes share a common type of 3-D structure with an open-sandwich topology in which two α-helices are packed against a twisted β-sheet. The more distantly-related thermolysin and other bacterial zinc metalloendopeptidases also have this type of 3D structure. The matrix metalloproteinases, however, appear to be unique among the metalloendopeptidases in that they contain an additional zinc atom which is not part of the active centre and which appears to play a structural role in the enzymes. Both the catalytic and structural zinc atoms are very tightly bound in the proenzymes [11]. The catalytic zinc atom is coordinated by three histidine imidazole groups and a thiol group from the propeptide whereas the second zinc atom is coordinated by three histidine imidazole groups and an aspartic acid carboxylate.

The matrix metalloproteinases possess domain structures. They all have a propeptide of approximately 80 amino acid residues which must be proteolytically removed in order to form active enzyme. In addition, each matrix metalloproteinase, with the exception of matrilysin, is comprised of at least two domains, the N-terminal domain, which contains all the features essential for catalytic activity, and a C-terminal domain, which is thought to be important in binding to physiological substrates and effectors. Truncated forms of the proenzymes lacking the C-terminal domain have been constructed by using DNA technology and these are the forms that were used to determine the published structures. Some kinetic studies have been performed on these domain deletion mutants and they have been shown to have catalytic and specificity characteristics similar to those of their full-length counterparts [12,13]. As part of a study designed to characterise more fully the full-length and short form enzymes, we have compared the zinc content of full-length prostromelysin-1 and progelatinase A with that of the truncated mutants, *N*-prostromelysin-1 and *N*-progelatinase A. Our results suggest that the C-terminal domain may play an additional role in stabilising the structure of the catalytic domain.

2. Materials and methods

All reagents were purchased from BDH and were of Spectrosol or AristaR grade wherever possible. Prepacked PD-10 columns were from Pharmacia and Chelex-100 was from Bio-Rad. The substrate, (7-ethoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 was a generous gift from Dr. C.G. Knight (Strangeways Research Laboratory, Cambridge, UK).

2.1. Enzyme preparation

Recombinant human prostromelysin-1 was purified from the medium of C127 cells expressing this enzyme [14,15]. *N*-Prostromelysin-1 was prepared from transfected NSO mouse myeloma cell conditioned medium as previously described [16]. Progelatinase A and *N*-progelatinase A were also expressed from NSO mouse myeloma cells and purified from the conditioned media as described previously [17]. Prostromelysin-1 was expressed from NSO cells with low productivity and therefore could not be obtained in sufficient quantity to be used in the present study. However, a comparison of the stability, specific activity and activation kinetics of enzyme from the two cell lines confirmed that they have identical properties. The purity and latency of each proenzyme was confirmed immediately before treatment for zinc analysis by using polyacrylamide gel electrophoresis (data not shown) in addition to a comparison of protein concentration and active site titration data (see Results).

2.2. Reagent preparation

The buffer (50 mM HEPES, pH 7.5) was purified of contaminating metal ions by passage through a column of Chelex-100 (70 cm × 7 cm^2, flow rate 30 ml/h). Calcium nitrate ('Spectrosol' grade) from a stock of 249 mM was added to give a final concentration of 2 mM Ca^{2+}. The buffers were equilibrated to 4°C before use. When gel filtration was

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used to purify the enzyme samples the columns were washed with *o*-phenanthroline followed by metal-free water and then equilibrated with the HEPES buffer at 4°C. Metal-free containers and dialysis tubing were prepared as described by Holmquist [18] and Auld [19].

2.3. Sample preparation

Proenzyme samples were rendered free from non-specifically bound metal ions either by dialysis overnight or by gel filtration using pre-packed PD-10 columns. The concentration of the samples varied from 0.5–7 μM. Acid hydrolysis was performed at 50°C in 20% nitric acid for 18 h. The zinc content of the buffers in which the samples were purified was used as a blank which was subtracted from the zinc concentration of the enzyme.

2.4. Zinc determination

Zinc determination was performed using inductively coupled plasma mass spectroscopy (ICP-MS) at the National Environment Research Council's ICP-MS Facility in the Centre for Analytical Research in the Environment, Imperial College, Berkshire, UK.

2.5. Determination of enzyme concentration

Enzyme concentration was determined by titration of active enzyme with TIMP as described previously [20]. Prostromelysin-1 was activated by using 10 μg/ml trypsin for 30 min at 37°C and progelatinase A was activated by incubation with 2 mM (4-aminophenyl)mercuric acetate for 1 h at 25°C. Protein concentration was determined from the absorbance of the samples at 280 nm and using the extinction coefficients of the proenzymes: prostromelysin-1 65,100 $M^{-1} \cdot cm^{-1}$; *N*-prostromelysin-1 34,380 $M^{-1} \cdot cm^{-1}$; *N*-progelatinase A 75,100 $M^{-1} \cdot cm^{-1}$ (all calculated from the amino acid sequences using method of Mach et al. [21]); progelatinase A 122,800 $M^{-1} \cdot cm^{-1}$ (determined experimentally [22]).

3. Results

Initially, methods of sample preparation were compared by using full-length prostromelysin-1 to assess whether acid hydrolysis was necessary to release of all the zinc from the protein and whether the proenzyme was denatured by either of the sample purification techniques. The results of the comparison, which are summarised in Table 1, demonstrated that each of the treatments gave the same final zinc content for the proenzyme. The dialysis treatment was used for the remaining samples as the process had fewer steps and therefore fewer opportunities for contamination by background zinc. The background zinc concentrations were always less than 5% of the sample concentration.

For each proenzyme sample the protein, activated proenzyme and zinc concentrations were determined. The comparison between protein concentration and active enzyme concentration provided a method for ensuring: (a) that each preparation was pure; and (b) that the proenzyme was fully activatable and therefore correctly folded. The latter is important because incorrectly folded proenzyme may have a different zinc content. The proenzymes were used for the zinc analysis to decrease the possibility of denaturation during dialysis in view of their known high conformational stability and tight binding of the catalytic zinc atom [11]. The activation conditions used have previously been demonstrated to give fully active enzyme [16,17].

Table 1
Effect of sample preparation method on the measured zinc content of stromelysin-1

Treatment	[zinc]:[protein] ±S.D. (n = 2)	[zinc]:[enzyme] ±S.D. (n = 2)
(1) Dialysis	0.93 ± 0.07	0.9 ± 0.08
(2) Gel filtration	1.13 ± 0.06	1.15 ± 0.09
(3) Acid hydrolysis	1.15 ± 0.05	1.12 ± 0.08

Protein concentration was calculated from the A_{280} of the proenzymes, enzyme concentration was determined by active site titration of the active enzymes and zinc concentration of the proenymes was determined using ICP-MS. The instrumental analytical precision was ±1% at the 1σ level. The values are the average of two separate determinations performed at different enzyme concentrations in the range 0.5–7 μM.

Table 2
The zinc contents of full-length and short-form stromelysin-1 and gelatinase A

Enzyme	[zinc]:[protein] ±S.D. (n = 3)	[zinc]:[enzyme] ±S.D. (n = 3)
Stromelysin-1	0.93 ± 0.04	0.9 ± 0.07
N-Stromelysin-1	1.9 ± 0.05	1.8 ± 0.08
Gelatinase A	1.0 ± 0.08	1.1 ± 0.18
N-Gelatinase A	2.1 ± 0.07	2.6 ± 0.24

Values are the average ratios obtained from three separate determinations. The larger errors for the [zinc]:[enzyme] ratio for gelatinase A and *N*-gelatinase A is a result of instability of the active enzyme during activation and titration.

Table 2 shows that for each enzyme there is a good correlation between the protein concentration calculated from measurements of A_{280} and the concentration of active enzyme determined by active site titration. Samples of each proenzyme which had not been dialysed were also activated and shown to contain concentrations of active enzyme similar to those of the dialysed samples, indicating that dialysis does not result in denaturation of the proenzyme and thus loss of zinc.

The data in Table 2 demonstrate that there is an important difference in zinc stoichiometry between the full-length wild-type enzymes and the truncated mutants. Whereas the latter contain two zinc atoms per molecule, the former contain only one zinc atom per molecule.

4. Discussion

As part of a study of the consequences of replacing the zinc of matrix metalloproteinases by other metals, we determined the zinc content of prostromelysin-1 to be one atom per molecule. The result of this preliminary experiment was surprising because published structures of the catalytic domains of stromelysin-1 and collagenase clearly contain two zinc atoms per molecule [2–6]. The activity of trypsin-treated prostromelysin-1 suggests that the single zinc atom performs a catalytic role. In the present work the validity of the analysis of one zinc atom per molecule suggested by the preliminary experiment on full-length prostromelysin-1 was confirmed by demonstrating the consistency of this result when different methods of sample preparation were used (Table 1). Evidence that the zinc contents of full-length matrix metalloproteinases differ from those of their short forms is provided by the analysis of the zinc contents of another full-length proenzyme, progelatinase A, and the domain deletion mutants of both proenzymes (Table 2).

Published values of zinc contents of the matrix metalloproteinases are somewhat conflicting. The only other published value for a full-length proenzyme, also for prostromelysin-1, is 1.5 atoms per molecule [11]. This result does not permit a

decision between 1 and 2 atoms per molecule. Promatrilysin, which is a naturally occurring 'short-form' enzyme, and the catalytic domain of fibroblast procollagenase have both been reported to contain 1 zinc atom per enzyme molecule [23,24]. These results, however, contrast with zinc contents of 2 atoms per molecule reported for the prostromelysin-1 catalytic domain expressed in *E. coli* [11,25] and for pro- and active matrilysin from both *E. coli* and mammalian cells [25]. The results of the present work shown in Table 2 demonstrate a real structurally relevant difference between the zinc contents of two full-length matrix metalloproteinases (1 atom per molecule of proenzyme) and their respective N-terminal short forms produced by molecular genetic techniques (2 atoms per molecule of proenzyme).

A structural role has been proposed for the second zinc atom on the basis of its position in the structure and its high affinity for the protein. The zinc atom is positioned approximately 12 Å from the catalytic zinc atom and, together with a calcium ion, it holds a large exposed loop against the β-sheet. It is surprising that the affinity of the zinc for the enzyme is so high because coordination by three histidine imidazoles and one aspartic acid carboxylate group has not been reported to be unusually strong in other proteins. Indeed, the zinc atom in superoxide dismutase, which is also coordinated to three histidines and one glutamic acid, can be removed readily [27]. An alternative explanation for the observation that the zinc atom cannot be removed from the domain is that it is inaccessible to chelating agents. This seems unlikely, however, as it is near the surface of the molecule. It is possible that the tight binding of this zinc atom may be due primarily to the nearby calcium atom which may maintain the conformation of the loop so effectively that the zinc is trapped in place. There is support for this possibility from a study of the roles of zinc and calcium in the stabilization of the conformation of the catalytic domain of human fibroblast collagenase, where it was noted that calcium is significantly more effective at stabilizing the structure than is zinc [24].

The residues that coordinate the second zinc atom in the short form enzymes are totally conserved throughout the members of the matrix metalloproteinase family, suggesting that they and the zinc atom play a crucial role in maintaining the correct position for the exposed loop. It is possible that the position of this loop affects properties of the enzymes other than catalytic activity. If so, the conflicting values for zinc contents of short form enzymes that have previously been published [11,23–25] may reflect the possibility that enzyme may exist in two forms (either with or without the second zinc atom) which have indistinguishable kinetic characteristics. A more detailed physicochemical investigation may be required to elucidate the role of the second zinc atom in the short form enzymes.

The absence of the second zinc atom in the full-length enzyme may be due to masking of the zinc binding site by the C-terminal domain during the folding process. As both the full-length and short forms of the enzymes are stable molecules, it seems possible that the role played by the second zinc atom in the truncated mutants is usually performed by the C-terminal domain in the full-length enzymes. It can be envisaged that in the full-length enzyme the loop that is exposed in the short forms could be held in place by binding to the C-terminal domain, thus eliminating the requirement for the second zinc atom. This may be a result of evolution from a simple single domain structure to a larger, more complex enzyme the activity of which can be highly regulated. The involvement of the residues in the exposed loop in binding to the C-terminal domain could be tested using mutagenesis studies of full-length enzymes. It would be interesting to know the zinc content of short forms of the matrix metalloproteinases which have been generated autolytically by proteolysis [15], as these forms were originally folded in the presence of the C-terminal domain. A crystallographic structure of a full-length matrix metalloproteinase should reveal whether the interactions involving the C-terminal domain predicted to mask the binding site of the second zinc atom do exist.

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