Identification of glutamate-169 as the third zinc-binding residue in proteinase III, a member of the family of insulin-degrading enzymes

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A novel active site has been identified in a family of zinc-dependent metalloendopeptidases that includes bacterial proteinase III, the human and *Drosophila* insulin-degrading enzymes, and the processing-enhancing protein subunit of the mitochondrial processing proteinase. None of these enzymes contains the conserved active site described in most other metalloendopeptidases, HEXXH; instead, all four contain an inversion of this motif, HXXEH. Prior mutagenesis studies of proteinase III indicate that the two histidines are essential for co-ordinating the zinc atom, while all three residues are required for enzyme activity. To identify the third zinc-binding residue in this protein family, three glutamates downstream from the active site were mutated to glutamine in proteinase III. The mutant proteins were expressed and their ability to degrade insulin was compared with the wild-type enzyme. The glutamate-204 mutant was as active as the wild-type protein, the glutamate-162 mutant retained 20% of the activity of the wild-type enzyme and the glutamate-169 mutant was completely devoid of insulin-degrading activity. The purified wild-type and glutamate-204 mutant enzymes were found to contain nearly stoichiometric levels of zinc by atomic absorption spectrophotometry, whereas the glutamate-162 mutant had a slight reduction in the level of zinc, and the glutamate-169 mutant retained less than 0.3 mol of zinc/mol of enzyme. These findings are consistent with glutamate-169 being the third zinc-binding residue in proteinase III.

INTRODUCTION

Most, if not all, of the varied effects of insulin appear to be mediated through a cell surface receptor (Houslay and Siddle, 1989; Roth, 1990). After insulin binds to its receptor, the receptor–insulin complex is internalized in endocytic vesicles (Pease et al., 1985; Levy and Olefsky, 1990; Doherty et al., 1990). As the endosomes are acidified, the insulin–insulin-receptor complex dissociates, with most of the receptors being recycled to the cell surface and the insulin being degraded inside of the cell. One enzyme which has been postulated to play a role in the process of insulin degradation has been given the name insulin-degrading enzyme (IDE) (Duckworth, 1988, 1990). There are numerous lines of evidence that are consistent with a role for IDE in this process, including: (1) mice with elevated levels of IDE are resistant to elevated levels of insulin (Beyer, 1955); (2) the degradation products of insulin from intact cells are nearly identical with those seen with purified IDE (Assoian and Tager, 1981; Duckworth, 1988, 1990; Hamel et al., 1988; Duckworth et al., 1988; Williams et al., 1990); (3) inhibitors of IDE also inhibit insulin degradation in intact cells (Kayalar and Wong, 1989); (4) microinjection of antibodies directed against IDE inhibit the degradation of insulin in intact cells (Shii and Roth, 1986); (5) insulin can be cross-linked to IDE in intact cells (Hari et al., 1987); (6) IDE has been reported to be present in endosomes (Hamel et al., 1991); and most recently (7) overexpression of IDE has been found to increase the rate of insulin degradation in intact cells (Kuo et al., 1991). However, it is possible that IDE plays other roles in the cell. In this regard, it is of interest that the enzyme can also degrade glucagon (Kirschner and Goldberg, 1983), atrial natriuretic factor (Muller et al., 1991), transforming growth factor α (Gehm and Rosner, 1991) and oxidatively damaged haemoglobin (Fagan and Waxman, 1991).

The IDE (also called insulinase) in mammals was described more than 35 years ago (Mirsky, 1957). However, difficulties in purifying this enzyme have hindered studies of its detailed molecular properties. With the isolation and sequencing of a cDNA clone encoding the human enzyme, it was realized that IDE is related to a bacterial proteinase called proteinase III or Pi (Affholter et al., 1988). This bacterial enzyme readily degrades insulin and cleaves the B chain of insulin at the same sites as IDE (Cheng and Zipser, 1979; Duckworth, 1988). Both enzymes are synthesized as a single polypeptide of similar molecular mass (approx. 100 kDa), although the active mammalian enzyme may be a homodimer while the bacterial enzyme is active as a monomer (Ding et al., 1992). Subsequent studies have shown that there is also a related proteinase in *Drosophila* (Kuo et al., 1990). The amino acid sequence of the bacterial proteinase is approximately 27% identical with the sequences of the two eukaryotic proteinases, whereas the human and *Drosophila* enzymes are 46% identical (Finch et al., 1986; Affholter et al., 1988; Kuo et al., 1990). These enzymes also share three regions of high homology containing greater than 50% sequence identity. All three proteinases in this family have an absolute requirement for bivalent cations for activity, characterizing them as metalloproteinases (Cheng and Zipser, 1979; Ding et al., 1992). In addition, purified proteinase III has been shown to contain stoichiometric amounts of zinc (Ding et al., 1992; Becker and Roth, 1992), whereas partially purified human IDE contains high levels of zinc and significantly lower levels of manganese (Ebrahim et al., 1991). Because of the much greater ease in isolating large amounts of pure bacterial proteinase III in

Abbreviations used: PEP, processing-enhancing protein; IDE, insulin-degrading enzyme; MPP, mitochondrial processing proteinase; the amino acid numbering corresponds to the sequence of bacterial proteinase III; the mutants are named using a single-letter amino acid code as well as indicating the number of the residue mutated: E162Q is glutamate-162 mutated to glutamine; E169Q is glutamate-169 mutated to glutamine; E204Q is glutamate-204 mutated to glutamine; and H92R is histidine-92 mutated to arginine.

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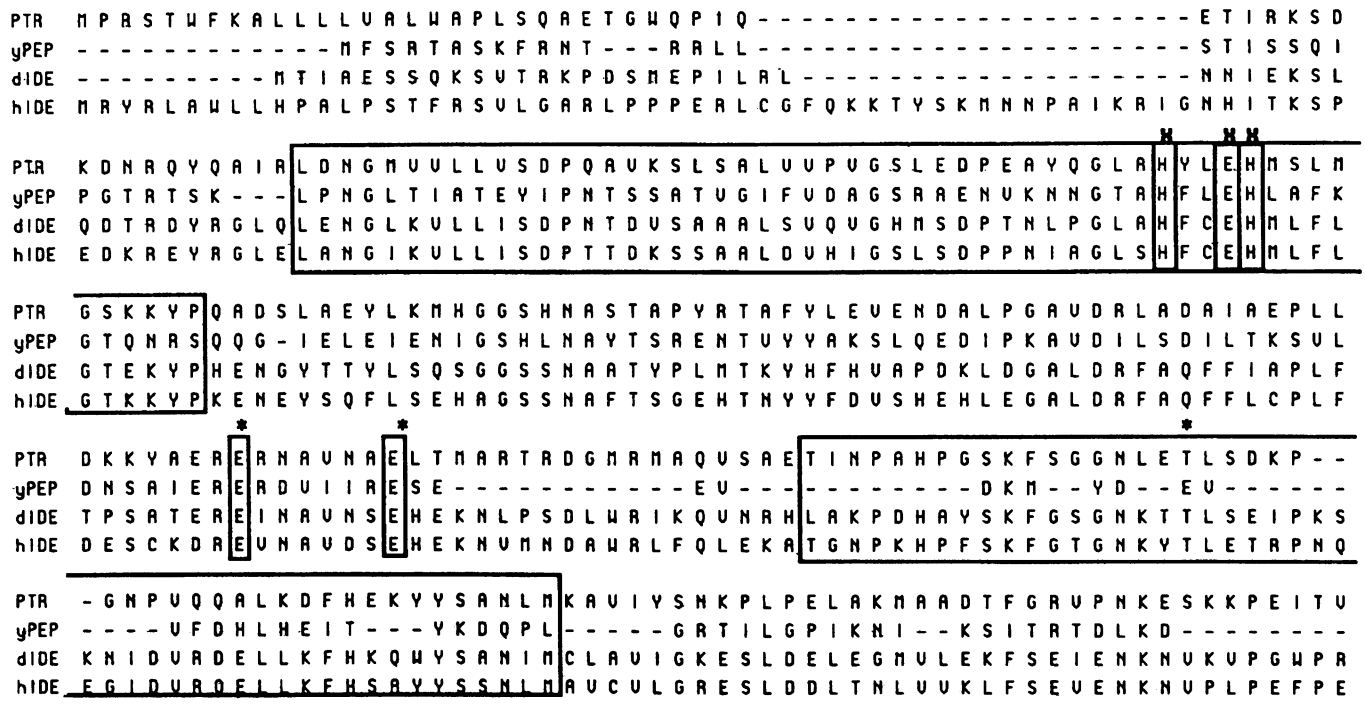

Figure 1 Sequence alignment of the first 267 amino acids of proteinase III (PTR) (Finch et al., 1986) and the homologous sequences of yeast processing-enhancing protein (yPEP) (Witte et al., 1988), *Drosophila* insulin-degrading enzyme (dIDE) (Kuo et al., 1990) and human insulin-degrading enzyme (hIDE) (Affholter et al., 1988) using the Tulla program developed by Subbiah and Harrison (1989)

The large boxes represent the first two highly conserved domains in the IDE family, the smaller boxes represent the five conserved histidines and glutamates in the four proposed family members. The (X) identifies residues previously mutated and analysed (Becker and Roth, 1992) and the (*) identifies residues mutated and analysed in this paper.

comparison with the two eukaryotic enzymes, we have begun studying the bacterial enzyme as a model for the others (Ding et al., 1992). Knowledge of the active site of this bacterial enzyme may help in the design of inhibitors of the mammalian enzyme.

Primary sequence analyses and X-ray crystallographic data have indicated that most metalloproteinases share a conserved active site (Vallee and Auld, 1990). This active site was first identified in the prototypic zinc metalloendopeptidase, thermolysin, and consists of the sequence, HEXXH, in which the two histidines co-ordinate the binding of the essential zinc atom and the glutamate acts as a general base in catalysis (Matthews, 1988). However, not all zinc-dependent metalloproteinases appear to contain this active-site motif. Among the metalloproteinases that do not contain this conserved active site are the carboxypeptidases (Vallee and Auld, 1990) and members of the IDE family.

Several conserved histidines and glutamates in the IDE family of proteinases have been proposed to play a role in zinc binding. Rawlings and Barrett (1991) have postulated that His-88, Glu-169 and His-283 may represent the three zinc-binding ligands in these proteinases. These residues were identified on the basis of an alignment of the sequences of the mitochondrial processing enzyme, three members of the IDE family and the two subunits of the mitochondrial processing proteinase (MPP) and processing-enhancing protein (PEP). Both of the latter proteins possess a high degree of sequence identity with the first highly conserved domain of the IDEs, and together form a complex of the same approximate molecular mass as the single polypeptide chains of the bacterial and human enzymes. The sequence analysis of Rawlings and Barrett presumes that both subunits of this proteolytic complex are catalytically active; however, it is thought that both proteins need to be present to obtain an active proteinase (Geli et al., 1990; Yang et al., 1991). Therefore it seems unlikely that both subunits would need to contain a complete active site. Using a sequence alignment of only the three confirmed members of the IDE family, we identified a different set of histidines and glutamates that could be functioning as the active site in these proteinases, His-88, Glu-91 and His-92 (Becker and Roth, 1992). We tested this hypothesis using site-directed mutagenesis of proteinase III and found that His-88 and His-92, but not Glu-91, were required for metal binding. However, all three of these residues were essential for activity. These results suggest that the active site in proteinase III consists of the sequence, HXXEH, an inversion of the active site normally identified in zinc-dependent metalloendopeptidases. This active-site sequence is present in the first highly conserved region in this protein family and is also found in the PEP subunit of the mitochondrial processing enzyme, but not MPP. This is consistent with the need for both subunits to form an active complex (Geli et al., 1990; Yang et al., 1991); however, it is inconsistent with the report that purified MPP appears to contain a low level of residual activity (Schneider et al., 1990).

It is known that zinc is a tetradentate molecule, requiring four binding sites. In most metalloproteinases, the four zinc-binding residues are comprised of three amino acids (either glutamates and/or histidines) and a water molecule which is transferred to the active-site glutamate for nucleophilic attack on the siscile bond of the substrate. In the case of thermolysin, the two histidines in the active site and a glutamate 19 amino acids downstream from the active site act as three of the zinc-binding

residues (Matthews, 1988; Vallee and Auld, 1990). As mentioned above, the two histidines in the active site of proteinase III, HXXEH, are also critical for zinc binding. In this paper we use site-directed mutagenesis of the proteinase III protein to identify the third zinc-binding residue. An alignment of the sequences of proteinase III, the human and *Drosophila* IDEs, and PEP, identified only two glutamates and no histidines outside of the active site that are conserved in all four proteins (Figure 1). We, therefore, mutated both of these glutamates to glutamine (residues 162 and 169). In addition, a glutamate present in the second highly conserved domain of the IDE family was also mutated (residue 204). This third glutamate is found in bacterial proteinase III and the human IDE, but not the *Drosophila* or PEP proteins. These three mutants along with wild-type proteinase III were expressed and purified to near homogeneity, and their catalytic activities and zinc content were compared.

MATERIALS AND METHODS

Materials

The following were purchased: standard SDS molecular-mass markers, Bradford protein assay reagents and BSA standards from Bio-Rad, EDTA from Sigma, phenyl-Sepharose from Pharmacia, Ultrogel AcA34 from LKB, 10 ml PD10 G-25 M columns from Pharmacia, and zinc standards for atomic absorption from Alfa Products, Thiokol-Ventron Division (Danvers, MA, U.S.A.). Insulin and purified proteinase III were labelled by the Iodogen method (Pierce).

Site-directed mutagenesis of proteinase III

Mutagenesis of Glu-204 was performed as previously described (Becker and Roth, 1992). The mutagenesis of Glu-162 and -169 was performed using a modification of the procedure accompanying the Muta-Gene M13 *in vitro* mutagenesis kit, Version 2 (Bio-Rad). A construct consisting of the proteinase III gene subcloned into the *Eco*RV and *Hin*dIII sites of the BlueScript (KS) vector (Stratagene) was used to transform competent CJ236 bacteria. Single-stranded phagemids of the BlueScript/proteinase III construct were rescued from the bacteria using K07 helper phage and then purified by a procedure described by Sambrook et al. (1989). Site-directed mutagenesis oligonucleotides were synthesized and used in conjunction with the protocol from the Muta-Gene Kit (Bio-Rad) to make site-directed mutants of proteinase III. Confirmation of mutagenesis was obtained by sequencing the mutant clones with Sequenase (Unites States Biochemical). The mutant proteinase III constructs were then subcloned into the *Bam*HI and *Hin*dIII sites of a modified isopropyl β-D-thiogalactopyranoside-inducible *Tac* vector for overexpression (Paluh and Yanofsky, 1986).

Overexpression and purification of mutant and wild-type proteinase III proteins

The procedure followed was identical with that previously described (Becker and Roth, 1992). In brief, the periplasmic space of overexpressing bacteria was extracted in a hypotonic medium. The proteins in the periplasmic 'shockates' were then adjusted to 2 M NaCl and applied to a 5–10 ml phenyl-Sepharose column equilibrated in a buffer consisting of 2 M NaCl in 10 mM Hepes, pH 7.6. The column was eluted with a decreasing salt gradient (2–0 M NaCl) and an increasing ethylene glycol gradient (0–50 %). The peak fractions, as determined by Coomassie Blue-stained gels, were concentrated in an Amicon chamber with a YM30 membrane followed by a Centricon 30 (Amicon), down to 1 ml. The concentrate was then applied to an Ultrogel AcA34 column (1.5 cm × 90 cm) equilibrated with a buffer containing 10 mM Hepes (pH 7.6) and 0.2 % sodium azide. Again, the peak fractions, as determined by Coomassie Blue-stained gels, were pooled and then concentrated in a Centricon 30 to 1–1.5 ml. The amount of protein present was determined by the Bradford assay with BSA as standard. Values obtained by this assay were approximately 70–100 % greater than those obtained by amino acid composition but were in good agreement with estimates of protein obtained by measuring absorbance at 280 nm.

Insulin-degradation assays

The indicated amount of purified protein (determined by Bradford analysis) was incubated with 50000 c.p.m. of ^{125}I-labelled insulin for 30 min at 37 °C in a final volume of 40 μl. The reactions were terminated by the addition of EDTA to a final concentration of between 1 and 1.25 mM, and the extent of degradation was assessed in a receptor-binding assay (Ding et al., 1992). All assays were performed in triplicate.

Radioimmunoassay of wild-type and mutant proteinase III proteins

The indicated amounts of each protein were incubated with 50000 c.p.m. of ^{125}I-proteinase III on microtitre wells previously coated with a monoclonal antibody directed against proteinase III (Ding et al., 1992). The binding was performed overnight at 4 °C and the decrease in binding of iodinated protein to the antibody relative to a control with no competitor was calculated as the percentage inhibition of binding. All assays were performed in triplicate.

Metal determinations

The amount of metal in each purified protein was determined by atomic absorption spectrophotometry (Perkin–Elmer, model 2380) in two ways. First, the concentrated peaks off the AcA34 sizing column were diluted between 10- and 40-fold using a buffer consisting of 10 mM Hepes (pH 7.6) which was shown by atomic absorption spectrophotometry to be virtually devoid of a zinc signal. The same buffer was used to dilute zinc standards specific for atomic absorption spectrophotometry and the machine was calibrated with this buffer. The diluted protein samples (containing 0.4 to 4 μg of protein) and the zinc standards were then examined by atomic absorption. The amount of zinc in each proteinase preparation was determined after subtracting the background readings from an equivalent amount of solution from the parental cell line prepared in the same way as the purified proteins. The amount subtracted represented 114 pg of zinc (88 nM).

Alternatively, separate 10 ml PD10 columns (Pharmacia) were treated with 100 ml of 10 mM EDTA in a 10 mM Hepes (pH 7.6) buffer followed by 100 ml of 10 mM Hepes (pH 7.6) without any chelating agent. Both buffers were virtually devoid of a zinc signal by atomic absorption spectrophotometry. After treatment of the columns, 228 μg of each purified protein from the AcA34 sizing column was applied in a final volume of 0.5 ml. Ten fractions (0.5 ml) were collected directly from the columns into sterile zinc-free plastic tubes. The 10 mM Hepes buffer without chelating agents was used as the running buffer. The amount of protein in each fraction was determined by Bradford analysis and the amount of zinc in the peak fraction was determined by atomic-absorption spectrophotometry. Again, zinc standards specific for atomic absorption spectrophotometry were used;

however, in these experiments the standards were diluted in the 10 mM Hepes buffer which had flowed through the PD10 column before application of the proteins. The spectrophotometer was also calibrated with this buffer. In these experiments, no background was subtracted from the zinc readings.

RESULTS AND DISCUSSION

The three mutant and wild-type proteinase III proteins were overexpressed in *Escherichia coli* after induction with isopropyl β-D-thiogalactopyranoside for 36–48 h. The expressed proteins were then purified from the periplasmic space of the engineered bacteria using a two-column procedure, a phenyl-Sepharose column eluted with a decreasing salt gradient and an increasing ethylene glycol gradient, followed by an AcA34 sizing column. Comparable amounts of each protein, as determined by Bradford analysis, along with the previously purified proteinase III mutant H92R (Becker and Roth, 1992), were analysed by SDS/PAGE (Figure 2). All five samples had one major protein band of approximate molecular mass 100 kDa. A parallel sample from the parental bacterial strain prepared in an identical manner with the overexpressed proteins did not contain any significant protein band, indicating that the major Coomassie Blue-stained protein bands were encoded by the expression vectors (Figure 2).

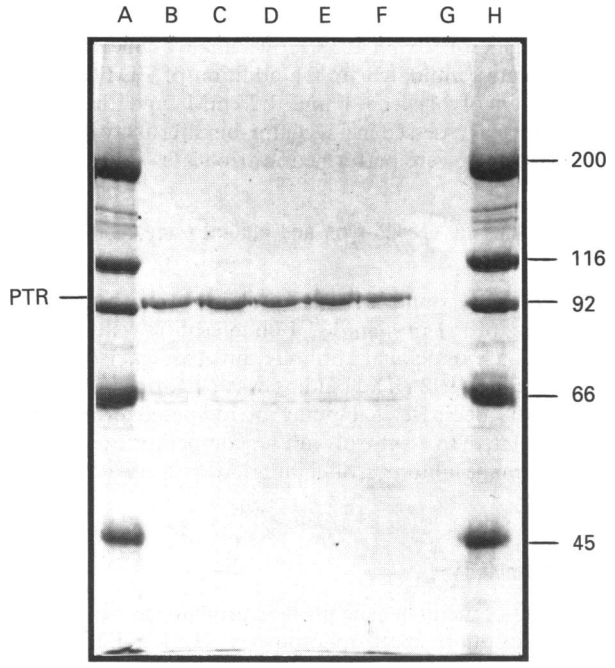

Figure 2 Coomassie Blue-stained SDS/polyacrylamide gels of purified wild-type and mutant proteinase III proteins

Lanes: A and H, molecular-mass markers (kDa); B, wild-type proteinase III; C, E162Q; D, E169Q; E, E204Q; F, H92R; G, endogenous proteins present in the preparation purified from the parental strain. The location of proteinase III (PTR) is indicated.

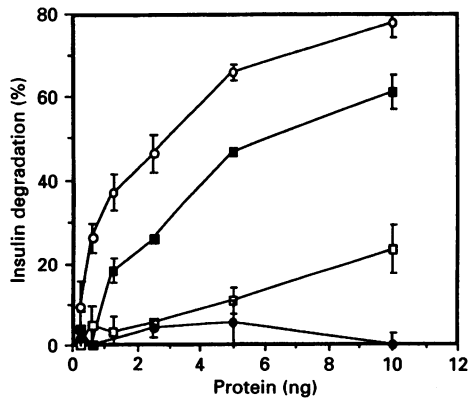

Figure 3 Degradation of ^{125}I-insulin by wild-type (■) and mutant proteinase III proteins (□, E162Q; ●, E169Q; ○, E204Q)

The indicated amounts of protein (determined by Bradford assays) were assayed for the ability to degrade ^{125}I-insulin as described. Results shown are means ± S.D. of triplicate determinations.

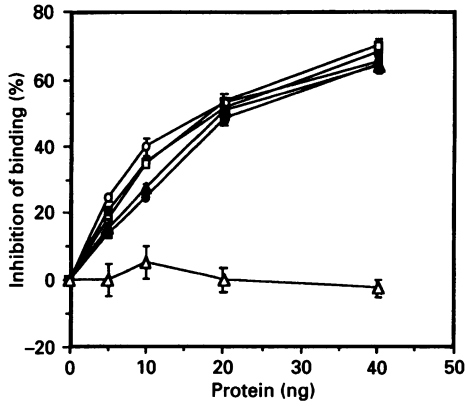

Figure 4 Radioimmunoassay of wild-type (■) and mutant proteinase III proteins (□, E162Q; ●, E169Q; ○, E204Q; ▲, H92R; △, endogenous)

The indicated amounts of each protein were incubated with 50000 c.p.m. of ^{125}I-proteinase III on microtitre wells previously coated with an antibody directed against proteinase III (Ding et al., 1992). Results shown are means ± S.D. of triplicate determinations.

The purified proteins were then assayed for their ability to degrade the exogenous substrate, insulin (Figure 3). Mutant protein E204Q was found to be as active as the wild-type enzyme in its ability to degrade insulin, whereas the E169Q mutant was completely devoid of activity. On the other hand, the E162Q mutant was partially active, retaining approximately 20% of the enzyme activity of the native protein.

The decreased activities of the E162Q and E169Q mutants could have been the result of changes in the gross three-dimensional structures of these proteins as a result of the mutagenesis. To examine this possibility, the mutant proteins were tested for their ability to compete with labelled native protein for binding to a monoclonal antibody directed against the native enzyme. This monoclonal antibody recognizes the native protein with high affinity in the radioimmunoassay (Becker and Roth, 1992) and only weakly recognizes the denatured proteinase on a Western blot (Ding et al., 1992). All three mutant proteins were found to compete equally with the wild-type protein in the radioimmunoassay (Figure 4). These results suggest that the three-dimensional structure of the three mutant proteins has not been drastically distorted by the mutagenesis. Additional evidence indicating that the mutagenesis has not greatly affected the protein structure of the mutants comes from the finding that all three mutant proteins and the native enzyme are expressed in the periplasmic space at comparable levels, since mutant proteins with distorted structures are often rapidly degraded. The mutant proteins are also purified identically with the native protein on the two columns, the phenyl-Sepharose column and the sizing

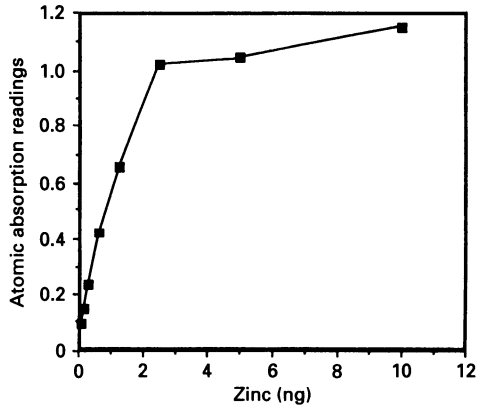

Figure 5 Zinc standard curve established by atomic-absorption spectrophotometry

Zinc standards designed specifically for atomic-absorption spectrophotometry were diluted in the same buffer which was used to calibrate the spectrophotometer and dilute the protein samples. The results shown are the means of at least three independent readings at each zinc concentration.

Table 1 Amount of zinc in wild-type proteinase III and four mutants as determined by atomic absorption spectrophotometry

The amount of zinc in each preparation was determined by atomic absorption spectrophotometry on three or four independent readings either immediately after purification on the sizing column (AcA34) or after passage over a desalting column (PD10). The amount of each protein was determined by Bradford analysis with BSA as standard. A zinc standard curve was established using a zinc standard solution designed for atomic absorption spectrophotometry. The spectrophotometer was calibrated with the same buffer as that in which the standards and the purified proteins were prepared. The abbreviation n.t. stands for not tested.

Enzyme	(mol zinc/mol of protein) (AcA34)	(mol zinc/mol of protein) (PD10)
Wild-type	0.77 ± 0.08	0.89 ± 0.14
Mutant E162Q	0.74 ± 0.22	0.47 ± 0.11
Mutant E169Q	0.27 ± 0.12	0.26 ± 0.15
Mutant E204Q	1.11 ± 0.22	0.94 ± 0.08
Mutant H92R	0.00 ± 0.006	n.t.

column. Finally, the change from glutamate to glutamine is relatively conservative, retaining the size of the residue and altering only its charge. The conservative nature of this change can be seen in the E204Q mutant which retains its complete activity, although this residue occurs in one of the three highly conserved regions of the enzyme.

Atomic absorption spectrophotometry was then used to measure the levels of zinc in the purified wild-type and mutant enzymes. First, a standard curve was established using zinc standards specific for atomic absorption spectrophotometry (Figure 5). All proteins were diluted such that the zinc readings would fall within the linear part of the curve. The levels of zinc in the various proteins were assayed directly in the purified samples and after passage over a desalting column (see the Materials and methods section for details). The values obtained by the two methods were generally in good agreement (Table 1). The wild-type proteinase and the fully active E204Q mutant protein were found to contain nearly stoichiometric amounts of zinc (Table 1). The E169Q mutant (which was devoid of enzyme activity) was found to have a significantly lower level of zinc than the wild-type enzyme, approximately 0.3 mol of zinc/mol of enzyme (Table 1). However, this mutant still contained more zinc than the H92R mutant protein, possibly indicating that mutation of one of the two proximal zinc-binding histidines in the active site has a greater effect on the affinity for zinc than mutation of the downstream glutamate (Table 1). Since residue 169 is absolutely required for activity and mutation of this residue results in a protein which contains a low level of zinc as compared with the wild-type enzyme, it appears that this glutamate represents one of the three zinc-binding residues in proteinase III.

Mutant E162Q, which has partial enzyme activity, was also found to contain less zinc than the wild-type enzyme after passage over the PD10 column; however, this mutant did retain more zinc than the E169Q mutant (Table 1). The finding that mutation of residue 162 results in a partially active enzyme as well as a decrease in zinc binding relative to wild-type protein may be explained by its close proximity to the essential residue at position 169. Mutation of amino acid 162 may result in a local perturbation of the protein structure in the area around the essential zinc-binding glutamate at position 169, thereby causing a slight decrease in the protein's affinity for zinc. Evidence for a lower zinc affinity in this mutant is seen in the decreased amount of zinc present after passage over the desalting column but not before passage over this column (Table 1). A similar result has previously been reported in an extracellular neutral proteinase from *Streptomyces cacaoi* in which the mutagenesis of a residue near the two zinc-binding histidines in the active site of the enzyme resulted in a significant decrease in zinc binding relative to the wild-type proteinase (Chang and Lee, 1992).

The identification of Glu-169 as the third zinc-binding residue in proteinase III indicates that the sequence, $HXXEH(X)_{76}E$, represents the active site and third zinc-binding residue in proteinase III. In this sequence, the two histidines and Glu-169 are essential for co-ordinating the zinc atom, while Glu-91 is not involved in zinc binding (Becker and Roth, 1992). However, all four residues are essential for catalytic activity. These four residues are conserved in the two other proteinases which comprise the IDE family (the human and *Drosophila* IDEs) and in the PEP (Figure 1). Thus, by analogy, these residues are likely to play a similar role in these other enzymes, although it will be necessary to test this hypothesis directly by further mutagenesis studies.

To date, the definitive identification of all three zinc-binding residues has been accomplished for only a limited number of metalloproteinases. Using site-directed mutagenesis, the active site and zinc-binding residues have been identified in endopeptidase 24.11 (Devault et al., 1988a,b; LeMoual et al., 1991), leukotriene A_4 hydrolase (Medina et al., 1991) and a neutral metalloproteinase from *Streptomyces cacaoi* (Chang and Lee, 1992). The active-site and zinc-binding residues have also been identified in several proteinases on the basis of X-ray crystallographic analysis, including thermolysin (Matthews, 1988), elastase (Thayer et al., 1991) and carboxypeptidase A (Faming et al., 1991). In all but the carboxypeptidases, the active sites and third zinc-binding residues consist of the sequence $HEXXH(X)_nE$ where n ranges from 19 to 34 amino acids. In the carboxypeptidases, the three zinc-binding residues are comprised of two histidines and a glutamate, $HXXE(X)_{123}H$, with the catalytically active glutamate being located 74 amino acids downstream from the third zinc-binding site (Faming et al., 1991).

In conclusion, the sequence comprising the active site and

third zinc-binding residue in proteinase III, $HXXEH(X)_{76}E$, is also found in the human and *Drosophila* IDEs and the PEP subunit of the MPP. This active site is distinct from that seen in all other metalloproteinases, and we propose that this motif represents the signature sequence for this proteinase family. A number of other proteinases share biochemical properties and inhibitor profiles with members of the IDE family (Resnick et al., 1991; Delporte et al., 1992). It remains for the amino acid sequences of these other proteinases to be elucidated in order to determine if they belong to the same class as the bacterial and eukaryotic IDEs and the MPP.

We thank Dr. Sidney Kushner for the original proteinase III construct, Dr. Paul Gallnick and Dr. Charles Yanofsky for the PTAC vector, and Dr. Edward Solomon for use of the atomic-absorption spectrophotometer. This work was supported by the National Institutes of Health Grant DK34926.

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Received 7 September 1992/26 November 1992; accepted 30 November 1992

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