Zinc Coordination and Substrate Catalysis within the Neuropeptide Processing Enzyme Endopeptidase EC 3.4.24.15

IDENTIFICATION OF ACTIVE SITE HISTIDINE AND GLUTAMATE RESIDUES*

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Endopeptidase EC 3.4.24.15 (EP24.15) is a zinc metalloendopeptidase that is broadly distributed within the brain, pituitary, and gonads. Its substrate specificity includes a number of physiologically important neuropeptides such as neuropeptides, bradykinin, and gonadotropin-releasing hormone, the principal regulatory peptide for reproduction. In studying the structure and function of EP24.15, we have employed in vitro mutagenesis and subsequent protein expression to genetically dissect the enzyme and allow us to glean insight into the mechanism of substrate binding and catalysis. Comparison of the sequence of EP24.15 with bacterial homologues previously solved by x-ray crystallography and used as models for mammalian metalloendopeptidases, indicates conserved residues. The active site of EP24.15 exhibits an HEXXH motif, a common feature of zinc metalloenzymes. Mutations have confirmed the importance, for binding and catalysis, of the residues (His473, Glu474, and His477) within this motif. A third putative metal ligand, presumed to coordinate directly to the active site zinc ion in concert with His473 and His477, has been identified as Glu502. Conserved alterations to these residues drastically reduces enzymatic activity against both a putative physiological substrate and a synthetic quenched fluorescent substrate as well as binding of the specific active site-directed inhibitor, N-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-p-aaminobenzoate, the binding of which we have shown to be dependent upon the presence, and possibly coordination, of the active site zinc ion. These studies contribute to a more complete understanding of the catalytic mechanism of EP24.15 and will aid in rational design of inhibitors and pharmacological agents for this class of enzymes.

Endopeptidase EC 3.4.24.15 (EP24.15)\(^1\) belongs to the family of zinc metalloendopeptidases that includes among its mem-

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The three-dimensional structure of EP24.15, or indeed any other mammalian metalloendopeptidase of this class, is currently unknown. Conversely, the structures of three related bacterial zinc metalloendopeptidases, thermolysin (22), bacterial elastase (23), and neutral protease (24), have previously been solved by x-ray crystallographic analysis to a resolution of greater than 2 Å. The mechanism of catalysis in these metalloenzymes centers upon an active site zinc ion coordinated to three amino acid side chains and a water molecule (1, 25–28). A zinc-binding nucleus is typically provided by two ligands separated by a short spacer of 1–3 amino acids. A third ligand, separated from the second by 20–120 amino acids, completes

bers ACE (angiotensin-converting enzyme), EP24.11 (neutral endopeptidase), EP24.16 (neurolisin), and bacterial thermolysin (1–3). Also known as thimet oligopeptidase (4), EP24.15 is a predominantly soluble, 77-kDa, thiol-sensitive enzyme that preferentially cleaves peptide bonds on the carboxyl side of hydrophobic amino acid residues. A hydrophobic or bulky residue in the P2 and P3’ positions relative to the scissile P1-P1’ bond (nomenclature of Schecter and Berger (5)) further contributes to substrate binding and catalytic efficiency (6, 7).

Most EP24.15 activity is known to be located in brain, pituitary, and gonads (8). Within the central nervous system, EP24.15 exhibits a widespread distribution with high levels observed in areas rich in neuropeptide content such as anterior pituitary, cerebellum, hippocampus, cortex, and hypothalamus (8, 9), thus suggesting a potential role for EP24.15 in the metabolism of bioactive peptides. Of particular interest to this laboratory is the decapetide substrate GnRH (gonadotropin-releasing hormone), the master regulatory peptide for reproduction. Several studies have previously documented the ability of EP24.15 to cleave GnRH at the Tyr5–Gly6 bond in vitro (consistent with its aforementioned substrate specificity) (6, 7, 10, 11). By using the competitive, specific active site-directed inhibitor, cFP-AAF-pAB (N-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-a-amino-benzoate) (12), workers (10, 13–15) have further demonstrated the potential importance of EP24.15 in the post-secretory regulation of GnRH signaling events in vivo. Other physiologically active peptides such as neuropeptides, bradykinin, and gonadotrophins are also cleaved at sites consistent with the substrate specificity of EP24.15 (6–8). This enzyme has also been shown to generate Leu- and Met-enkephalin from larger prodynorphin- and prodynorphin-derived precursors such as dynorphin ϐ-8, α- and β-neoendorphin, metorphamide, and Leu-enkephalin-Arg-Gly-Leu (8, 16, 17) and to cleave the endogenous opioid receptor-like ligand, nociceptin/orphanin FQ, a recently identified heptadecapeptide structurally resembling dynorphin A (18). EP24.15 has further been implicated in the secretase processing of the wild type and mutant forms of β-amylloid precursor protein in Alzheimer’s disease (19–21). This latter implication is unusual given its general preference for oligopeptides.
In column one, the first letter of the mutant designation indicates the amino acid present in the wild type protein; the number is the position in the sequence, and the last letter represents the newly substituted amino acid. The second and third columns indicate base changes (bold and underlined) used within mutant primers. The superscript numbers in column two indicate cDNA base position. Base changes (column three) were designed to obey prokaryotic codon usage rules to obviate use of rare codons which may hinder subsequent expression of the mutants.

QFS was obtained from Auspep (Victoria, Australia); Mca-Pro-Leu standard was from Calbiochem; DTT (dithiothreitol) was purchased from Roche Molecular Biochemicals; Chameleon mutagenesis kit was purchased from Stratagene (La Jolla, CA); all reaction buffers, restriction and modification enzymes, were purchased from New England Biolabs (Beverly, MA); T7 Sequenase was purchased from Amersham Pharmacia Biotech; QIAprep spin plasmid preparation kit was from Qiagen (Chatsworth, CA); unless otherwise indicated, all other chemicals were purchased from Sigma.

Sequence Alignments—Utilizing the computer algorithms, CLUSTAL (34) and BESTFIT (35), the protein sequences of EP24.15 and the bacterial homologues, thermolysin, bacterial elastase, and neutral protease, were compared as described previously2 to obtain homology between functional domains and key conserved catalytic residues. Mutagenesis and Protein Expression—Double-stranded site-directed mutagenesis of rat EP24.15 was performed on the EP24.15 expression vector pGEX-24.15, modified for rapid screening of mutations by addition of a unique restriction endonuclease site (EcoRI) that replaces the vectors sole Apal restriction site (36). Oligonucleotide primers were synthesized with mismatches coding for the appropriate amino acid change (Table I) but following prokaryotic codon usage rules to obviate the use of rare codons that may hinder subsequent protein expression. Mutant and selection primers, phosphorylated at the 5′-end, were annealed to the double-stranded expression vector before being extended and ligated in a single reaction. The resulting plasmid DNA was selected for the Apal to EcoRI mutation, transformed into repair-deficient XL Mut5 bacterial cells, and selected for ampicillin resistance. Plasmid DNA was subsequently purified (QIAprep spin plasmid kit) and subcloned to a further round of selection for the Apal to EcoRI mutation to be confirmed before being transformed into XL1-Blue bacterial cells and screened for ampicillin resistance. Purified plasmid DNA (37) was cleaved with EcoRI to screen for desired mutations. Putative positives were confirmed by double-stranded template dideoxy sequencing (38). Expression and purification of the mutant proteins for biochemical characterization were as described previously (36), with all enzymes stored at 90°C for subsequent analysis. The reproducibility of the mutagenesis procedure was also addressed in this study. To this end, EP24.15 wild type and a selection of mutants were re-prepared from bacterial glycerol stocks and assayed (as described below), yielding very similar kinetic trends.

Native Gel Electrophoresis—Assessment of purification to homogeneity, yield, and proper folding of expressed proteins was by native polyacrylamide gel electrophoresis (PAGE) on an 8% gel under reducing conditions as described previously (39). Yields of expressed protein were similar for all of the mutations.

Circular Dichroism (CD)—To determine if gross structural alterations occurred during mutagenesis and subsequent protein expression, selected mutants displaying a substantial decrease in catalytic and inhibitor binding capacity were examined by CD spectroscopy. CD spectra were collected in the wavelength range of 300 to 185 nm at 0.2-nm intervals with an Aviv 60DS spectropolarimeter (AVIV Instruments, Lakewood, NJ). The instrument wavelength was checked with benzene vapor. Optical rotation was calibrated by measuring the ellipticity of d12-camphorsulfonic acid at 192.5 and 290 nm. Measurements of optical ellipticity were made at 25°C using a 0.1-cm path length quartz cell. At least eight reproducible scans were collected for each

### Table I

| Mutation | Oligonucleotide primer (5′ to 3′) | Base change |
|----------|---------------------------------|-------------|
| HEXXH motif | | |
| H473A | 1404GTTGAGACCTACTTCCGGGACCCACGC | CA ⇒ GC |
| H473Q | 1404GTTGACCTACTTCCGGGACCCACGC | G ⇒ C |
| E474D | 1409CCTACTTCCGGGACCCACGC | G ⇒ C |
| E474Q | 1409CCTACTTCCGGGACCCACGC | G ⇒ C |
| H477A | 1413CTTCCAGAGGGGGCCCTATGCACCCACGC | G ⇒ C |
| H477Q | 1413CTTCCAGAGGGGGCCCTATGCACCCACGC | G ⇒ C |
| Third zinc ligand | | |
| E497D | 1475GTGGGACCCACGTCGCAGTTGTGG | G ⇒ C |
| E497Q | 1475GTGGGACCCACGTCGCAGTTGTGG | G ⇒ C |
| E502D | 1492CGGGACTTTGTGG1504 | G ⇒ C |
| E502Q | 1492CGGGACTTTGTGG1504 | G ⇒ C |
| E537Q | 1508CTAGGACCCCGTCGTAGACACCTTA | G ⇒ C |

### EXPERIMENTAL PROCEDURES

**Materials**—Bradford protein assay reagent and Bio-Gel HT hydroxyapatite were purchased from Bio-Rad; BSA was obtained from Pierce;
FIG. 1. Sequence alignment of EP24.15 with bacterial homologues. A comparison of specific residues within the catalytic site region of EP24.15 (rat) with those of thermolysin (Bacillus thermoproteolyticus), bacterial elastase (Pseudomonas aeruginosa), and neutral protease (Bacillus cereus), bacterial homologues of mammalian metalloendopeptidases that have been solved to atomic resolution. The sequences of mammalian EP24.11 and ACE have also been included for comparison. Numbers in superscript indicate residue positioning relative to each protein sequence. Distances between amino acids are indicated in parentheses.

**Thermolysin**

H$^{42}$ - E$^{43}$ - L - T - H$^{146}$ - [20] - E$^{166}$

**Bacterial Elastase**

H$^{40}$ - E$^{41}$ - V - S - H$^{144}$ - [20] - E$^{164}$

**Neutral Protease**

H$^{43}$ - E$^{44}$ - L - T - H$^{147}$ - [20] - E$^{167}$

EP24.15

H$^{73}$ - E$^{74}$ - F - G - H$^{77}$ - [20] - E$^{97}$ - [5] - E$^{502}$ - [35] - F$^{537}$

EP24.11

H$^{583}$ - E$^{584}$ - I - T - H$^{587}$ - [20] - T$^{607}$ - [9] - E$^{516}$ - [30] - E$^{646}$

ACE

H$^{529}$ - E$^{696}$ - M - G - H$^{693}$ - [20] - E$^{983}$ - [4] - E$^{878}$

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**Active Site-directed Inhibitor Binding Assay**—Binding of the competitive, specific active site-directed inhibitor, cFP-AAP-pAB (12), was quantitatively determined in triplicate for all mutant and wild type enzymes using a modification of the method of Shrimpton et al. (44).

Briefly, 2 μg of enzyme was incubated for 30 min at 37 °C with 10 nM cFP-AAP-pAB containing 20,000 cpm of cFP-AA$^{125}$I-Y-pAB in a final buffer volume of 200 μl (0.3 mM DTT, 5 mM Sørensen’s buffer, pH 7.2). Iodinated inhibitor was prepared via the ODO-GEN method (45). Following incubation, an equal volume of hydroxyapatite resin, prepared and equilibrated as described previously (44), was added to the reaction mixture and then placed immediately on ice for 30–45 min with frequent mixing to facilitate maximal protein binding to the resin. Each sample was subsequently centrifuged at 6500 × g for 5 min, the supernatant decanted, and the pellet washed in 350 μl of the above buffer. This process was repeated a further two times. Total radioactive counts were determined in both the pellet and supernatants (3 supernatants) collected for each sample using a Packard Multi-Priam 4 gamma counter (Meriden, CT). Percentage inhibitor binding was calculated as (cpm$^{125}$I in pellet + supernatant) × 100. Nonspecific inhibitor binding was estimated by replacing EP24.15 in the binding assay with a molar equivalent of BSA (bovine serum albumin) and was found to be less than 2%.

**Protein Determination**—Protein was quantitated according to the method of Bradford (46) with BSA as standard.

**RESULTS**

**Sequence Alignment with Bacterial Homologues**—Computer-aided homology searching between functional domains of EP24.15 with those of thermolysin, bacterial elastase, and neutral protease, bacterial homologues previously solved by x-ray crystallography, reveals conserved structural and catalytic elements. These include an HEXXH motif conserved within an active α-helix (His$^{472}$, Glu$^{474}$, and His$^{477}$ in EP24.15, equivalent to His$^{142}$, Glu$^{144}$, and His$^{146}$ in thermolysin), and a glutamate residue, 20 amino acids carboxyl to this motif (Glu$^{497}$ in EP24.15, equivalent to Glu$^{666}$ in thermolysin) (Fig. 1). A C-terminal four-helix bundle is also conserved among these metalloenzymes. Site-directed mutagenesis of the cDNA encoding rat EP24.15 (32) was subsequently employed to prepare mutants in which the above histidine and glutamate residues were genetically substituted. Two additional glutamate residues carboxyl to the HEXXH motif, Glu$^{502}$ and Glu$^{537}$, were also mutated. The close proximity of Glu$^{502}$ to Glu$^{527}$, coupled with the earlier identification of Glu$^{537}$ as the third zinc ligand in EP24.11 (equivalent to Glu$^{646}$ in EP24.11) (30), warranted their investigation as putative third zinc ligands.

**Site-directed Mutagenesis and EP24.15 Expression**—Following expression and affinity purification, wild type and all mutants were subjected to native PAGE under reducing conditions.
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Comparison of EP24.15 Wild Type and Mutant Binding to cFP-AAY-pAB—The importance of both the HEXXH motif and the third zinc-coordinating ligand to inhibitor binding is clearly evident (Table III). Substitution of His573 and His577 for either alanine or glutamine completely eliminates inhibitor binding. Similarly, replacement of Glu474 with either aspartate or glutamine reduces inhibitor binding by 100 and 80%, respectively. A complete loss of binding was also noted when Glu502 was substituted for either aspartate or glutamine, whereas substitution of Glu537 (equivalent to Glu646 in EP24.11) for a glutamate residue had virtually no effect, despite reduced activity with this mutation. Of particular interest was the observation of enhanced inhibitor binding when Glu497 was substituted for either aspartate or glutamine (154 and 197%, respectively), a finding that mirrors the increased QFS hydrolizing activity noted above with mutations at this position.

Effect of ZnCl2 on EP24.15 Wild Type and Mutants—The possibility that some of the mutations examined may lower the affinity of the active site for the catalytically essential zinc was investigated. EP24.15 wild type and mutants displaying a substantial decrease in catalytic and inhibitor binding capacity (i.e. substitutions to His473, Glu474, His477, and Glu502) were further subjected to analysis by circular dichroism spectroscopy, a technique that is sensitive to detecting net changes (~2%) in secondary structure (47). No significant alteration in the CD spectra of any of these mutants relative to that of EP24.15 wild type was observed (Fig. 2, panel B).

Kinetic Analyses of EP24.15 Wild Type and Mutants—The effects of individual mutations on EP24.15 catalytic activity were assessed using the substrates GnRH1–9 and QFS (Table II). Complete abolition of EP24.15 activity was observed with both substrates when different substitutions were made for His473, Glu474, and His477. In addition, of the three glutamates (Glu497, Glu502, and Glu537) postulated to function as the third zinc ligand, only Glu502 demonstrated a complete abolition of activity when substituted with either aspartate or glutamine. By contrast, similar substitutions for Glu497, only five residues away, substantially increased the catalytic efficiency of the enzyme relative to wild type for QFS but not GnRH1–9. Substitution of Glu537 for a glutamine residue demonstrated some activity losses with both substrates.

Effect of Metal-chelating Agents on EP24.15 Wild Type Binding to cFP-AAY-pAB—The highly potent, specific active site-directed inhibitor, cFP-AAY-pAB, was employed in this study to probe the three-dimensional integrity of the catalytic site in EP24.15 wild type and mutants. Orlowski and co-workers (12) have previously shown that the iodinated AAY inhibitor displays very similar inhibition kinetics ($K_i = 24$ nm) with the non-iodinated AAF and AAY species ($K_i = 27$ and 16 nm, respectively). These researchers have also demonstrated that the carboxyl group of the N-(1-carboxy-3-phenylpropyl) moiety of this inhibitor specifically coordinates with the active site zinc of EP24.15 (12). Based on this information, we specifically addressed the requirement of an active site zinc in EP24.15 for inhibitor binding. Assays were subsequently performed in which EP24.15 wild type was preincubated for 30 min with various metal-chelating agents to remove active site zinc. Results indicate a drastic, dose-dependent decrease of inhibitor binding following enzyme pretreatment with 1 and 2 mM 1,10-phenanthroline. Pretreatment with 10 mM EDTA also reduced inhibitor binding by over 85% (Fig. 3). These effects could be reversed following reincubation of EP24.15 with 1 mM ZnCl2. These results confirm the dependence of inhibitor binding on the presence of an active site zinc as previously suggested (12). Consequently, this binding assay serves as a useful tool for assessing the effects of specific point mutations postulated to alter the ability of EP24.15 to coordinate properly the catalytically essential active site zinc.

Comparison of EP24.15 Wild Type and Mutant Binding to cFP-AAY-pAB—The importance of both the HEXXH motif and the third zinc-coordinating ligand to inhibitor binding is clearly evident (Table III). Substitution of His573 and His577 for either alanine or glutamine completely eliminates inhibitor binding. Similarly, replacement of Glu474 with either aspartate or glutamine reduces inhibitor binding by 100 and 80%, respectively. A complete loss of binding was also noted when Glu502 was substituted for either aspartate or glutamine, whereas substitution of Glu537 (equivalent to Glu646 in EP24.11) for a glutamate residue had virtually no effect, despite reduced activity with this mutation. Of particular interest was the observation of enhanced inhibitor binding when Glu497 was substituted for either aspartate or glutamine (154 and 197%, respectively), a finding that mirrors the increased QFS hydrolizing activity noted above with mutations at this position.

Effect of ZnCl2 on EP24.15 Wild Type and Mutants—The possibility that some of the mutations examined may lower the affinity of the active site for the catalytically essential zinc was investigated. EP24.15 wild type and mutants displaying a substantial decrease in catalytic and inhibitor binding capacity (i.e. substitutions to His473, Glu474, His477, and Glu502) were further examined by addition of ZnCl2 over a broad concentration range (0.001–10 mM), via a discontinuous QFS assay.3 Results demonstrated that activity of the wild type enzyme was unaffected after zinc was added back. Concentrations ≥0.5 mM ZnCl2, however, tended to inhibit the enzyme, a finding previously reported by Orlowski and co-workers (7) while studying the native rat testes enzyme. Furthermore, the mutants tested demonstrated no significant activation following incubation with zinc over this concentration range. Any direct effect of ZnCl2 on product (Mca-Pro-Leu) fluorescence was also tested in these experiments and found to be non-existent.

DISCUSSION

Coordination of a zinc ion within the catalytic site of metalloenzymes is typically affected by an HEXXH motif, a third zinc ligand distantly located carboxyl to this motif, and a water molecule. Previous studies with zinc metalloendopeptidases isolated from bacterial (1, 25–28, 48, 58, 59) and mammalian (29, 49) species have all identified a similar role for the histidine residues within this motif, to coordinate directly to the active site zinc ion, whereas the HEXXH glutamate has been shown to coordinate weakly to zinc via an activated water

3 P. J. Crack, T. J. Wu, P. M. Cummins, J. W. Tullai, E. S. Ferro, M. J. Glucksman, and J. L. Roberts, submitted for publication.
molecule, thus facilitating the acid-base catalytic mechanism. The absolute requirement for EP24.15 function of both an HEXH motif and a glutamate 25 residues distant is evident from these investigations. Consequently, we propose similar roles for His473, Glu 474, and His 477 in EP24.15. We further propose a role for Glu 502 as the third zinc ligand in EP24.15. This differs from the expected candidate, Glu 497, which is highly conserved in the bacterial metalloendopeptidases thermolysin, bacterial elastase, and neutral protease.

In support of this hypothesis is the following experimental evidence. (i) Replacement of any one of the aforementioned residues (see Table I) results in ablation of enzyme activity with both GnRH$_{1-9}$ and QFS utilized as substrates. (ii) These substitutions are also characterized by a drastic reduction in the ability of EP24.15 to bind the active site-directed inhibitor, cFP-AAY-pAB. We have demonstrated in this study that binding of this inhibitor is dependent on the presence of zinc within the catalytic site of EP24.15, in agreement with previous observations by Orlowski and co-workers (12). The loss of inhibitor binding in these instances, however, may also reflect a distortion of the zinc coordination sphere following substitution of a metal ligand. Previous studies with the ACE inhibitors, enalaprilat and trandolaprilat, for example (also cFP-related inhibitors), demonstrate the importance of both zinc presence and coordination geometry to site-directed inhibitor binding (31, 50, 51, 57). In this regard, Lesburg et al. (56) have also clearly demonstrated how substitution of a zinc-coordinating ligand in the metalloenzyme carbonic anhydrase II alters zinc coordination geometry with subsequent detrimental effects on catalytic and inhibitor binding properties. (iii) The results obtained were similar for both substitutions employed (Glu to Asp/Gln, His to Ala/Gln). Amino acid substitutions were chosen which attempted to preserve side chain charge and/or space-filling conformation in order to minimize substantial three-dimensional changes to the mutant protein. Glutamates were therefore substituted for either aspartate (side chain length reduction by 1.5 Å) or glutamine (loss of the negative charge but retaining space-filling geometry). Histidines were substituted for either an alanine or glutamine residue. Replacement of histidine for alanine is frequently employed in scanning
Mutagenesis to assess the putative function of histidine residues within a protein (52), whereas replacement with glutamine is a common occurrence in nature, reflecting the similarity of the two residues in terms of hydrophilic and hydrophobic properties as well as partial specific volume and accessible surface area (53).

With respect to the proposed third zinc ligand, one notes that Glu502 is located further carboxyl to the HEXXH motif in EP24.15 (25 residues) than its counterpart in thermolysin (20 residues) or, indeed, the other bacterial homologues (see Fig. 1). However, the sequences of EP24.15 and the bacterial metalloendopeptidases also reveal a highly conserved DXXH motif, located equidistant to the glutamate third zinc ligand (Asp561-XXX-His565) in EP24.15 numbering. In thermolysin, these residues have been shown to form hydrogen bonds with the incoming substrate, thereby stabilizing the hydrated peptide in the transition state (54). Consequently, one would expect the spatial position of these two residues relative to the third zinc ligand to be crucial to catalysis. Assuming a similar function for these residues in EP24.15 (currently under investigation), it is significant, therefore, that the amino acid spacing between Glu502 and Asp561/His565 in EP24.15 is virtually identical to that observed (Glu497 and Asp229/His311) in thermolysin.

A previous report by Le Moual and co-workers (30) shows that mutation of the glutamate third zinc ligand in EP24.11 (Glu466) to an aspartate residue results in a marked but incomplete decrease in catalytic activity. The present study however, demonstrates a complete ablation of activity following an identical substitution of the proposed glutamate third zinc ligand in EP24.15 (Glu502). The greater effect on the catalytic activity of this mutation in EP24.15 compared with EP24.11 can most likely be explained in terms of the distance between the second and third zinc ligands. In EP24.15, this distance is 25 amino acid residues compared with 59 in EP24.11. It has been postulated that the length of this spacer sequence is directly related to the flexibility of the zinc coordination sphere (55). Based on this hypothesis, therefore, one would expect the coordination sphere to be less flexible in EP24.15 compared with EP24.11, subsequently reflecting more rigid requirements for the spatial positioning of the active site zinc. Similar observations have also been reported for human ACE by Williams and co-workers (31). This latter research group reports that substitution of the glutamate third zinc ligand in the ACE carboxyl domain (Glu987 to Asp987) virtually eliminates enzyme activity. Interestingly, Glu987 in ACE is located a distance of 24 residues carboxyl to the HEXXH motif (compared with the 25 amino acid spacing between Glu502 and His477 in EP24.15).

The possibility that the results observed with any of the substitutions examined stems from global alterations to the protein folding pattern is unlikely. (i) All of the proteins examined were depicted with an equivalently high level and exhibit an identical native PAGE migration profile, indicative of normal protein folding. (ii) CD analysis of selected mutants displaying substantial alterations in catalytic and inhibitor binding properties (i.e. substitutions to His473, Glu474, His477, and Glu502) indicated no significant change in their global secondary structure relative to EP24.15 wild type. (iii) Other mutations introduced in the same area of the EP24.15 molecule, such as the substitution of Val468 for either alanine or leucine, had no significant effect on either the enzymes Km for GnRH1–9 or its ability to bind cFF-pAAY-pAB.4 (iv) Substitution of Glu497 generates EP24.15 mutants (E497D, E497Q) displaying increased QFS-hydrolyzing efficiency and inhibitor binding capacity.

The possibility that mutations causing a decrease in catalytic and inhibitor binding capacity may lower the affinity of the active site for the catalytically essential zinc was also investigated in these studies. Both EP24.15 wild type and mutants with substitutions to His473, Glu474, His477, and Glu502 amino acids that comprise the proposed zinc coordination model, were subsequently examined by addition of ZnCl2 over a broad concentration range (0.001–10 mM) via QFS assay. Results demonstrated that activity of the wild type enzyme was unaffected after zinc was added back, indicating no significant metal loss during the expression/purification process. Furthermore, the mutants tested demonstrated no significant activation following incubation with zinc over this concentration range, suggesting a reduction in affinity of the metal for the active site. These observations are consistent with a previous study by Lesburg et al. (56) who demonstrate that substitution of any one of the zinc-coordinating ligands in the metalloenzyme carbonic anhydrase II drastically reduces catalytic and inhibitor binding capacity, in parallel with reduced affinity of the enzyme active site for zinc. Similar findings have also been reported by Le Moual and co-workers (30) while identifying the third zinc ligand in the metalloenzyme, EP24.11. One also notes that in both of these latter studies, zinc is still bound within the active site, although improperly coordinated.

As mentioned previously, mutation of Glu987 to either glutamine or aspartate increases both QFS-hydrolyzing efficiency and inhibitor-binding capacity. By contrast, these mutations had relatively minor effects on GnRH1–9 catalysis. Given the close proximity of Glu987 to Glu502, these results may possibly be explained by a small distortion in the geometry of the zinc-coordinating residues (or possibly a loop of undetermined secondary structure), subsequently improving accessibility of the catalytic zinc and/or activated water molecule to an incoming substrate (QFS) or inhibitor (cFF-pAAY-pAB) moiety. The lack of significantly increased GnRH1–9 hydrolysis in this instance may reflect subtle differences in its interaction with catalytic subsites. Minor alterations to the active site tertiary structure may also explain the reduced activity observed when Glu537 was mutated to glutamine. The normal levels of inhibitor binding observed with this substitution suggest that zinc coordination is unaffected. It is possible, however, that this mutation induces a conformational change that alters the distance between the bound substrate and the aforementioned Asp561/His565 residues (postulated to stabilize the transition state

4 P. M. Cummins, A. Pabon, and M. J. Glucksman, unpublished observations.
complex by forming hydrogen bonds with a carboxyl oxygen), thus reducing the hydrolitic efficiency of the enzyme.

In conclusion, the spatial organization of the proposed zinc-coordinating ligands in EP24.15 (Fig. 4) is consistent with observations for other zinc metalloenzymes (1) that a zinc-binding nucleus is provided by two ligands (His$^{473}$ and His$^{477}$) separated by a 1–3-amino acid spacer. A third zinc ligand (Glu$^{505}$), separated from the second by a distance of 20–120 amino acids, completes the coordination sphere. A fourth residue (Glu$^{474}$), located between the first and second ligands, subsequently provides a hydrogen-bonded water molecule that weakly coordinates via oxygen to the active site zinc ion, thereby creating a nucleophilic center for catalysis. The observations made in this investigation are indicative of the absolute requirement of these residues to binding and catalysis, although definitive interpretations await the availability of the solution of the enzyme structure by x-ray diffraction.

EP24.15 is one of the most extensively characterized soluble endopeptidases with respect to physiological regulation and the neural environment. We report here an integrated approach of structural, genetic, and biochemical methodologies to assess the effects of specific point mutations on EP24.15 function. Unlike similar investigations with other mammalian zinc metalloendopeptidases that address the functionality of one or two residues, this study details the comprehensive examination of six residues postulated to function in EP24.15 active site zinc coordination. Each residue under investigation has been examined via two appropriate amino acid substitutions, the structural integrity of all enzymes subsequently assessed by native PAGE and CD spectroscopy, and their catalytic efficiencies determined with multiple substrates. Furthermore, the radiiodinated, site-directed inhibitor, cFP-AAY-pAB, serves as a useful, zinc-sensitive probe to evaluate the binding efficiency of each mutant. Using this strategy, further experimentation is currently underway to identify other functional residues within the active site of EP24.15. This will be coupled with information gleaned from ongoing experiments to determine the three-dimensional structure of the enzyme. A detailed understanding of the molecular architecture of EP24.15 will lead to a more complete understanding of the mechanism of substrate binding and the action of site-directed inhibitors as well as providing a useful template for the rational design of pharmacological agents for this prototypical member of the mammalian family of zinc metalloendopeptidases.

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