Rat endothelin-converting enzyme-1 forms a dimer through Cys^{412} with a similar catalytic mechanism and a distinct substrate binding mechanism compared with neutral endopeptidase-24.11

Kohei SHIMADA*, Masaaki TAKAHASHI*, Anthony J. TURNER† and Kazuhiko TANZAWA*‡

*Biological Research Laboratories, Sankyo Co. Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan, and †Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Endothelin-converting enzyme-1 (ECE-1) is involved in the conversion of big endothelins (big ETs) into endothelins (ETs) and shows sequence similarity with neutral endopeptidase-24.11 (NEP). Unlike NEP, ECE-1 exists as a disulphide-linked dimer. Here we reveal that Cys^{412} is solely responsible for the dimerization of rat ECE-1. The C412S mutant enzyme, which existed as a monomer, showed no difference in glycosylation level, subcellular localization or clustering structure formation, but showed a higher K_m and lower k_{cat} for big ET-1 compared with the wild-type enzyme. These results indicate that dimerization of ECE-1 is preferential for effective conversion of big ETs into ETs. In addition, complete loss of activity in the mutants E592Q, E651Q and H716Q confirmed that these residues are responsible for catalytic activity, zinc binding and stabilization of the intermediate during the transition state respectively. In contrast, the catalytic properties of mutant enzymes containing a substitution at Arg^{129} or Glu^{752} were not markedly different from those of the wild-type enzyme, suggesting that these residues play only a minor role, if any, in substrate binding, in contrast with their role in NEP.

INTRODUCTION

Endothelins (ETs) are potent vasoconstrictive 21-amino-acid peptides originally isolated from the supernatant of cultured porcine aortic endothelial cells [1]. ETs are generated by a unique proteolytic cleavage of big endothelins (big ETs) at the Trp^{21}–Val^{22} or Trp^{21}–Ile^{22} bond catalysed by an endothelin-converting enzyme (ECE) [2].

Purification and cDNA cloning studies of rat ECE-1 [3,4], which was subsequently defined as ECE-1α [5,6], revealed it as a highly glycosylated type II membrane-bound neutral metalloproteinase. ECE-1 has two isoforms, designated ECE-1α and ECE-1β, with different N-terminal amino acid sequences which are probably produced by alternative splicing; the isoforms show similar enzymic properties, tissue distribution and subcellular localization [5]. More recently, ECE-2 cDNA has been cloned and characterized [7]. It is also a membrane-bound metalloproteinase which is structurally related to ECE-1. Both ECE-1 and ECE-2 show sequence similarity to neutral endopeptidase-24.11 (NEP) and Kell blood group protein over the whole sequence [4,7], and all of these proteins are contained in a subfamily of the M13 protein family [8]. Since 10 out of the 14 cysteine residues in ECE-1 are conserved in NEP, it is suggested that ECE-1 shows sequence similarity to NEP not only in primary but also in tertiary structure.

Although the primary and tertiary structures of ECE-1 seem to be similar to those of NEP, the substrate and inhibitor specificity of ECE-1 is much more limited than that of NEP. Thiorphan inhibits NEP but not ECE-1. Phosphoramidon inhibits both ECE-1 and NEP, but its IC_{50} value for ECE-1 is approximately three orders of magnitude greater than that for NEP. In the case of NEP, many amino acid residues involved in zinc binding or substrate binding have been identified and characterized using site-directed mutagenesis. It was revealed that Glu^{584} participates in the catalytic mechanism and that His^{583}, His^{587} and Glu^{646} are the three ligands for the zinc atom [9,10]. Asp^{650} is also crucial for hydrolytic activity [11]. His^{711} was shown to be involved in the stabilization of the tetrahedral intermediate during the transition state [12]. In addition, Arg^{102} and Arg^{747} are responsible for substrate and inhibitor binding [13,14]. Almost all of these amino acid residues are conserved in rat ECE-1α, i.e. Glu^{584}, His^{583}, His^{587}, Glu^{646}, Asp^{650}, His^{711} and Arg^{102} in NEP correspond to Glu^{592}, His^{591}, His^{595}, Glu^{651}, Asp^{655}, His^{716} and Arg^{129} respectively in ECE-1. However, Arg^{747} in NEP is replaced by a residue of opposite charge (Glu^{752}) in ECE-1.

It has been reported that NEP forms a dimeric structure in the kidney [15], but its electrophoretic mobility is the same under either reducing or non-reducing conditions. On the other hand, the molecular mass of ECE-1 was estimated to be 130 kDa under reducing conditions, but 300 kDa under non-reducing conditions, suggesting that ECE-1 is composed of two disulphide-linked subunits [16,17]. This is further confirmed by cross-linking of ECE-1 from rat lung microsomes [16].

In the present study, mutations were introduced in amino acid residues in ECE-1 which correspond to the sites for catalytic activity, zinc binding or substrate binding in NEP, and the enzymic properties of these mutant enzymes were investigated. Mutations were also introduced to identify the cysteine residue(s) responsible for the formation of the dimeric structure of ECE-1 and to investigate the function of the dimeric structure in ECE-1 activity.

EXPERIMENTAL

Site-directed mutagenesis and vector construction

For site-directed mutagenesis, a 2.5 kb *Bam*HI–*Bam*HI fragment containing the full-length rat ECE-1α coding sequence was

Abbreviations used: ECE, endothelin-converting enzyme; ET, endothelin; NEP, neutral endopeptidase-24.11.

‡ To whom correspondence should be addressed.

excised from the rat ECE-1α expression vector pcDL-SRα296/rECE-1α [4,5] and subcloned into the *Bam*HI site of M13mp18 RF DNA. Site-directed mutagenesis was performed according to the method described by Taylor et al. [18] using the Sculptor™ *in vitro* mutagenesis system (Amersham). Recombinant M13-ECE-1 phages carrying the mutations were screened directly by dideoxy sequencing [19]. *Bam*HI–*Bam*HI fragments of 2.5 kb containing the mutated region were substituted for the equivalent non-mutated fragment in pcDL-SRα296/rECE-1α [4]. The presence of the mutation in each expression vector was confirmed by sequencing of the mutated region.

Transfection of COS-1 cells and assay of ECE-1

COS-1 cells were transfected with the wild-type or a mutated cDNA by electroporation (600 V, 30 μs; two times) and cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. At 60 h after transfection, the cells were collected, washed with PBS and homogenized in a buffer consisting of 20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM PMSF, 20 μM pepstatin A and 20 μM leupeptin. The homogenates were centrifuged at 100000 ***g*** for 45 min. The membrane fractions thus obtained were solubilized with Triton X-100 as described previously [4]. The enzyme reaction was carried out for 2 h at 37 °C in 100 μl of assay mixture consisting of 100 mM Tris/HCl (pH 7.0), 0.1% (w/v) BSA, 0.1 mM PMSF, 20 μM pepstatin A, 20 μM leupeptin, various amounts of human big ET-1, and the solubilized membrane protein. For the determination of K_m and k_{cat} values, human big ET-1 was used at concentrations of 0.33, 0.5, 0.67, 1, 2 and 5 μM. The reaction was terminated by the addition of 100 μl of 5 mM EDTA, and the product ET-1 was determined by sandwich ELISA [20].

Immunological analysis

The membrane fractions obtained from COS-1 cells transfected with wild-type or mutant ECE-1 cDNA were boiled with SDS/PAGE sample buffer [21] in the presence or absence of 1% β-mercaptoethanol, and separated on a 4–20% SDS/polyacrylamide gel. The membrane fractions were also separated on a 2–15% native polyacrylamide gel [22]. Immunoblot analysis using the anti-(rat ECE-1) monoclonal antibody, AEC27-121 [4,16], was carried out with the enhanced chemiluminescence detection system (Amersham) as described previously [16], and the immunoreactivity in each sample was determined by measurement of chemiluminescence using the ARGUS system (Hamamatsu Photonics) using purified rat lung ECE-1 as a standard [3].

Indirect immunofluorescent analysis was performed as described previously [16]. Briefly, COS-1 cells transfected with either wild-type or C412S mutant ECE-1 cDNA were cultured on glass coverslips for 48 h as described above. The cells were fixed with formaldehyde, quenched with glycine, and incubated in saponin for permeabilization. The samples were blocked with normal goat serum and then incubated with AEC27-121, washed in PBS, and finally incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Samples were viewed under a confocal fluorescence microscope and photographed.

RESULTS

Substitution of amino acid residues responsible for catalytic activity

In the case of NEP, several amino acid residues involved in catalytic activity have been identified and characterized. Of these, Glu^{584}, Glu^{646} and His^{711} are responsible for catalytic activity, zinc binding and stabilization of the transition state during catalysis respectively. These are conserved as Glu^{592}, Glu^{651} and His^{716} respectively in ECE-1 (Figure 1). In order to clarify the roles of these amino acid residues in the enzymic activity of ECE-1, plasmids for E592Q, E651Q and H716Q mutant enzymes were constructed and expressed in COS-1 cells. Immunoblot analysis of the solubilized membrane fractions prepared from these cells showed that each mutant enzyme had the same apparent molecular mass as the recombinant wild-type enzyme, and thus appeared to be similarly glycosylated (results not shown). All of these mutant enzymes (E592Q, E651Q and H716Q) exhibited a complete loss of ECE-1 activity (results not shown). These results indicate the essential roles of these residues for ECE-1 activity, as in the case of NEP.

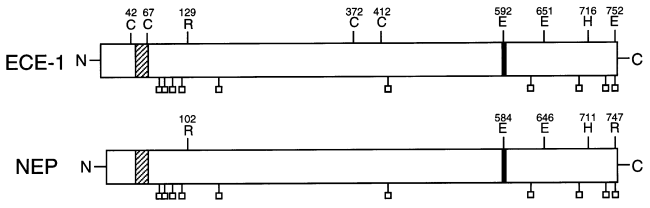

Figure 1 Schematic representation of the primary structures of ECE-1 and NEP

Single-letter codes indicate amino acid residues in ECE-1 mutated in this study (upper) and corresponding amino acids residues in NEP (lower). Cysteine residues conserved in both ECE-1 and NEP are indicated by open squares. The closed box indicates a zinc binding motif, and the hatched box indicates a deduced transmembrane domain.

Table 1 Kinetic parameters of wild-type and mutant ECE-1, and wild-type and mutant NEP

The NEP data are from [14]. Specific activities were determined at a concentration of 0.1 μM big ET-1. For IC_{50} determinations, the inhibitor was phosphoramidon (μM) for ECE-1 or thiorphan (nM)* for NEP.

Enzyme	Specific activity (nmol/h per mg)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	IC_{50} (μM or nM*)
Wild-type ECE-1	156 ± 6.3	0.55 ± 0.06	1.50 ± 0.22	2.73	0.69 ± 0.07
R129Q ECE-1	264 ± 9.5	1.68 ± 0.26	7.12 ± 0.58	4.24	1.34 ± 0.12
E752Q ECE-1	134 ± 6.7	0.93 ± 0.11	2.18 ± 0.12	2.34	1.28 ± 0.25
E752D ECE-1	110 ± 1.1	1.40 ± 0.23	3.12 ± 0.18	2.23	1.55 ± 0.35
E752R ECE-1	92 ± 9.5	0.87 ± 0.12	1.26 ± 0.08	1.45	1.08 ± 0.30
Wild-type NEP	–	35 ± 6	35.7 ± 8.5	1.02	2.1 ± 1.2*
R102M NEP	–	210 ± 15	30.1 ± 9.4	0.13	13.5 ± 1.8*
R747M NEP	–	105 ± 6	27.3 ± 7.5	0.29	8.5 ± 1.8*

Substitution of amino acid residues corresponding to substrate binding sites in NEP

In the case of NEP, it is reported that two arginine residues are located at the active site and participate in substrate binding. Thus Arg^{102} binds to the free carboxy group of a P2′ residue, which explains the dipeptidylcarboxypeptidase activity, and Arg^{747} binds to the carbonyl amide group of the P1′ residue, which explains the endopeptidase activity [14]. Alignment of NEP and ECE-1 revealed that Arg^{102} in NEP is conserved as Arg^{129} in ECE-1, whereas Arg^{747} in NEP is replaced in ECE-1 by

Table 2 Substrate specificities of wild-type and R129Q ECE-1

Specific activities were determined at a concentration of 0.1 μM big ETs. Values in parentheses indicate the activity as a percentage of that with big ET-1 as substrate.

Enzyme	Specific activity (nmol/h per mg)		
	Big ET-1	Big ET-2	Big ET-3
Wild-type	156 ± 6.3 (100%)	38 ± 0.7 (24%)	74 ± 0.9 (47%)
R129Q	264 ± 9.5 (100%)	62 ± 1.0 (23%)	119 ± 19.1 (45%)

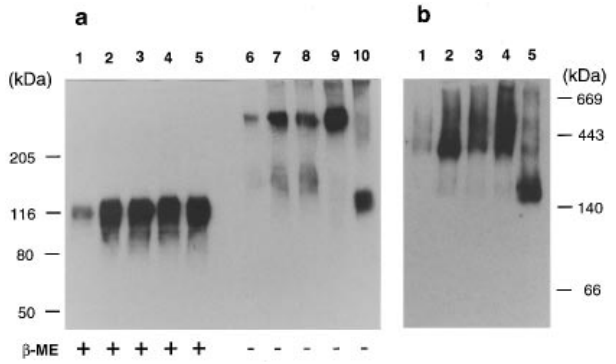

Figure 2 Immunoblot analyses of wild-type and cysteine mutant enzymes

(**a**) Immunoblot of 5 μg portions of membrane fractions obtained from COS-1 cells transfected with wild-type (lanes 1 and 6), C42S (lanes 2 and 7), C67S (lanes 3 and 8), C372S (lanes 4 and 9) and C412S (lanes 5 and 10) ECE-1 cDNA. The samples were boiled in SDS gel sample buffer in the presence (+) or absence (−) of β-mercaptoethanol (β-ME), and were subjected to SDS/PAGE and analysed by immunoblotting using AEC27-121. (**b**) Immunoblot of 5 μg portions of membrane fractions obtained from COS-1 cells transfected with wild-type (lane 1), C42S (lane 2), C67S (lane 3), C372S (lane 4) and C412S (lane 5) ECE-1 cDNA. The samples were subjected to native PAGE (2–15%) and analysed by immunoblotting using AEC27-121.

Glu^{752}, a residue of opposite charge (Figure 1). We hypothesized that this replacement might explain the difference in substrate specificity between ECE-1 and NEP. To clarify the roles of these amino acid residues in ECE-1 substrate binding, plasmids for R129Q, E752Q, E752R and E752D mutant enzymes were constructed and expressed in COS-1 cells.

R129Q showed similar specific activities to those of the wild-type enzyme (Table 1). Both the K_m and k_{cat} were increased 3–5-fold compared with those of the wild-type, but the degree of change in k_{cat}/K_m was small compared with that seen in the case of the R102Q NEP or R102M NEP mutant enzymes [13,14]. The substrate specificity of R129Q was unchanged from that of the wild-type enzyme, i.e. it preferred big ET-1 to big ET-2 and big ET-3 (Table 2).

For the three mutations of Glu^{752}, the kinetic parameters did not change markedly in comparison with those of the wild-type enzyme. Although the E752R mutant has an arginine residue at this site, as in NEP, it neither had NEP activity nor showed sensitivity to thiorphan of the ECE-1 activity as in the case of the wild type (results not shown). Similar results were obtained with the other mutant enzymes. The IC_{50} values for phosphoramidon of all mutant enzymes were approximately 2-fold higher than that of the wild-type enzyme. The substrate specificities of these mutant enzymes towards big ET-1, -2 and -3 were also unchanged from that of the wild-type enzyme (results not shown).

Table 3 Kinetic parameters of cysteine mutants of ECE-1

Specific activities were determined at a concentration of 0.1 μM big ET-1. IC_{50} values were determined with phosphoramidon as inhibitor. N.D., not determined.

Enzyme	Specific activity (nmol/h per mg)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min$^{-1}\cdot\mu$M^{-1})	IC_{50} (μM)
Wild type	156 ± 6.3	0.55 ± 0.06	1.50 ± 0.22	2.73	0.69 ± 0.07
C42S	179 ± 5.2	N.D.	N.D.	N.D.	N.D.
C67S	150 ± 2.6	N.D.	N.D.	N.D.	N.D.
C372S	150 ± 5.0	N.D.	N.D.	N.D.	N.D.
C412S	32 ± 1.1	2.91 ± 0.48	0.96 ± 0.16	0.33	3.31 ± 0.60

ECE-1 forms a dimeric structure through Cys^{412}

From the alignment of the amino acid sequences of ECE-1 and NEP, we speculated that ECE-1 forms a dimer through at least one of the four cysteine residues Cys^{42}, Cys^{67}, Cys^{372} and Cys^{412}, which are not conserved in NEP (Figure 1). Therefore four mutant plasmids for C42S, C67S, C372S and C412S were constructed by site-directed mutagenesis and expressed in COS-1 cells. Solubilized membrane fractions prepared from cells expressing the wild-type or mutant enzymes were analysed by immunoblotting. When these fractions were electrophoresed under non-reducing conditions, only the C412S mutant enzyme appeared to have a molecular mass of 130 kDa, whereas the wild-type and three other mutant enzymes appeared to be of molecular mass 300 kDa (Figure 2a). The same result was obtained when these fractions were analysed by native PAGE (Figure 2b). Under reducing conditions with 1% β-mercaptoethanol, a 130 kDa immunoreactive protein was observed in all fractions. These results clearly indicate that only Cys^{412} contributes to the formation of the disulphide bond required for the dimeric structure of ECE-1.

The specific activity of C412S was 5-fold lower than that of wild-type enzyme, whereas those of C42S, C67S and C372S were similar to that of the wild type at a substrate concentration of 0.1 μM (Table 3). Further kinetic analyses revealed that the K_m for big ET-1 of C412S was 5-fold higher than that of the wild-type enzyme, and the k_{cat} of C412S was half of that of the wild-type enzyme, resulting in a 8.5-fold lower k_{cat}/K_m value than that of the wild type. The IC_{50} for phosphoramidon of C412S was 5-fold higher than that of the wild-type enzyme.

From confocal microscopic observations, we previously reported that most ECE-1 was found to be localized on the plasma membrane, with a large clustering structure in endothelial cells and COS-1 cells transfected with ECE-1 cDNA [16]. When COS-1 cells transfected with either wild-type or C412S mutant cDNA were observed by confocal microscopy, both the wild-type and C412S mutant enzymes mainly clustered along the plasma membrane, and no significant difference was detected (Figure 3).

DISCUSSION

In the present study we have identified and characterized several amino acid residues responsible for catalytic or structural features of ECE-1. Mutation of Glu^{592}, Glu^{651} or His^{716} in ECE-1 resulted in a complete loss of ECE-1 activity, as in the case of NEP. Glu^{592} in the consensus zinc binding motif HEXXH is the most important residue for catalytic activity, and two histidine residues around Glu^{592} act as zinc co-ordinating amino acids. The third

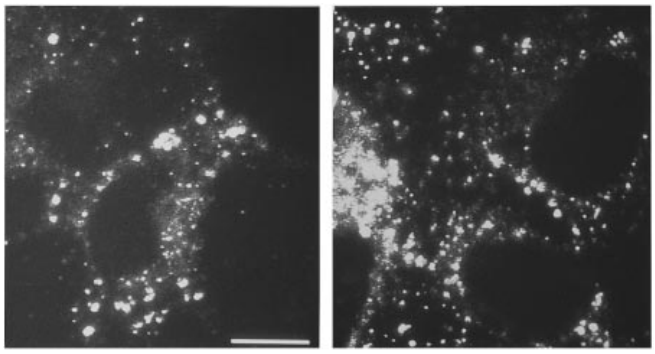

Figure 3 Subcellular localization of ECE-1 by confocal laser immunofluorescent analysis

COS-1 cells transfected with wild-type (left) or C412S (right) ECE-1 cDNA were fixed and permeabilized for both cell-surface and intracellular staining. Bar = 10 μm.

ligand for the zinc atom is shown to be Glu^{651}, which is equivalent to Glu^{646} in NEP. His^{716} is involved in the stabilization of the tetrahedral intermediate during the transition state. Thus the fundamental proteolytic mechanism of ECE-1 is similar to that of NEP.

Chemical modification studies, molecular modelling and site-directed mutagenesis of NEP have combined to indicate a role for Arg^{102} and Arg^{747} in substrate binding. Arg^{102} interacts with the free carboxy group of the P2′ residue of some substrates (e.g. enkephalins), explaining the peptidyldipeptidase activity of NEP, and mutations in this residue cause an 8-fold decrease in the k_{cat}/K_m [14] (Table 1). Arg^{102} in NEP is equivalent to Arg^{129} in ECE-1; however, the mutant R129Q ECE-1 exhibited only a 1.5-fold increase in k_{cat}/K_m. This change is not substantial and is in the opposite direction compared with R102Q or R102M NEP. The activity of R129Q ECE-1 increased to the same extent when big ET-2 or big ET-3 was used as a substrate, and there was no difference between the substrate specificities of the wild-type and R129Q enzymes. These results coincide well with the observations that ECE-1 does not have peptidyldipeptidase activity and that big ETs, the substrates of ECE-1, do not contain a free carboxy group in their P2′ residue. Therefore it can be concluded that Arg^{129} in ECE-1 plays little role in substrate binding.

Arg^{747} in NEP interacts with the carbonyl amide group of the P1′ residue, explaining the endopeptidase activity of this enzyme [14]. Among the amino acid residues that are important for NEP activity, only Arg^{747} is not conserved in ECE-1, where it is replaced by Glu^{752}. This led us to assume that Glu^{752} might be responsible for the unique substrate specificity of ECE-1. However, the degree of change in the enzymic properties of mutants E752Q, E752D and E752R was nowhere near as marked as that observed in the R747M NEP mutant enzyme. These results strongly suggest that Glu^{752} plays little, if any, role in substrate binding to ECE-1. Slight changes in kinetic parameters or IC_{50} values of phosphoramidon can be explained by an alteration in conformation and/or polarity caused by a substitution of the residue. Even when Glu^{752} was mutated to Arg^{752}, to mimic wild-type NEP, neither NEP activity nor sensitivity to thiorphan of ECE-1 activity was detected. Thus the substrate recognition mechanisms of ECE-1 and NEP seem to differ considerably, although the possibility remains that ECE-1 contains other arginine residue(s) playing a similar role to Arg^{747} in NEP. In addition, Arg^{129} and Glu^{752} are also conserved in bovine ECE-2 as Arg^{172} and Glu^{786} respectively, yet the reported sensitivity of ECE-2 to phosphoramidon is 250-fold higher than that of ECE-1 [7], indicating that there could also be other amino acid residues responsible for sensitivity to phosphoramidon.

ECE-1 is composed of two disulphide-linked subunits, whereas NEP forms a dimeric structure without covalent associations. The alignment of the amino acid sequences of ECE-1 and NEP suggested that four cysteine residues in ECE-1 which are not conserved in NEP are candidates for the residue(s) responsible for intra-chain disulphide linkage. Analyses using site-directed

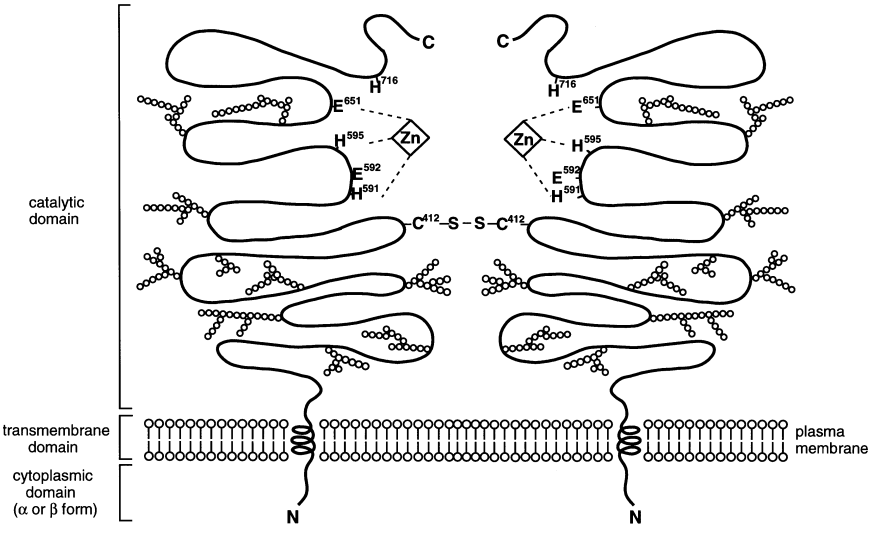

Figure 4 Molecular model of ECE-1

The large catalytic domain projects outside the plasma membrane, leaving the small N-terminal region in the cytoplasm. The enzyme forms a disulphide-linked dimeric structure through Cys^{412}, and each subunit has a zinc atom co-ordinated by three zinc ligands, His^{591}, His^{595} and Glu^{651}, in the active site pocket. Glu^{592} acts as a catalytic site, and His^{716} contributes to the stabilization of the intermediate during the transition state. There are 10 putative N-linked glycosylation sites in the extracellular domain. ECE-1 has two isoforms (α and β) which are different only in their N-terminal regions [5].

mutagenesis of these cysteine residues and subsequent immunoblotting revealed that ECE-1 forms a disulphide-linked dimeric structure through Cys^{412}. The C412S mutant enzyme was also detected as a monomeric form in native PAGE, suggesting that other bonds such as ionic or hydrophobic interactions do not contribute to the dimeric structure formation of ECE-1. Increases in both the K_m for big ET-1 and the IC_{50} value for phosphoramidon of C412S indicate that the monomerization of ECE-1 decreases its affinity for substrate and inhibitor. These results, together with the fact that the k_{cat} of C412S was decreased compared with that of wild-type enzyme, indicate that the dimeric structure of ECE-1 is preferential, although not essential, for effective conversion of big ETs into ETs. The changes in the kinetic parameters of C412S may reflect a relaxation of conformational restraint on the enzyme. The dimeric structure of ECE-1 was not required for plasmalemmal localization and clustering structure formation, as no significant difference was observed in the staining patterns of the wild-type and C412S mutant enzymes by confocal immunofluorescent microscopy. In the case of three other cysteine mutant enzymes, similar electrophoretic mobilities and specific activities to those of the wild-type enzyme were observed. These results strongly suggest that these cysteine residues are not involved in either an inter-chain disulphide bond or an intra-chain bridge in ECE-1.

Among the plasma membrane-bound metalloproteinases that occur as ectoenzymes, only a few are known to have covalent association of subunits. To date, meprin has been the only established example [23]. More recently, it was shown that membrane dipeptidase forms a dimeric structure through a single disulphide bond (S. Keynan, N.T. Habgood, N.M. Hooper and A.J. Turner, unpublished work). The present results show that ECE-1 is another disulphide-linked dimeric enzyme among plasma membrane-bound metalloproteinases.

Cys^{412} in rat ECE-1 is conserved in bovine ECE-2 (Cys^{445}), strongly suggesting that ECE-2 is also composed of two disulphide-linked subunits. Of three other cysteine residues mutated in the present study, two are not conserved in ECE-2 and one conserved residue is located in the transmembrane domain. These facts also support the idea that these cysteine residues do not contribute to inter-chain disulphide bonds. Furthermore, all of the 10 cysteine residues conserved between ECE-1 and NEP are also conserved in bovine ECE-2. At least some these residues may contribute to intra-chain covalent bonds and the conformational restraint on these enzymes.

From the results obtained in the present study, together with our previous observations [4,5,16], we propose a putative structural model for the ECE-1 molecule (Figure 4). The catalytic domain projects outside the plasma membrane of endothelial cells [16]. ECE-1 has two isoforms, designated α and β, whose sequences differ only in the N-terminal cytoplasmic tail [5]. Cys^{412} contributes to formation of the dimeric structure of ECE-1, which may exist as either a homodimer ($\alpha\alpha$ or $\beta\beta$) or a heterodimer ($\alpha\beta$) [5], through a single disulphide bond. Each subunit has a zinc atom co-ordinated by three zinc ligands (His^{591}, His^{595} and Glu^{651}) in its active pocket. His^{716} is also important for catalytic activity. There are 10 putative N-linked glycosylation sites in the C-terminal extracellular domain [4].

The model of ECE-1 presented here clarifies its fundamental proteolytic mechanism, although the mechanism of the restricted substrate specificity of ECE-1, which is distinct from that of NEP, is still unclear. Identification of the amino acid residues responsible for substrate binding of ECE-1 might be exploitable in the development of ECE-1-selective inhibitors, which might be useful for treatment of some diseases associated with augmented production of ETs.

A.J.T. thanks the British Heart Foundation for support.

REFERENCES

1 Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature (London) **332**, 411–415
2 Opgenorth, T. J., Wu-Wong, J. R. and Shiosaki, K. (1992) FASEB J. **6**, 2653–2659
3 Takahashi, M., Mastushita, Y., Iijima, Y. and Tanzawa, K. (1993) J. Biol. Chem. **268**, 21394–21398
4 Shimada, K., Takahashi, M. and Tanzawa, K. (1994) J. Biol. Chem. **269**, 18275–18279
5 Shimada, K., Takahashi, M., Ikeda, M. and Tanzawa, K. (1995) FEBS Lett. **371**, 140–144
6 Turner A. J. and Murphy, L. J. (1996) Biochem. Pharmacol. **51**, 91–102
7 Emoto, N. and Yanagisawa, M. (1995) J. Biol. Chem. **270**, 15262–15268
8 Rawlings, N. D. and Barrett, A. J. (1995) Biochem. J. **290**, 205–218
9 Le Moual, H., Devault, A., Roques, B. P., Crine, P. and Boileau, G. (1991) J. Biol. Chem. **266**, 15670–15674
10 Le Moual, H., Roques, B. P., Crine, P. and Boileau, G. (1993) FEBS Lett. **324**, 196–200
11 Le Moual, H., Dion, N., Roques, B. P., Crine, P. and Boileau, G. (1994) Eur. J. Biochem. **221**, 475–480
12 Dion, N., Le Moual, H., Crine, P. and Boileau, G. (1993) FEBS Lett. **318**, 301–304
13 Bateman, R. C., Jr, Jackson, D., Slaughter, C. A., Unnithan, S., Chai, Y. G., Moomaw, C. and Hersh, L. B. (1989) J. Biol. Chem. **264**, 6151–6157
14 Beaumont, A., Le Moual, H., Boileau, G., Crine, P. and Roques, B. P. (1991) J. Biol. Chem. **266**, 214–220
15 Kenny, A. J. and Maroux, S. (1982) Physiol. Rev. **62**, 91–128
16 Takahashi, M., Fukuda, K., Shimada, K., Barnes, K., Turner, A. J., Ikeda, M., Koike, H., Yamamoto, Y. and Tanzawa, K. (1995) Biochem. J. **311**, 657–665
17 Schmidt, M., Kroger, B., Jacob E., Seulberger, H., Subkowski, T., Otter, R., Meyer, T., Schmalzing, G. and Hillen, H. (1994) FEBS Lett. **356**, 238–243
18 Taylor, J. W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. **13**, 8764–8785
19 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
20 Suzuki, N., Matsumoto, H., Kitada, C., Kimura, S., Miyauchi, T. and Fujino, M. (1990) J. Immunol. Methods **127**, 165–170
21 Laemmli, U.K., (1970) Nature (London) **227**, 680–685
22 Davis, B. J. (1964) Ann. N. Y. Acad. Sci. **121**, 404–427
23 Marchand, P., Tang, J. and Bond, J. S. (1994) J. Biol. Chem. **269**, 15388–15393

Received 11 December 1995/5 February 1996; accepted 9 February 1996