Protein Engineering vol.11 no.12 pp.1235–1241, 1998

Molecular modelling and site-directed mutagenesis of the active site of endothelin-converting enzyme

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Mammalian endothelin-converting enzyme is a membrane-bound metalloprotease; its C-terminal domain contains sequence motifs characteristic of zinc metalloproteases. We examined residues expected from molecular modelling to be important for substrate binding using selectively mutated recombinant rat ECE-1α expressed in CHO cells. A conserved N–A–Ar–Ar (Ar = aromatic) motif is likely to be important for substrate binding. Mutating N550 to Gln or Y552 to Phe reduces V_{max}/K_m by 8- and 18-fold, respectively. The equivalent residue to Y553 in thermolysin binds the inhibitor through its NH group. Removing this putative interaction by mutating Tyr to Pro destroys activity, but mutating it to Ala or Phe also removes most activity. Mutating G583 (in a conserved GGI motif N-terminal of the zinc-binding helix) to Ala has no measurable effect, but mutating G584 to Ala destroys activity. Changing V583 in the zinc-binding helix to Met, to mimic the sequence pattern in bovine ECE-2, increases V_{max}/K_m to 1.7-fold that of the wild-type. Assays of phosphoramidon binding follow the pattern of those of substrate binding, but the IC_{50} of the more potent ECE inhibitor CGS 26303 was not significantly altered by any of these mutations, suggesting that this compound may bind to ECE in a different mode from phosphoramidon.

Keywords: active site/endothelin-converting enzyme/molecular modelling/site-directed mutagenesis/thermolysin

Introduction

Mammalian endothelin-converting enzymes (ECE-1 and ECE-2) are zinc metalloproteases and members of the M13 subfamily which also contains neprilysin (NEP; endopeptidase 24.11) and the Kell group glycoprotein (Turner and Murphy, 1996; Turner and Tanzawa, 1997). These proteases are ecto-enzymes, anchored to the plasma membrane by a single transmembrane helix (type II topology); the catalytic groups are located in the large C-terminal extracellular domain. All these proteins are active as dimers, but only ECE forms covalent dimers linked through a single disulphide bridge. The cysteine residue implicated in disulphide bond formation has been shown to be Cys412 in rat ECE-1 (Shimada *et al*., 1996) and the equivalent Cys416 in human ECE-1 (Hoang *et al*., 1997). *In vivo*, ECE cleaves the hormone endothelin from its inactive precursor, big endothelin. Interest in ECE stems largely from the fact that this reaction is the rate-determining step in the biosynthesis of endothelin, which is one of the most potent vasoconstrictors identified to date. Control of endothelin biosynthesis may lead to important advances in therapy against many diseases of the cardiovascular system.

Most ECEs synthesized to date are of the form known as ECE-1, which is active at neutral pH. There is over 90% amino acid identity between ECE-1 from different mammalian species. Human and bovine ECE-1 both occur in two isoforms, differing only in the short N-terminal cytoplasmic domain. We have termed the short form, with 51–52 cytoplasmic residues, α and the longer form, with 56 cytoplasmic residues, β (Turner and Murphy, 1996). A similar enzyme, known as ECE-2, has also been sequenced from cattle (Emoto and Yanagisawa, 1995). ECE-2 has a very similar specificity to ECE-1, but has a very narrow pH profile; it is most active at about pH 5.5 and almost inactive at neutral pH. There is about 60% identity between bovine ECE-2 and any of the ECE-1 sequences.

Both ECE-1 and ECE-2 are highly related to neutral endopeptidase E-24.11 (NEP, neprilysin) in substrate selectivity and are likely to be so in three-dimensional structure (Turner and Tanzawa, 1997). ECE and NEP share 35–37% sequence identity and covalent dimerization is also observed in rabbit NEP when mutating Glu403 to Cys (Hoang *et al*., 1997). This strongly suggests that they both have the same fold. In addition, ECE has recently been shown to hydrolyse the vasodilator bradykinin (Hoang and Turner, 1997). This is so structurally unrelated to big ET that it suggests that ECE, like NEP, has broad substrate specificity. However, ECE may not contribute significantly to bradykinin metabolism *in vivo* because of its high K_m (1 mM). Under physiological conditions, NEP is involved in inactivating peptides through degradation whereas ECE preferentially cleaves the peptide bond between Trp21–Val22 of big endothelin, to form active endothelin and the so-called 'C-terminal fragment'. In fact, although endothelin occurs in at least three isoforms (ET-1 to ET-3), both ECE-1 and ECE-2 preferentially cleave big ET-1. There are no known proteases which preferentially cleave big ET-2 or big ET-3. Although ECEs are inhibited by phosphoramidon, they are not inhibited by many other metalloprotease inhibitors (such as another NEP inhibitor, thiorphan). The construction of a three-dimensional model of the ECE active site is essential for a full understanding of the mechanism of action of this enzyme and for the eventual design of potent and specific ECE inhibitors. No structural data are yet available for any membrane-bound metalloprotease. This is partly due to the heavy glycosylation of these enzymes (ECE-1 has 10 *N*-glycosylation sites). Cluster analysis has suggested important similarities between the active site of NEP and the bacterial metalloprotease thermolysin (TL) (Benchetrit *et al*., 1988). We have described the sequence alignment of ECE-1 with NEP and TL, leading to the identification of key residues (Sansom *et al*., 1995).

Here we describe the model of the active site of rat ECE-1 in detail and use this to suggest a number of mutants of residues believed to lie in the active site. These mutagenesis results, in addition to those reported by Shimada *et al.* (1996), lead to some important insights into the active site structure and mechanism of this enzyme. This will, in turn, lead to further refinement of our molecular model.

Materials and methods

Materials

Tissue culture media, serum containing L-glutamate and D-glucose, trypsin, EDTA, penicillin, streptomycin and transfection reagent (LipofectAmine) were purchased from Gibco-BRL (Paisley, UK). The restriction enzyme *Bst*XI was obtained from New England Biolabs (Hitchin, UK). Sequenase kit (version 2.0) and the enhanced chemiluminescence (ECL) kit were supplied by Amersham International (Amersham, Bucks, UK). [^{35}S]-dαATP (1000 Ci/mmol) was obtained from DuPont (Stevenage, UK). T4 DNA ligase and restriction enzyme *Rsr*II were purchased from Boehringer-Mannheim (Sussex, UK). DNA polymerase *pfu* was purchased from Stratagene (Cambridge, UK). Of the inhibitors, phosphoramidon was purchased from Peptide Institute (Osaka, Japan); thiorphan was obtained from Sigma-Aldrich, (Poole, Dorset, UK). CGS 26303 and CGS 24592 were generous gifts from Dr S. De Lombaert, Novartis (formerly Ciba-Geigy, NJ, USA). The synthetic substrate [Phe^{22}]big ET-1-(18–34), was obtained from Multiple Sclerosis Peptide Laboratory, Oxford Brookes University (Oxford, UK). Plasmid pcDL-SRα296/rECE (containing rat ECE-1α cDNA) and monoclonal antibody AEC27–121 were gifts from Dr K.Tanzawa (Sankyo, Tokyo, Japan). All other chemicals were of analytical grade and obtained from Sigma-Aldrich.

Molecular modelling

All protein sequences were extracted from the OWL comprehensive sequence database (Bleasby and Wootton, 1990). Automatic multiple sequence alignment was performed using Clustal W (Higgins *et al*., 1992) and pairwise alignment using the GCG program GAP (Deveraux *et al*., 1984); the alignment was modified by inspection using Vistas (Perkins and Attwood, 1995) (Figure 1). Table I shows equivalent residues in the metalloproteases studied here. The structure of *Bacillus thermoproteolyticus* thermolysin with phosphoramidon bound (Tronrud *et al*., 1986; PDB code 1TLP) was extracted from the PDB (Bernstein *et al*., 1977). Residues in the active site of TL were changed into the equivalent residues in ECE using the program O (Jones *et al*., 1991), with the side-chain positions chosen from a database of frequently observed amino acid rotamers to maintain observed attractive interactions while minimizing steric hindrance. Residues I585 to H595, including the insertion of G586, were modelled as a standard α-helix, with the zinc-binding histidines H595 and H598 superimposed on the equivalent residues in TL (Figure 2).

Site-directed mutagenesis

Point mutations of rat ECE were introduced using a 'mega-primer' of the PCR approach described by Reikofski and Tao (1992).

The following primers were used for constructing different mutants.

Forward primer: 5′-TTCGACGATCAAGGCCG-3′

Reverse primer: 5′-GCGGGAGGGGCTCTGCGGATCGG-TG-3′

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ECE  550 ---------- -NAYYSPTKN EIVFPAGILQ APFYTRSSPN ALNFGGIGVV 588
NEP  542 ---------- -NAFYSSGRN QIVFPAGILQ PPFFSAQQSN SLNYGGIGMV 580
TLN  101 RSSVHYSQGY NNAFWNGSEM VYGDGDGQTF IPLS------ ----GGI-DV 139

ECE  589 VGHELTHAFD DQGREYDKDG NLRPWWKNSS VEAFKQQTEC MVQQYNNYS- 637
NEP  581 IGHEITHGFD DNGRNFNKDG DLVDWWTQQS ASNFKEQSQC MVYQYGNSFW 630
TLN  140 VAHELTHAVT D--------- ---------- --------YT AGLIYQNES- 161

ECE  638 -VNGEPVNG- RHTLGENIAD NGGLKAAYRA YQNWVKKNGA EQILPTLGLT 685
NEP  631 DLAGGQHLNG INTLGENIAD NGGLGQAYRA YQNYIKKNGE EKLLPGLDLN 680
TLN  162 ---------- -GAINEAISD IFGTLVEFYA NKNPDWEIGE DVYTPGISGD 200

ECE  686 SNQLFFLGFA QVWCSVRTPE SSHEGLITDP HSPSRFRVIG SLSNSKEFSE 735
NEP  681 HKQLFFLNFA QVWCGTYRPE YAVNSIKTDV HSPGNFRIIG TLQNSAEFSE 730
TLN  201 SLRSMSDPAK YGDPDHYSKR YTGTQDNGGV HINSGIINKA AYLISQGGTH 250

ECE  736 HFRCPLGSPM NPRHKCEVW  754
NEP  731 AFHCRKNSYM NPEKKCRVW  749
TLN  251 YGVSVVGIGR DKLGKIFYRA LTQYLTPTSN FSQLRAAAVQ SATDLYGSTS 300


TLN  301 QEVASVKQAF DAVGVK  316
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Fig. 1. Alignment of part of the C-terminal catalytic domains of ECE-1 and NEP with thermolysin. Part of the C-terminal catalytic domain of ECE-1 (ECE; Shimada *et al*., 1996) and human NEP (NEP; Malfroy *et al*., 1988) was aligned with *B.thermoproteolyticus* thermolysin (TLN; O'Donohue *et al*., 1994) as described in Materials and methods. The conserved motifs or residues referred to in the text (N–A–Ar–Ar; GGI; HExxH; ExxxD) are shown in bold. Also indicated in bold are (a) V589 of ECE-1 as the only residue differing from ECE-2 in the region of the zinc-binding motif, (b) a conserved tyrosine (Y633 in ECE-1) which may hydrogen bond to inhibitor, (c) a putative stabilizing histidine (H716 in ECE-1) and (d) R747 of NEP, which has been implicated in substrate binding. Residues constituting a triad proposed as critical for hydrolytic activity in NEP and thermolysin (Marie-Claire *et al*., 1997) are shown in bold and underlined. The present alignment reveals these residues are conserved between NEP and ECE-1. Conserved residues and motifs referred to in the text and shown in the figure were maintained in the model.

Table I. Equivalent residues in zinc metalloproteases

Residue function		THER	NEP	ECE-1α
Binds inhibitor C-terminus		None	R102	R129
Inhibitor binding motif (motif 1)		N112	N542	N550
		A113	A543	A551
	(S_1)→	F114	F544	Y552
	(S_2)→	W115	Y545	Y553
Conserved region close to zinc-binding site		G135	G575	G583
		G136	G576	G584
		I137	I577	I585
	(S'_1)→	V139	V580	V588
		V140	I581	V589
		A141	G582	G590
'HExxH' zincin motif (motif 2)	(Zn^{2+})→	H142	H583	H591
	(Glu cat)→	E143	E584	E592
		L144	I585	L593
		T145	T586	T594
	(Zn^{2+})→	H146	H587	H595
H-bonds to inhibitor (3)		Y157	Y625	Y633
Zn^{2+} binding H-bond to His (motif 4)	(Zn^{2+})→	E166	E646	E651
		D170	D650	D655
Stabilizing His (5)		H231	H711	H716

The values were obtained as follows: thermolysin from Tronrud *et al.* (1986), neprilysin from Devault *et al*. (1987) and endothelin-converting enzyme from Shimada *et al*. (1996).

Antisense mutagenic primers were constructed:

N550Q: (**AAC**→**CAG**) 5′-GCGAGTAGTAGGC**CTG**CACC-ATGGGCGGG-3′

Y552F: (**TA**C→T**T**C) 5′-GTGGGCGAGTAG**AA**GGCCGTT-CACCA-3′

Y553P: (**TA**C→**CC**C)5′-TCTTGGTGGGCGAG**GG**CTAGG-CGTTCACCA-3

Y553A: (**TA**C→T**T**C) 5′-TTGGTGGGCGAG**AA**GTAGGCG-TTCA-3′

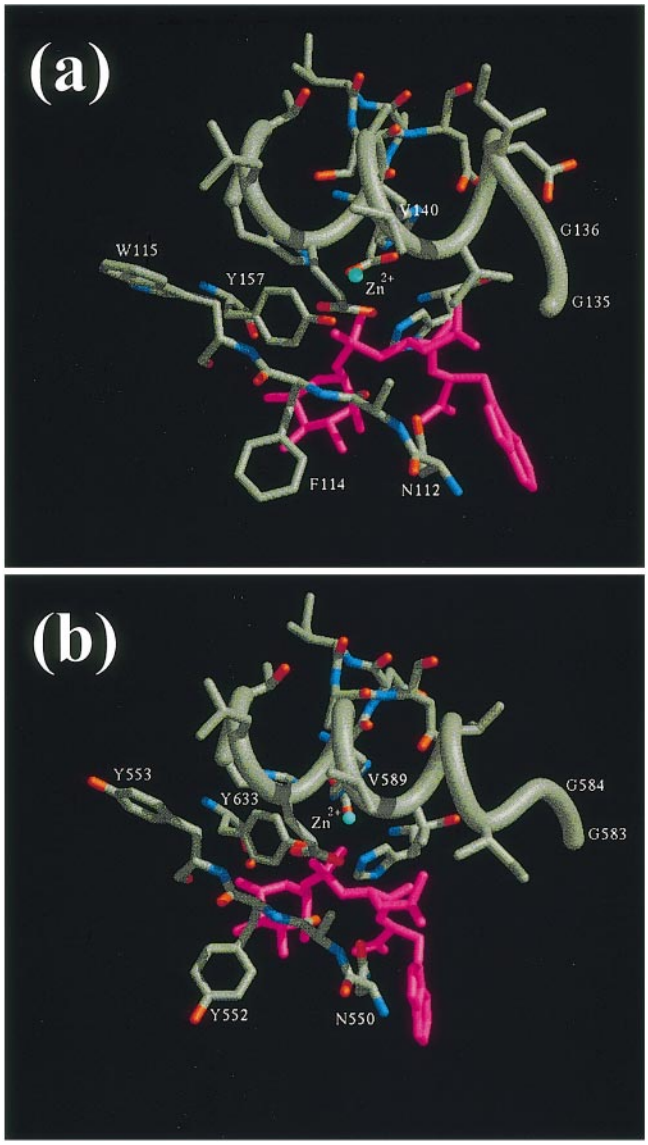

Fig. 2. Active site of (**a**) thermolysin and (**b**) endothelin-converting enzyme. (a) X-ray crystal structure of the active site of thermolysin from *B.thermoproteolyticus* (Tronrud *et al*., 1986) with phosphoramidon bound. (b) Model of the active site of rat ECE-1, also with phosphoramidon bound.

Y553F: (T**A**C→G**C**C) 5′-CTTGGTGGGCGAG**GC**GTAGGC-GTTCACC-3′
G583A: (G**G**T→G**C**T) 5′-ACGACCCCGATA**GC**ACCAAA-GTTCAAGG-3′
G584A: (G**G**T→G**C**T) 5′-GACCCCGATACC**AGC**AAAGT-TCAAGGC-3′
V589M: (**G**TG→**A**TG) 5′-GCTCGTGCCC**CAT**AACGACCC-CGAT-3′
Y663F: (T**A**T→T**T**T) 5′-CTGTAGTTGTTA**AA**ACTGCTGTA-CCA-3′

Mutagenesis was performed in two PCR reactions, each of 32 cycles. The final PCR product was restricted by *Bst*XI and *Rsr*II then replaced for the equivalent non-mutated fragment in rat ECE-1α expression vectors, respectively. The fidelity of the mutants was confirmed by sequencing the full length of the subcloned fragment on both strands.

Cell growth and expression

Chinese hamster ovary (CHO) cells were cultured in Glasgow minimum essential medium, supplemented with 10% fetal calf serum (heat inactivated), 2 mM nucleosides, 10 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine/asparagine at 37°C in 5% CO_2. The expression vectors pcDL-SR296/rECE (containing wild-type or mutant rat ECE-1α cDNA) were used to transfect CHO cells. For transient expression, CHO cells were plated in 150 cm^2 flasks of ~2×10^6 cells. After 24 h growth, the cells were washed twice with OptI Mem and transfected (5.0 μg DNA per flask) by using LipofectAmine as cationic lipid (DNA:lipid = 1:10). The cells were incubated for 3 h prior to adding Glasgow minimum essential medium containing 10% fetal calf serum; 24 h after transfection, the medium was aspirated and the cells were replaced with fresh medium. After a further 24 h the cells were harvested and membrane fractions prepared as described previously (Hoang and Turner, 1997). Isolated membranes were solubilized with 1% Triton X-100 then subjected to Western blotting or enzyme assay.

Protein determination

Protein concentrations were determined by using the bicinchoninic acid method of Smith *et al*. (1985) modified for use in 96-well microtitre plates with bovine serum albumin as standard.

SDS–PAGE and immunoblotting

PAGE was performed as described by Laemmli (1970) using a 5% stacking and a 5–15% gradient separating gel. The gels were blotted (Towbin *et al*., 1979) using a semi-dry blot and the detection of protein was performed by using chemical luminescence. Pre-stain molecular weight standards were run in parallel.

ECE assay

For the determination of the enzyme activity of expressed rat ECE-1 cDNA (wild-type or mutants), solubilized transfected and non-transfected CHO cell membranes (10 μg protein), were preincubated with 10 μM amastatin, 10 μM leupeptin and 1 mM DipF for 1 h in 100 mM Tris–HCl, pH 7.0, with or without the inhibitor phosphoramidon (100 μM in a total volume of 100 μl). The reaction was started by adding substrate [Phe^{22}]big ET-1-(18–34) to 200 μM final concentration. After incubation for 30 min at 37° C, samples were heated at 100°C for 4 min and then centrifuged at 2×10^4 *g* for 20 min at 4°C. The supernatant was retained and then the N-terminal fragment was quantified by HPLC on a μBondapak C_{18} column, using a linear gradient of acetonitrile from 15 to 85% in 0.02% trifluoroacetic acid, pH 2.5, with detection at 214 nm. Kinetic assay conditions were selected so that the variation of product formation with time and protein concentration used was linear. The substrate concentrations used for the determination of K_m and V_{max} were in the range 50–500 μM.

Results and discussion

Sequence analysis

The degree of similarity between ECE, neprilysin and the Kell group glycoprotein (e.g. 39.3% sequence identity between rat ECE-1 and human neprilysin) is sufficient to indicate statistically that these proteins share a common origin and, almost certainly, a common fold. Furthermore, although there is no significant overall sequence similarity between ECE and thermolysin, there is statistically significant similarity between thermolysin (from *B.thermoproteolyticus*) and the carboxy-terminal domain of ECE (which contains sequence motifs characteristic of zinc metalloproteases). It was possible to

identify, from sequence alignment (Figure 1), the crystal structure of thermolysin and functional and modelling studies of neprilysin, residues in ECE thought to be important for substrate and inhibitor binding (Sansom *et al*., 1995).

Three characteristic sequence motifs and two individual residues were identified which were likely to form part of the active site of rat ECE-1. The N–A–Ar–Ar motif (motif 1; Ar = aromatic residue) in thermolysin is conserved in ECE (residues 550–553 of rat ECE-1). The two aromatic residues (Ar) line the S1 and S2 subsites in thermolysin (Tronrud *et al*., 1986). The whole of the zinc-binding helix (motif 2) is well conserved and there is a region containing two 100% conserved glycine residues immediately N-terminal of this helix. The third characteristic motif (ExxxD; motif 3) is found from E651 to D655 in rat ECE-1. Residue H 716 in rat ECE-1 has been accepted as the stabilizing histidine in ECE. In the crystal structure of TL, Tyr157 makes a strong hydrogen bond to phosphoramidon (Tronrud *et al*., 1986). We chose to maintain this interaction, aligning this tyrosine with the only tyrosine in the appropriate region which is conserved between NEP and ECE: Y633 in rat ECE-1. However, it was impossible to gain any further information about two charged residues, known to bind a substrate or peptide-mimetic inhibitor in ECE. Arg129 in rat ECE-1 is equivalent to R102 in NEP, which is known to bind to the C-terminus of a substrate or inhibitor (Beaumont *et al*., 1991; Kim *et al*., 1992). This residue is N-terminal of the thermolysin-like domain in the ectoenzymes. In NEP, R747 has been proposed to play a role in substrate and inhibitor binding (Beaumont *et al*., 1991), possibly equivalent to R203 in TL. In all ECEs, including ECE-2, this arginine at the extreme C-terminus is replaced by Glu, which has opposite charge. Obviously, this residue cannot bind substrate in an equivalent manner, although it may well have some other important role. However, mutagenesis of this glutamate (E752 in rat ECE-1) to glutamine, aspartate or arginine resulted in no significant change in kinetic parameters and the arginine mutation (E752R) did not introduce NEP-like characteristics into ECE-1 (Shimada *et al*., 1996), thus questioning the significance of R747 in NEP. A more recent mutagenesis and modelling study of NEP has, however, implicated R717 as the equivalent of R203 in thermolysin (Marie-Claire *et al*., 1997) and proposes that both enzymes conserve a triad of residues which provide a hydrogen bond network and are crucial for hydrolytic activity (H142, D170, R203 in thermolysin and H583, D650 and R717 in NEP). The alignment in Figure 1 reveals that these same residues are conserved in rat ECE-1 (H591, D655 in our proposed ExxxD motif and R722).

Molecular model of the active site

The model of the active site of rat ECE-1, based on the structure of a complex between thermolysin from *B.thermoproteolyticus* (TL) and phosphoramidon (Tronrud *et al*., 1986) is shown in Figure 2. It uses residues 550–553 (motif 1), 584–595 (motif 2), 633 (the tyrosine thought to be equivalent to Y157 in TL), 651–655 (motif 3) and 716 (the stabilizing histidine). It was possible to maintain the attractive interactions between these residues and between the active site residues and the bound phosphoramidon without introducing serious steric hindrance. There was only one insertion, of a single residue, G586, at the extreme N-terminus of the long zinc-binding helix. This was modelled as an extension of the helix; insertions and deletions are more often found at the termini of secondary structure elements.

Both thermolysin and ECE may cleave peptide substrates between large hydrophobic residues; in its primary function of endothelin biosynthesis, ECE is specific for a W–V/I cleavage. It cleaves bradykinin between residues Pro7 and Phe8. The active sites of these proteins are necessarily large, in order to accommodate these substrate residues. Differences in the specificity of the zinc metalloproteases will be indicated by differences in the shape of the hydrophobic parts of the active site cleft. In particular, the tryptophan in position P2′ in phosphoramidon has been shown to be necessary for ECE inhibition (Bigaud *et al*., 1994). Hydrophobic residues in equivalent positions to F130 and L202 of TL, which constitute the limit between the S1′ and S2′ subsites, are therefore expected to play an important part in determining ECE specificity. It was not possible to identify these residues in ECE from sequence alignment. In the absence of any structural information, structure–activity relationships of a range of ECE inhibitors and further site-directed mutagenesis experiments will be necessary to determine which residues are likely to form the walls of the S2′ subsite. These residues should be important determinants of ECE specificity.

Rationale for selection of mutants

Motif 1. Motif 1 is in an extended conformation in TL. There is a hydrogen bond between N112 in TL and the inhibitor which is present in many crystal structures of TL with different inhibitors. Site-directed mutagenesis has shown that N542 of human neprilysin is involved in binding the P2′ residue of substrates and inhibitors in an equivalent manner (Dion *et al*., 1995). The two conserved aromatic residues (FW in TL; YY in ECE) point away from the inhibitor, so neither aromatic group can interact with substrate or inhibitor. However, the backbone NH of W115 in TL can form hydrogen bonds to TL inhibitors, although in many structures including the complex with phosphoramidon (Tronrud *et al*., 1986) this interaction is mediated via a water molecule. Asn550, which is equivalent to N542 of neprilysin (this residue is 100% conserved in thermolysin and the M13 sub-family), was mutated into its most conservative substitution (Gln). Tyr552 was mutated into Phe, which occurs in this position in both neprilysin and thermolysin. In order to remove the only direct contact between Tyr 553 and the inhibitor—through the backbone NH group—it was necessary to mutate this residue to Pro. However, such a mutation (Y553P) not only removes the backbone binding, but is also likely to introduce vigorous changes into the local chain conformation. To distinguish between these effects, it is also necessary to mutate Y553 into Phe (the most conservative change, removing only the hydroxyl group) and into Ala (to remove both the hydroxyl and the aromatic groups).

Motif 2: the zinc-binding helix. A conserved –GGI– motif occurs immediately N-terminal of the alpha helix (in TL) which contains the conserved zinc-binding –HELTH– motif. Conserved patterns of glycines are likely to indicate either an unusual backbone conformation (as glycine can occupy positions on the Ramachandran map which are disallowed for any other residue) or a conformation where other residues approach the protein chain too closely to cause serious steric hindrance with any side-chain at this position. In thermolysin (Tronrud *et al*., 1986) the (ϕ,ψ) values for G135 and G136 fall within the acceptable ranges for amino acids other than Gly. However, the main chain in this region lies close to residues V13, D191 and V192. This part of the chain is likely to lie in a similarly ‘crowded’ environment in the ectoenzymes.

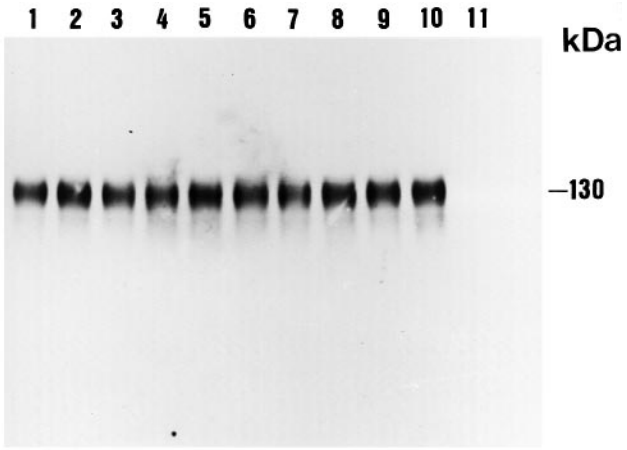

Fig. 3. Immunoblotting of the CHO cells transfected with wild-type and mutant rat ECE-1α. Portion (5 μg protein) of membrane fractions obtained from CHO cells transfected with wild-type and several mutants of rat ECE-1α were subjected under reducing conditions to the 5–15% gradient SDS–PAGE and analysed by immunoblotting by using AEC27-121. Lanes: 1, wild-type; 2, N550Q; 3, Y552F; 4, Y553P; 5, Y553A; 6, Y553F; 7, G583A; 8, G584A; 9, V589M; 10, Y633F; 11, control CHO cells.

To test this hypothesis, both G583 and G584 in rat ECE-1 were mutated to Ala.

There is one residue change between all known ECE-1 sequences and bovine ECE-2: Val589 (rat ECE-1 numbering; two residues N-terminal of the first zinc-binding histidine) is Met in ECE-2. This residue lies within the zinc-binding helix and will point away from the zinc ion. This change is therefore unlikely to affect inhibitor binding directly, although it may well subtly alter the position of the zinc-binding helix with respect to the rest of the active site. Val589 was altered to Met to mimic the active site of bovine ECE-2.

Tyrosine 633. The hydroxyl group of Tyr157 in TL can form hydrogen bonds both to an inhibitor (as in its complex with phosphoramidon (Tronrud *et al*., 1986)) and to the side-chain of Glu 166. This residue was mutated into Phe to remove these attractive interactions.

Expression of wild-type and active site mutant ECEs in CHO cells

To obtain wild-type and active site mutated enzymes, CHO cells were transfected with vector pcDL-SRα296/rECE-1α (Shimada *et al*., 1996). This is a eukaryotic expression vector (Takabe *et al*., 1988) carrying the full length of the wild-type or active site mutant ECE cDNA (residues described above). The expression of the enzymes was confirmed by immunoblot analysis using the anti-(rat ECE-1) monoclonal antibody AEC27-121 (Figure 3). This monoclonal antibody recognizes part of the extracellular polypeptide chain of ECE-1, rather than any portion of the carbohydrate (Takahashi *et al*., 1995). Figure 3 shows the protein level of wild-type and nine active site mutants of ECE-1α under reducing conditions. In all cases, similar amounts of protein with identical molecular weight (130 kDa) were detected (lanes 1–10) and no ECE was observed in non-transfected CHO cells (lane 11). Under non-reducing conditions, a similar protein level with double (also identical) molecular mass (280 kDa) was observed (result not shown). Taken together, these results imply that mutations did not interfere with the post-translational modification (including glycosylation and dimerization) and distribution of the ECE in the cells.

Enzymic properties of the active site mutant ECEs compared with wild-type

Motif 1 mutations. The K_m values of the substrate analogue (18–34)[F^{22}]big ET-1 for N550Q, Y552F, Y553A and Y553F were almost identical with the K_m for the wild-type enzyme, indicating that such mutations do not provoke any major changes in ground-state binding. For the mutant enzymes, however, V_{max} values were lower, leading to decreases in V_{max}/K_m of 8-, 16-, 76- and 26-fold for N550Q, Y552F, Y553A and Y553F, respectively (Table II). The decrease in V_{max} may be due to indirect effects because there is no evidence for the direct participation of these residues in catalysis from modelling or other studies. Both N542 in NEP and N112 in TL have been shown to bind to the backbone of the P2′ substrate via hydrogen bonds (Dion *et al*., 1995). The involvement of N542 in substrate binding is shown in a decrease in K_m for N542Q mutant NEP compared with wild-type. This could be one of the major differences between ECE and NEP. When residue Y552 is changed into Phe, which is the most conservative substitution and is present in both TL and NEP, there is a significant loss of activity. As its side-chain is predicted to point away from the substrate or inhibitor, this reinforces the fact that the decrease in V_{max} must be due to secondary effects.

Mutation of Y553 into Pro results in complete loss of activity. This must be due to an indirect effect in addition to simply loss of a hydrogen bond from the backbone NH to substrate. This mutation may alter the local chain conformation due to its rigid side-chain, which could lead to the loss of activity. However, this change cannot be significant enough to prevent the correct folding of the protein as it is expressed at the cell surface. Although we cannot exclude that substrate and/or inhibitor still bind to this mutant, this is very unlikely since, from the modelling, this mutation causes considerable changes and would alter the central α-helix containing the HELTH active site motif. Mutating Y553 into A, however, which removes its side-chain but not the putative hydrogen bond from the backbone, results in a highly significant 76-fold decrease in activity and even the most conservative change, Y553F, causes a 26-fold decrease in activity. These changes are entirely due to decreases in V_{max}; K_m is increased slightly in both cases, indicating that the substrate binds less tightly than to the wild-type enzyme.

Motif 2 mutations. Mutating the N-terminus of the two conserved glycines in this motif, G583, to Ala has no significant effect on either binding or hydrolysis, but mutating G584 into Ala completely destroys activity. A requirement for glycine in this position is most likely to indicate a ‘crowded’ local molecular environment where other residues approach this part of the chain too closely to allow the presence of a side-chain. However, in thermolysin, the N-terminus of the equivalent two glycine residues is slightly more sterically constricted.

Changing V589 to Met, to mimic the active site of ECE-2, results in an increase in activity, to 1.7 times that of the wild-type, due to slight changes in both V_{max} and K_m. This residue is within the zinc-binding helix and must point away from the substrate if the two histidine residues in this helix are to coordinate the zinc ion. The most likely effect of this mutation is a slight shift in the position of this helix with respect to the substrate. The main difference in activity between ECE-1 and ECE-2 is a shift in pH optimum from 7 to 5.5, but this change, replacing one neutral, hydrophobic residue with another, is unlikely to be responsible for this shift in pH optimum. (All assays reported here were carried out at pH 7.0.)

Table II. Kinetic parameters of wild-type and the mutant forms of the active site of rat ECE-1α expressed in CHO cells

	Clones									
	Wild type	N550Q	Y552F	Y553P	Y553A	Y553F	G583A	G584A	V589M	Y633F
Specific activity (nmol/min.mg)	17.8 ± 1.4	3.4 ± 0.2	2.0 ± 0.1	0.0	0.5 ± 0.1	2.3 ± 0.1	17.9 ± 1.3	0.0	21.7 ± 1.7	14.7 ± 0.9
K_m (μM)	201 ± 12	136 ± 8	125 ± 6	ND	265 ± 13	312 ± 15	236 ± 19	ND	136 ± 19	220 ± 10
V_{max} (nmol/min.mg)	35.2 ± 1.5	3.0 ± 0.2	1.4 ± 0.1	ND	0.6 ± 0.1	2.1 ± 0.2	31.1 ± 1.8	ND	40.5 ± 2.4	32.0 ± 1.7
V_{max}/K_m (ml/min.mg)	175.1 ± 19.3	22.1 ± 2.0	11.2 ± 1.0	ND	2.3 ± 0.4	6.7 ± 0.7	131.8 ± 10.8	ND	297.8 ± 45.2	145.5 ± 10.2
IC_{50} (μM)										
Phosphoramidon	3.3 ± 0.2	148.3 ± 15.9	88.0 ± 5.0	ND	31.2 ± 2.4	42.0 ± 3.2	3.4 ± 0.2	ND	7.6 ± 1.3	14.4 ± 1.0
CGS 26303	1.03 ± 0.09	0.83 ± 0.07	0.97 ± 0.03	ND	3.77 ± 0.22	5.3 ± 0.33	0.87 ± 0.07	ND	2.93 ± 0.15	1.23 ± 0.14

Membrane fractions were prepared from CHO cells transfected with either wild-type or mutant rat ECE-1α as described in Materials and methods. The results were determined from three independent transfections and represent mean ± SEM. ND: not detectable.

Tyr633. Mutating Y633, which, by analogy with thermolysin is believed to be able to form a hydrogen bond to substrate or inhibitor, to Phe (removing the potential hydrogen bond donor) causes a very slight but probably insignificant decrease in activity. This mutation has a greater effect on phosphoramidon binding, increasing IC_{50} (the concentration of inhibitor giving 50% inhibition of enzyme activity) 4-fold. This is compatible with the loss of a single hydrogen bond. This residue may well form a hydrogen bond to phosphoramidon, as thermolysin does in its complex with phosphoramidon (Tronrud *et al*., 1986), but not to the substrate.

Inhibition of the wild-type and active site mutants of ECE

The effects of these mutations of active site residues on ECE–inhibitor interactions were examined using the inhibitors phosphoramidon, CGS 26303, thiorphan and CGS 24592 (see Figure 4 for structures). All these inhibitors have a common hydrophobic residue in the P1′ position, to fit into the S1′ subsite and a strong zinc-chelating group. However, phosphoramidon and CGS 26303, having extra residues at P2′ to fit into the S2′ subsite, are both ~200-fold more potent than either thiorphan or CGS 24592. The rationale for selecting these particular mutants of ECE was based on the assumption that phosphoramidon could bind to the ECE active site in a similar manner to its binding to thermolysin (Figure 2). Hence the difference in affinity binding between wild-type and mutant ECEs to phosphoramidon should represent similarities between the ECE and thermolysin active sites. Indeed, even the most conservative substitutions for residues in the 'NAArAr' motif in ECE produced significant differences in phosphoramidon binding affinity from the wild-type. In contrast, there was no difference in the binding affinity for the most potent compound, CGS 26303, between the wild-type and any of the active site mutants (Table II). There was no significant change in binding potency for the weak inhibitors, thiorphan or CGS24592 (data not shown). These results strongly suggest that the actual interaction of phosphoramidon with the active site of ECE is similar to its interaction with that of thermolysin. In other words, ECE and thermolysin do have very similar active site structures, although the fold of the proteins may well be different.

The crystal structure of thermolysin shows the 'NAFW' motif as a beta-sheet strand (residues 112–116) which can act as a 'jaw' to hold phosphoramidon in the active site (Holland *et al*., 1992). In particular, N112 forms two hydrogen-bonds with the NH and CO groups of the P2′ residue. Site-directed

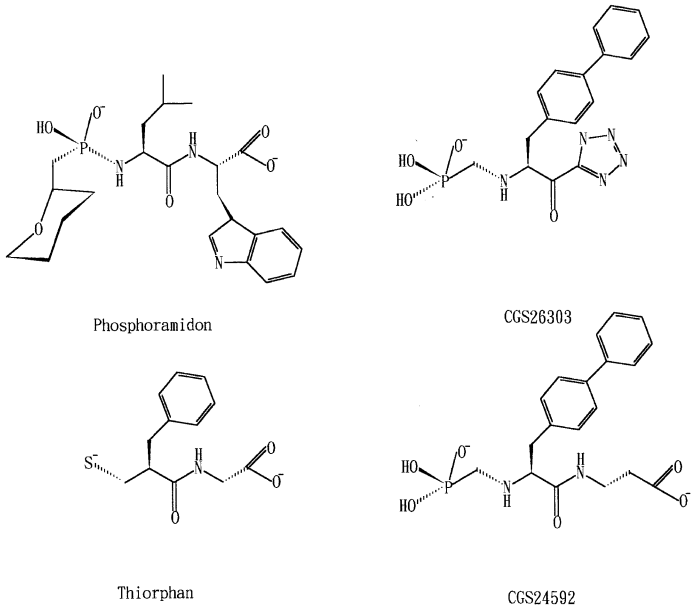

Fig. 4. Structures of four ECE inhibitors used in this study.

mutagenesis has shown that the equivalent residue N542 in human NEP interacts with the inhibitor in a similar manner (Dion *et al*., 1995). In ECE, mutating N550 into Gln (introducing only one extra CH_2 group into the side-chain) results in a significant 50-fold decrease in phosphoramidon binding. Thus, N550 in ECE is very likely to interact with phosphoramidon in the active site in a similar manner to N112 in thermolysin or N552 in human NEP. The crystal structure of thermolysin shows that the two aromatic residues, Y114 and W115, point away from the phosphoramidon, but the side-chain of W115 binds to phosphoramidon via a water molecule. The two equivalent residues, Y552 and Y553, may not have the same orientation, but they do seem to play an important role in inhibitor binding as even mutating each of these into Phe (the most conservative change, which only removes the OH group from the Tyr side-chain) reduces phosphoramidon binding 30- and 15-fold, respectively. Mutating Y553 into Ala results in a 10-fold decrease in binding affinity, but mutating it into Pro (removing the hydrogen bond from its main chain NH group) completely destroys activity. In the ECE active site, these residues may well point away from the inhibitor, as F115 and Y116 do in thermolysin, but in each situation the mutants are

likely to alter the local conformation of this beta-strand and to change the binding affinity of phosphoramidon as a result.

The second motif, 'GGI', may not play any role in phosphoramidon binding as there is no observed difference in binding affinity associated with mutations in this motif. Mutating V589 into methionine to mimic ECE-2 also does not alter phosphoramidon binding affinity. Hence this may not alter the 250-fold more potent binding affinity for phosphoramidon of ECE-2 over ECE-1.

The binding affinity of all mutants for thiorphan and CGS 24592 is not significantly different from that of wild-type ECE. This is consistent with the fact that both thiorphan and CGS 24592 are weak inhibitors of ECE and that the mutations of the ECE active site studied could not provoke any unexpected changes in the binding of such weak inhibitors.

Conclusions

Molecular modelling and site-directed mutagenesis of the active site of endothelin-converting enzyme, using the X-ray structure of thermolysin with phosphoramidon bound and structure–function studies of neprilysin as templates, have indicated that the active site of ECE is very similar to those of thermolysin and neprilysin in spite of the low sequence similarity of thermolysin to the C-terminal domains of ECE and NEP. Except for Y553P and G584A, which result in complete inactivation of ECE, most of the mutants lead to a decrease in specific activity. The major changes in specific activity are due to decreases in V_{max}, although small alterations to K_m are also noted. This implies that these mutations generally affect catalytic activity rather than substrate binding. Hence there is unlikely to be a single specific structural explanation for these effects.

Directed mutants of the residues in the 'NAYY' motif show that phosphoramidon binds to the ECE active site in a very similar manner to that of the equivalent motif in thermolysin or neprilysin. In particular, the Asn residue in this motif (N112 in thermolysin, N454 in neprilysin and N660 in ECE) is strictly conserved and is very likely to play a similar role in NEP and ECE to the one that it plays in thermolysin, forming two hydrogen bonds with the NH and CO groups of the P2′ residue. The two aromatic residues (FW in thermolysin, FY in neprilysin and YY in ECE) are also critical to phosphoramidon binding, although they may not have the same orientation in the active site of the three enzymes. Indeed, this motif may well act as a 'jaw' to hold a substrate or inhibitor in the active site in ECE, as it does in thermolysin.

Although these results have shown significant similarities between the ECE, NEP and thermolysin active sites, the enzymes differ in their sensitivity towards phosphoramidon (ECE is inhibited by phosphoramidon at the μM scale, compared with the nM scale for NEP and thermolysin) and the other inhibitors studied. As substitution of phosphoramidon and CGS 26303 at the P1 position by hydrophobic residues leads to increased inhibitor potency, hydrophobic residues in the S1 subsite are expected to play a critical role in defining the specificity of these enzymes towards substrates and inhibitors, as Bigaud *et al.* (1994) have indicated. It was not possible to identify the residues in this subsite using the present model.

Acknowledgements

We thank the BBSRC, the MRC and the British Heart Foundation for support for this work. V.M.H. was in receipt of an MRC studentship. We thank Dr K.Tanzawa (Tokyo) for generous provision of ECE-1α cDNA and antibody AEC27-121.

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Received March 11, 1998; revised July 27, 1998; accepted August 5, 1998