An unusual active site identified in a family of zinc metalloendopeptidases

(Insulin degrading enzyme/mitochondrial processing protease)

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ABSTRACT An unusual active site has been identified in a family of zinc metalloendopeptidases that includes bacterial protease III and the human and Drosophila insulin-degrading enzymes. All of these enzymes have been characterized as metalloendopeptidases and purified protease III has been shown to contain stoichiometric levels of zinc. However, all three proteases lack the consensus sequence (HEXXH) described in the active site of other zinc metalloendopeptidases. Instead, these proteases contain an inversion of this motif, HXXEH. To determine whether this region could represent the active site in these proteins, the two histidines in protease III were individually mutated to arginine and the glutamate was mutated to glutamine. All three mutants were devoid of proteolytic activity toward an exogenous substrate, insulin, as compared to the wild-type protease. Three lines of evidence indicate that this loss of activity in the mutants is not due to distortion of the three-dimensional structure of the protein: (i) the mutants are secreted into the periplasmic space and chromatograph normally; (ii) all three mutants are expressed at levels nearly identical to wild-type protein and do not appear to have an increased susceptibility to proteolysis in the bacteria; and (iii) the mutants compete equally with wild-type protein in a radioimmunoassay. The purified wild-type and glutamate mutants were found to contain stoichiometric amounts of zinc by atomic absorption spectrophotometry, whereas both histidine mutants had negligible zinc signals. These findings are consistent with this region being the active site in this protein, with the histidine residues coordinating the essential zinc atom and the glutamate involved in catalysis.

Proteases play a variety of roles in many physiological processes, including fibrinolysis, blood homeostasis, complement activation, digestion, and hormone processing (1). Based on common active sites and mechanisms of action as determined by primary amino acid sequence, inhibitor and substrate profiles, and x-ray crystallographic analyses (2), these proteins are generally classified into one of four families. Representative members of each protease class (i.e., cysteine, serine, aspartyl, and metallo-) have been crystallized and their active sites elucidated (2). For example, the bacterial enzyme thermolysin is the prototypic zinc metalloendopeptidase. X-ray crystallographic studies have shown that this enzyme contains two histidines (His-142 and His-146) and a glutamic acid (Glu-143) at its active site, with the histidines coordinating the binding of a zinc atom and the glutamate acting as a general base in catalysis (3). Based on studies in thermolysin, it has been proposed that the active site sequence in metalloendopeptidases is HEXXH (where X is any amino acid, H is histidine, and E is glutamate). This sequence has been identified in well over 15 zinc-dependent metalloproteases, including aminopeptidases and metalloendopeptidases, many of which show little overall sequence identity to thermolysin outside of this domain (4, 5). Recently, through mutagenesis studies, the sequence HEXXH has been confirmed to be at the active site of several of these zinc-dependent proteases, including neutral endopeptidase and angiotensin-converting enzyme (6–8). Moreover, this consensus sequence has even been used to predict the peptidase activity of proteins not previously considered to be proteases, such as leukotriene A4 hydrolase (9).

However, some proteases, based on their amino acid sequence, do not fit into the currently recognized classification system. One example is the carboxypeptidases, zinc-dependent metalloexopeptidases, in which the metal binding domain consists of the motif HXXE(X)123H (4) as opposed to the more common active site seen in thermolysin. Another example is the bacterial enzyme protease III, or pi, from Escherichia coli (10–13). This periplasmic enzyme shows 27% overall sequence identity with a human enzyme, called insulin-degrading enzyme (14), and 28% identity with a homologous enzyme from Drosophila melanogaster (15). In addition, three regions in these enzymes show >50% sequence identity (10, 14, 15). Although the physiological roles of these three enzymes are not known, they all degrade insulin (10–17). All three proteases also have molecular masses of ∼100 kDa and, based on their requirement for divalent cations, have been classified as metalloendopeptidases. Moreover, protease III has recently been shown to contain zinc at stoichiometric levels (18). However, none of these enzymes contains the consensus active site sequence HXXH described above for zinc metalloendopeptidases.

A careful alignment of the sequences of these three proteases reveals the conservation of 12 histidines and glutamates, which could function in zinc binding (10, 14, 15). In fact, a number of motifs resembling that of the carboxypeptidases can be identified in all three enzymes, including HXXE(X)134H. In addition, all three enzymes contain a sequence, HXXEH, which could be considered an inversion of the active site consensus sequence seen in thermolysin and other metalloendopeptidases (10, 14, 15). This sequence is found in one of the three highly conserved regions present in these enzymes. The surrounding residues of this sequence also appear to be inverted in comparison to other zinc metalloendopeptidases (Table 1). The spacing of the two histidines and the first histidine to the glutamate in this region are consistent with the one to three amino acids separating the zinc binding residues in more than a dozen zinc enzymes whose structures have been analyzed (4) and are the only two histidines and/or glutamates conserved among all three proteases that exhibit the requisite spacing.

To test whether the residues in the apparently inverted sequence HXXEH comprise the active site of protease III, the two histidine residues were individually mutated to arginine and the glutamate was changed to glutamine by site-directed mutagenesis. (The mutants were named by using the single-letter amino acid code and indicating the number of the residue that was mutated—H88R, histidine-88...
Table 1. Comparison of the proposed active site residues in bacterial protease III, the human (hIDE) and Drosophila (dIDE) insulin-degrading enzymes, and several other zinc metalloendopeptidases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sequence</th>
<th>Residues</th>
<th>Ref(s).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease III</td>
<td>GLA</td>
<td>H Y L E E MSL</td>
<td>85-95</td>
</tr>
<tr>
<td>hIDE</td>
<td>GLS</td>
<td>H F C E MLF</td>
<td>106-116</td>
</tr>
<tr>
<td>dIDE</td>
<td>GLA</td>
<td>H Y L E MEF</td>
<td>78-88</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>VVA</td>
<td>E E LT</td>
<td>139-149</td>
</tr>
<tr>
<td>Collagenase</td>
<td>VAA</td>
<td>E E LG</td>
<td>215-225</td>
</tr>
<tr>
<td>Serratia protease</td>
<td>TPT</td>
<td>H E G</td>
<td>89-99</td>
</tr>
<tr>
<td>Endopeptidase 24.11</td>
<td>VIG</td>
<td>E E IT</td>
<td>580-590</td>
</tr>
<tr>
<td>Leukotriene A4 hydrolase</td>
<td>VIA</td>
<td>E E IS H SW</td>
<td>292-302</td>
</tr>
</tbody>
</table>

The three active site residues, two histidines and a glutamate, in a number of proteases are marked with an asterisk. Underlined residues indicate residues adjacent to the active site that are inverted in the insulin-degrading enzymes and protease III relative to other metalloproteases. The diamond is used to mark residues that are invariably hydrophobic in the different metalloendopeptidases.

to arginine; H92R, histidine-92 to arginine; E91Q, glutamate-91 to glutamine.) The mutant proteins were then compared to wild-type protein in terms of activity and zinc content to ascertain whether this region is the active site in this protein family.

MATERIALS AND METHODS

Materials. The following were purchased: standard SDS molecular weight markers from Bio-Rad, bacitracin from Sigma, phenyl-Sepharose from Pharmacia, Ultrogel AcA-34 from LKB, and zinc standards for atomic absorption spectrophotometry from Alfa Products, Thikol-Ventron Division (Danvers, MA). Insulin and purified protease III were labeled by the Iodo-Gen method (Pierce).

Site-Directed Mutagenesis of Protease III. The protease III gene (10) was subcloned into the EcoRV and HindIII sites of the BlueScript (KS) vector (Stratagene) and then used to transform the bacterial strain TG1. Single-stranded phagemids of the BlueScript/protease III construct were rescued from the bacteria by using K07 helper phage and then purified by a procedure accompanying the T7-Gen in vitro mutagenesis kit (United States Biochemical). Site-directed mutagenesis oligonucleotides were synthesized and used in conjunction with the T7-Gen kit to make site-directed mutants of protease III. Confirmation of mutagenesis was obtained by sequencing the mutant clones with Sequenase (United States Biochemical).

Overexpression and Purification of Mutant and Wild-Type Protease III Proteins. The wild-type and mutant protease III genes were subcloned into the EcoRV and HindIII sites of a modified Tacterm vector and were used to transform competent JM101 bacteria as described (18). Expression of the proteins was induced with the addition of 2 mM isopropyl β-D-thiogalactopyranoside for 24–48 hr in 4 liters of modified M9 medium (18). Bacteria were shifted from hypertonic to hypotonic media to release periplasmic proteins containing the wild-type and mutant protease III proteins as described (18).

The periplasmic extracts were then brought up to 2 M NaCl and applied to a 5- to 10-ml phenyl-Sepharose column previously equilibrated with 2 M NaCl in 10 mM Hepes buffer (pH 7.6). The column was washed extensively with 2 M NaCl buffer and then eluted with a decreasing salt gradient (2–0 M NaCl) and an increasing ethylene glycol gradient (0–50%) in a 10 mM Hepes buffer. Approximately 60 (3 ml) fractions were collected and the peak fractions, as determined by Coomassie blue-stained gels, were pooled. The protease peak was concentrated to 1 ml in an Amicon chamber with a YM-30 membrane (Amicon) and then applied to an Ultrogel AcA34 column (1.5 × 90 cm) equilibrated with a buffer containing 10 mM Hepes (pH 7.6) and 0.02% sodium azide. Again, the peak fractions as determined by Coomassie blue-stained gels were pooled and then concentrated in a Centrifcon 30 (Amicon) to 1–1.5 ml.

Insulin-Degradation Assays. The indicated amounts of protease relative to wild-type protease III (determined by radioimmunooassays and Coomassie blue-stained gels) were incubated in microtiter wells previously coated with an antibody directed against protease III (18). The wells were washed and the immunocaptured enzyme was tested for its ability to degrade 125I-labeled insulin (125I-Insulin). Degradation of insulin was assessed by its loss of ability to bind to the insulin receptor as described (18). All assays were performed in triplicate using 40,000 cpm of 125I-insulin in a final vol of 35 μl for 30 min at 37°C. The assays were terminated by addition of bacitracin to a final concentration of 1 mg/ml, and the extent of degradation was assessed in a receptor binding assay (18).

Radioimmunooassay of Wild-Type and Mutant Protease III Proteins. The indicated amounts of each protein were incubated with 50,000 cpm of 125I-protease III on microtiter wells previously coated with an antibody directed against protease III (18). 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 percent inhibition of binding. All assays were performed in triplicate.

Metal Determinations. The amount of zinc in each purified protein preparation was determined by atomic absorption spectrophotometry (Perkin–Elmer, model 2380). All assays were performed in triplicate and a zinc standard curve was established by using a zinc standard solution designed for atomic absorption spectrophotometry. The amount of zinc in each protease preparation was determined after subtracting the background readings from an equivalent amount of solution from the parental cell line prepared in the same manner as the purified proteins. The amount subtracted represented ≈ 500 pg of zinc (385 nM).

RESULTS AND DISCUSSION

The three mutant and wild-type protease III proteins were expressed using a bacterial expression plasmid containing a Tac promoter, and periplasmic shockates containing these proteins were prepared as described (18). Comparable amounts of wild-type protease III and the three mutant proteins were assayed for proteolytic activity against an exogenous substrate, insulin, after immunoinmobilization (Fig. 1). The amount of each protein in the periplasmic shockates was determined relative to the other proteins by a radioimmunooassay using a monoclonal antibody directed against protease III (18) and these data were subsequently confirmed with Coomassie blue-stained gels (Figs. 2 and 3A). The wild-type protease readily degraded iodinated insulin; however, the three mutants were devoid of activity (Fig. 1). Similar data were obtained when purified proteins in a solution-phase assay were used.

The loss of enzymatic activity in the mutated protease III proteins could have resulted from a general distortion of their three-dimensional structure as a result of mutagenesis. Several lines of evidence argue against this possibility. First, the mutant proteins are secreted into the periplasmic space of E. coli and chromatographed identically to wild-type protease III on hydrophobic and sizing columns. The mutant proteins are also expressed at levels comparable to the wild-type protein and do not appear to have an increased susceptibility to
proteolysis in the bacteria. Finally, the mutant proteins compete equally with wild-type protease in a radioimmunoassay with $^{125}$I-protease III and a monoclonal antibody directed against the protease (Fig. 2).

The wild-type and mutant proteases were purified to near homogeneity by a two-column purification procedure, a phenyl-Sepharose column followed by an ACA34 sizing column (Fig. 3B). The amount of each purified protein was then determined by quantitative amino acid determination of 3-7 µg of each preparation. The calculated amount of each amino acid as determined through this procedure was nearly identical in all preparations and matched closely the amino acid composition predicted by the DNA sequence for this protein (10). The amount of zinc in each purified protein was then determined by atomic absorption spectrophotometry on two different and independent preparations of each protein (Table 2). The wild-type and glutamate mutant both contained stoichiometric amounts of zinc, while the two histidine mutants had negligible zinc signals.

These results indicate that the two histidine residues in the conserved motif HXXEH are involved in binding the essential zinc molecule in the active site. In contrast, the glutamate did not appear to be required for zinc binding. However, all three amino acids do appear to play an important function in the catalytic activity of this protein. These findings are consistent with the hypothesis that HXXEH represents an inverted active site sequence in comparison to that present in thermolysin. However, definitive confirmation of these findings must await crystallization and elucidation of the structure of at least one member of this unusual class of proteases.
Table 2. Amount of zinc in purified wild-type protease III and three mutants as determined by atomic absorption spectrophotometry

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Zinc, (\mu)M</th>
<th>Protein, (\mu)M</th>
<th>mol of zinc per mol of protein</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>0.068</td>
<td>0.55</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>Mutant H88R</td>
<td>&lt;0</td>
<td>1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Preparation 1</td>
<td>&lt;0</td>
<td>1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Preparation 2</td>
<td>&lt;0</td>
<td>1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Mutant E91Q</td>
<td>1.12</td>
<td>1.1</td>
<td>1.02 ± 0.13</td>
</tr>
<tr>
<td>Preparation 1</td>
<td>1.24</td>
<td>1.1</td>
<td>1.09 ± 0.08</td>
</tr>
<tr>
<td>Preparation 2</td>
<td>&lt;0</td>
<td>1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Mutant H92R</td>
<td>&lt;0</td>
<td>1.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

The amount of zinc in each preparation of purified protein was determined by atomic absorption spectrophotometry on three independent samples. The amount of each protein was determined by amino acid quantitation and a zinc standard curve was established by using a zinc standard solution designed for atomic absorption spectrophotometry. ND, not determined because the zinc readings were below the parental cell background.

While residues other than those studied in this report have been proposed as the metal binding ligands in this class of proteases (24), it is clear that the two histidines in the proposed active site are essential for zinc binding. However, if these proteins are analogous to other classes of zinc metalloendopeptidases, then one can anticipate that another glutamate or histidine residue downstream from the active site will also contribute to zinc binding (3, 4). There are several glutamate and histidine residues conserved between all members of this protein family that are a suitable distance from the proposed active site to be this third zinc binding residue. Which of these residues comprises the third zinc binding site remains to be determined.

When the residues adjacent to the active site histidines and glutamate in over a dozen zinc-dependent metalloendopeptidases were analyzed, it was found that in a region of 10 amino acids, from 3 upstream to 3 downstream of the conserved active site, there were no charged amino acids except for the 3 active site residues (5). However, 2 recently cloned metalloendopeptidases, meprin A and N-benzoyl-tyrosyl-aminobenzoic acid hydrolase, are exceptions to this rule; each contains a charged glutamate residue adjacent to the first histidine of the active site (25). In addition, further analysis of the different metalloendopeptidases indicates that there is invariably a hydrophobic residue 2 amino acids downstream from the second active site histidine (5). This hydrophobic residue is conserved in meprin A and in papain hydrolase (25). These findings suggest that the signature sequence at the active site of most zinc-dependent metalloendopeptidases is UUZHEUUHUHU (where U represents uncharged residues, B signifies hydrophobic residues, and Z represents a residue that can be either charged or uncharged). In the proposed active site of protease III and the insulin-degrading enzymes, these 10 amino acids are all uncharged except for the 3 active site residues. However, the position of the adjacent invariant hydrophobic residue is also inverted in the protease III active site relative to the active site seen in other metalloendopeptidases, with the hydrophobic residue being 2 amino acids upstream from the first histidine (Fig. 1). These findings lead to a completely inverted signature sequence in protease III and the related insulin-degrading enzymes relative to other known zinc metalloendopeptidases.

The proposed active site, HXXEH, is conserved in a bacterial enzyme (protease III) and in the human and DrosoPhila insulin-degrading enzymes. Another potential mem-

ber of this class of zinc metalloendopeptidases is the mitochondrial-processing protease (26, 27). This protease is composed of two separate subunits, the mitochondrial-processing protease (MPP) and the processing-enhancing protein (PEP) (26, 27). Both of these peptides have a significant degree of homology to the first conserved region between insulin-degrading enzyme and protease III (24). However, only PEP has the proposed active site motif HXXEH (24, 26, 27). A role for this sequence as the active site of the mitochondrial enzyme is consistent with the reported requirement of both subunits for activity (28, 29) but is inconsistent with some claims that a low residual activity is found in preparations of purified MPP (30). Additional studies are therefore required to test the role of these residues in the enzymatic activity of the mitochondrial enzyme.

Based on biochemical characteristics such as molecular mass and inhibitor profiles, several as yet uncloned metalloendopeptidases may also be members of the same class of proteases as the mammalian and bacterial insulin-degrading enzymes. These include enzymes that have been proposed to play a role in antigen processing in lymphocytes (31), differentiation of myoblasts (32), exocytosis in mast cells (33), intracellular membrane fusion in fertilization in the sea urchin (34), degradation of atrial natriuretic factor in kidney (35), and, most recently, degradation of the antimicrobial magainin peptides found in Xenopus (36). Elucidation of the amino acid sequences of these enzymes will be necessary to determine whether these molecules also belong to the same class of proteases as bacterial protease III and the human and DrosoPhila insulin-degrading enzymes and whether they contain the proposed active site sequence HXXEH.

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