Mutagenesis of Glu^{403} to Cys in rabbit neutral endopeptidase-24.11 (neprilysin) creates a disulphide-linked homodimer: analogy with endothelin-converting enzyme

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Neutral endopeptidase-24.11 (NEP; neprilysin; EC 3.4.24.11) and endothelin-converting enzyme (ECE) are related zinc metallopeptidases involved in the processing of biologically active peptides. Only ECE, however, exists as a disulphide-linked homodimer. The covalent linkage in rat ECE is between Cys^{412} in each subunit, which is equivalent to Glu^{403} in rabbit NEP. Here we report that directed mutagenesis of Glu^{403} to cysteine in rabbit NEP creates a disulphide-linked homodimer, as revealed by transient transfection in COS-1 cells and SDS/PAGE of a membrane fraction. Under reducing conditions, both the mutant (E403C) and the wild-type NEP migrate as a polypeptide of 92 kDa. However, under non-reducing conditions, the M_r of the wild type remains unchanged, whereas that of the mutant is doubled. Co-transfection of wild-type ECE and E403C NEP cDNA did not result in the production of a NEP–ECE heterodimer. Comparison of the kinetic constants for wild-type and E403C mutant NEP with either [D-Ala^2,Leu^5]enkephalin or 3-carboxypropanoyl-alanyl-alanyl-leucine-4-nitroanilide (Suc-Ala-Ala-Leu-NH-Np) as substrate show a decrease of approx. 50% in V_{max}/K_m for the mutant form. The IC_{50} value for inhibition of the mutant by phosphoramidon or thiorphan is increased 3-fold and 5-fold respectively. Although NEP and ECE exhibit only about 40% identity and differ substantially in substrate specificity and some other characteristics, these data indicate that they have considerable similarity in three-dimensional structure, allowing dimer formation in the mutant NEP with the disulphide link probably occurring in a hydrophilic surface loop.

INTRODUCTION

Neutral endopeptidase-24.11 (NEP; neprilysin; EC 3.4.24.11) and endothelin-converting enzyme (ECE) are mammalian membrane zinc peptidases, and members of a subfamily of the M13 peptidase family [1]. They, and the erythrocyte Kell group glycoprotein [2], show sequence similarity along their whole length. Each of these proteins has a short N-terminal cytoplasmic domain, followed by a single transmembrane helix and a large C-terminal extracellular domain. The C-terminal region of the extracellular domain contains the HEXXH zincin motif characteristic of many zinc metalloproteases [3]. NEP is widely distributed in mammalian tissues and has a broad substrate specificity, being mainly involved in the inactivation of biologically active peptides such as the enkephalins, tachykinins and atrial natriuretic peptides (see, e.g., [4,5] for review). ECE is a highly specific peptidase, its only known physiological function being the production of the potent vasoconstrictor, endothelin, from its inactive precursor, big-endothelin [5,6]. Both ECE and NEP have been considered therapeutic targets, because of their respective involvements in the metabolism of vasoactive peptides [6,7].

Two distinct forms of ECE exist (ECE-1 and ECE-2), which represent different gene products, although only ECE-1 has been characterized at the protein level in any detail [6,8]. ECE-1 can also exist in two alternatively transcribed forms that differ in part of the N-terminal cytoplasmic domain (ECE-1α and ECE-1β [6,9]). There is approx. 40% sequence identity between ECE-1 and NEP, and 35% between ECE-2 and NEP. This is sufficient to indicate that ECE and NEP will almost certainly share the same fold. Furthermore, there is sufficient sequence identity between the C-terminal domain of both these enzymes and the well characterized bacterial metalloprotease thermolysin (including the presence of the zincin motif HEXXH) to allow us to propose a model for the active site of human ECE-1 based on the structure of thermolysin [10].

NEP and ECE-1 differ, however, in quaternary structure. ECE-1 is functionally active as a disulphide-linked dimer [11,12], whereas, in most species, NEP forms non-covalent dimers [13], although in the rabbit it is monomeric, as judged by gel-filtration and ultracentrifugation analysis [14]. On structural grounds, the candidate cysteine producing dimerization of ECE-1 is likely to reside in the extracellular domain and to occur in a surface loop region, in a relatively hydrophilic environment. It should also represent a residue that is not conserved in NEP. Only two cysteine residues in rat ECE-1α fulfil these criteria (Cys^{372} and Cys^{412}), and site-directed mutagenesis has shown that the only residue involved in dimerization of rat ECE-1α is Cys^{412} [15]. ECE-2 conserves this residue [8] and may well therefore also exist as a disulphide-linked dimer. However, NEP possesses a glutamyl residue in this position (Glu^{403} in the rabbit enzyme; Figure 1). Here we show that mutagenesis of this glutamine to cysteine in NEP followed by expression of the mutant in COS-1 cells produces a fully functional enzyme at the cell surface that now exists as a covalent dimer. Additionally, Cys^{416} (equivalent to Cys^{412} in rat ECE-1) is established as the site of dimer formation in human ECE-1β. Thus, although no structural data are available on any member of this membrane-protein family, the mutagenesis data imply that their three-dimensional structures must be highly similar to permit dimerization of the E403C

Abbreviations used: ECE, endothelin-converting enzyme; ECL, enhanced chemiluminescence; NEP, neutral endopeptidase-24.11; Np, 4-nitroanilide; Suc, succinyl.

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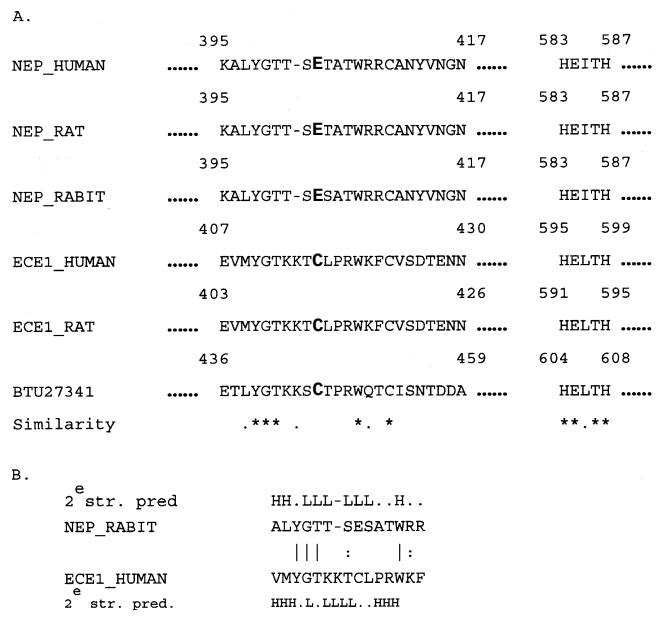

Figure 1 Alignment of amino acid sequences (A) and secondary structure prediction (B) of NEP and ECE

The target mutation site, which is located within a loop by secondary structure prediction (2^estr. pred), is shown in bold. The number of residues in each sequence is shown. RABIT, rabbit; ECE1-HUMAN, human ECE-1β; ECE1-RAT, rat ECE-1α; BTU27314, bovine ECE-2. The sequence numbering for human and rat ECE-1 is based on that in [32] and [25] respectively. (**A**) * indicates identical residues between two sequences; . indicates similar residues. (B) H, helix; L, loop; | indicates identical residues between two sequences; : indicates similar residues; . indicates ambiguous prediction results.

mutant of NEP and its production in an active form. However, there was no evidence for the formation of NEP/ECE heterodimers when both cDNAs were co-expressed.

EXPERIMENTAL PROCEDURES

Materials

Tissue-culture media, serum, L-glutamine, D-glucose, trypsin/EDTA, penicillin, streptomycin and transfection reagent (Lipofect-Amine) were purchased from Gibco–BRL (Paisley, U.K.). The restriction enzymes *Bst*XI and *Bst*EII were obtained from New England Biolabs (Hitchin, U.K.). Sequenase kit (version 2.0) and the enhanced chemiluminescence (ECL) kit were from Amersham International (Amersham, Bucks., U.K.). [α-^{35}S]dATP (1000 Ci/mmol) was from Du Pont (Stevenage, U.K). T4 DNA ligase was from Boehringer-Mannheim (Sussex, U.K.). Phosphoramidon was from the Peptide Institute (Osaka, Japan). Thiorphan was obtained from Sigma–Aldrich (Poole, Dorset, U.K.). [D-Ala^2,Leu^5]Enkephalin was purchased from Cambridge Research Biochemicals (Northwich, Cheshire, U.K.). 3-Carboxypropanoyl-alanyl-alanyl-leucine-4-nitroanilide (Suc-Ala-Ala-Leu-NH-Np) and *Staphylococcus griseus* aminopeptidase were gifts from Dr. S. Blumberg (Tel Aviv University, Tel Aviv, Israel). Plasmid pcDL-SRα296/hECE (containing human ECE-1 cDNA) and anti-ECE-1 monoclonal antibody AEC32-236 were gifts from Dr. K. Tanzawa (Sankyo, Tokyo, Japan). Plasmid pSVNK19 (containing rabbit NEP cDNA) and the anti-(rabbit NEP) monoclonal antibodies 23B11 and 18B5 were generously provided by Professor P. Crine (University of Montréal, Montréal, Canada). All other chemicals were of analytical grade.

Sequence analysis

Sequences of human and rat ECE-1, bovine ECE-2 and human, rabbit and rat NEP were extracted from the OWL composite sequence database [16]. Multiple sequence alignment was performed using Clustal W [17] and modified by inspection using VISTAS [18]. All pairwise sequence alignments were carried out using the GCG program GAP [19]. Three-state secondary-structure prediction (helix, strand and rest) of rabbit NEP and human ECE-1 was performed using PredictProtein [20]. This is a novel algorithm using multiple sequence alignment and neural networks that can predict the secondary structure of globular proteins with over 70% per-residue accuracy.

Cell growth and expression

COS-1 cells were cultured in Dulbeccos's modified Eagle's medium supplemented with 10% (v/v) foetal-calf serum (heat-inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37 °C in 5% CO_2 as described previously [21]. The expression vectors pcDL-SR296/hECE (containing human ECE-1β) and/or pSVNK19 (containing rabbit NEP) were used to transfect COS-1 cells. For transient expression, COS-1 cells were plated in 24-well plates at 33% confluence or in 150 cm^2 flasks at approx. 2×10^6 cells. After 24 h growth, the cells were washed twice with OptI Mem and transfected by using Lipofect-Amine as cationic lipid (DNA/lipid, 1:10; 0.2 μg of DNA/well or 5 μg of DNA/flask) for a single transfection. Half the amount of each clone was used for the double transfection. The cells were incubated for 3 h, and Dulbeccos's modified Eagle's medium containing 10% (v/v) foetal-calf serum was added. After 24 h the medium was replaced with fresh medium and the cells were incubated for another 24 h. After transient expression, membranes were isolated and solubilized with 1% (w/v) Triton X-100 as described previously [21] and then subjected to Western blotting or enzyme assay.

Site-directed mutagenesis

Point mutations were introduced using a 'megaprimer' for rabbit NEP and, for human ECE-1β, by 'overlap extension' of the PCR approach described by Reikofski and Tao [22].

The following primers were used for constructing the different mutants. Rabbit NEP: forward primer, 5′-CTCTATAACAAA-ATGACATTGGCCCAG-3′; reverse primer, 5′-ATCCTTTTC-GCAATAACCTAATTTCAT-3′; mutagenic primer E403C (**GAA** → **TGT**), 5′-CCAAGTTGCAGA**ACA**TGATGTGGTG-CCAT-3′ (antisense). Human ECE-1β: forward primer, 5′-ATCGAGGAGCTCAGGGCCAAACCTC-3′; reverse primer, 5′-GGGGGCTTTCCTGAGCTGATCGGC-3′; mutagenic primers C376S (**TGC** → **AGC**), 5′-ACCACCGACAGA **AGC**CTGCTCAACA-3′ (sense) and 5′-TGTTGAGCAG**GCT** TCTGTCGGTGGT-3′ (antisense); C416S (**TGT** → **AGT**), 5′-ACCAAGAAGACC**AGT**CTTCCTCGCT-3′ (sense) and 5′-AGCGAGGAA**ACT**GGTCTTCTTGGT-3′ (antisense); C416E (**TGT** → **GAG**), 5′-ACCAAGAAGACC**GAG**CTTCCTCGCT-3′ (sense) and 5′-AGCGAGGAA**CTC**GGTCTTCTTGGT-3′ (antisense). Bold type indicates the mutated nucleotides; mutated codons are underlined.

Mutagenesis was performed in two PCR reactions. PCR I involved 32 cycles (at 95 °C for 45 s, 50 °C for 90 s and 72 °C for 90 s), using forward and mutagenic antisense or reverse and

mutagenic sense primers, incorporating the desired base changes. This material was then purified on an agarose gel and used for the second PCR (32 cycles at 95 °C for 45 s, 55 °C for 90 s and 72 °C for 90 s), which made a full-length product. The PCR II product was restricted by *Bst*XI and *Bst*EII, then used to replace the equivalent non-mutated fragment in rabbit NEP or human ECE-1 expression vectors respectively. The fidelity of the mutants was confirmed by sequencing of both nucleotide strands.

SDS/PAGE and immunoblotting

PAGE was performed as described by Laemmli [23], using a 5 %-stacking and a 5–15 %-gradient separating gel. The gels were blotted [24] using a semi-dry blot with primary antibody AEC32-236, which recognizes the C-terminal domain of human ECE-1 [25], and 18B5, which recognizes a linear epitope of the rabbit NEP sequence [26]. For immunocytochemistry, antibody 23B11 was used [27]. The detection of protein was performed by using chemical luminescence (ECL kit; Amersham). Pre-stain molecular-mass standards were run in parallel.

Protein determination

Protein concentrations were determined using the bicinchoninic acid method of Smith et al. [28] modified for use in 96-well microtiter plates [29] with BSA as standard.

Enzyme assay

NEP activity and IC_{50} values for inhibitors were measured using either 150 μM [D-Ala^2,Leu^5]enkephalin [30] or 250 μM Suc-Ala-Ala-Leu-NH-Np as substrate [31]. Product formation was linear with time, and protein concentration in the range used with either substrate and no background hydrolysis of substrates was observed in non-transfected COS-1 cell membranes. With [D-Ala^2,Leu^5]enkephalin, assays were carried out in 100 μl of 100 mM Tris/HCl buffer, pH 7.4, at 37 °C for 2 h, and the reaction was stopped by boiling at 100 °C for 4 min. The product Tyr-D-Ala-Gly was separated and quantified by HPLC as described previously [30]. K_m and V_{max} values were determined using substrate concentrations in the range 25–250 μM substrate and 5 μg of solubilized membrane fraction.

With Suc-Ala-Ala-Leu-NH-Np as substrate, solubilized membrane protein (5 μg) was incubated with or without inhibitors in 20 μl of reaction buffer (50 mM Tris/HCl, 100 mM NaCl and 10 mM $CaCl_2$, pH 7.4) for 30 min at 37 °C. The reaction was started by adding 180 μl of reaction buffer containing substrates and *S. griseus* aminopeptidase. The rate of reaction was measured by following the increase in absorbance at 405 nm. K_m and V_{max} values were measured in the range 40–400 μM of substrate concentration.

RESULTS AND DISCUSSION

Sequence analysis

Cys^{412} in rat ECE-1α [25] aligns with Cys^{416} in human ECE-1β [32] and with a glutamic acid residue in NEP (Figure 1A; Glu^{403} in all species) [33]. There is a conserved YGT motif six or seven residues N-terminal to this residue and a conserved tryptophan four residues on the C-terminal side. It is most likely that a residue involved in intermolecular covalent bonding will lie in a surface loop. As this part of the sequence is not part of the thermolysin-like domain of these proteins, no information can be gained about its likely structure from similarity with the bacterial protein. However, prediction with the PredictProtein algorithm [20] shows that in both human ECE-1 and rabbit NEP this residue is most likely to lie within a loop between two helices (Figure 1B). However, it should be noted that, especially in ECE, this potential dimerization site is located within a region rich in positively charged residues. In order for dimerization to take place, the protein fold in this region will need to keep these charges from approaching each other and so destabilizing the intermolecular interaction. In order to test these predictions, Glu^{403} in NEP was changed to Cys, and Cys^{376} and Cys^{416} in human ECE-1 have been mutated either to Glu (equivalent to wild-type NEP) or to Ser.

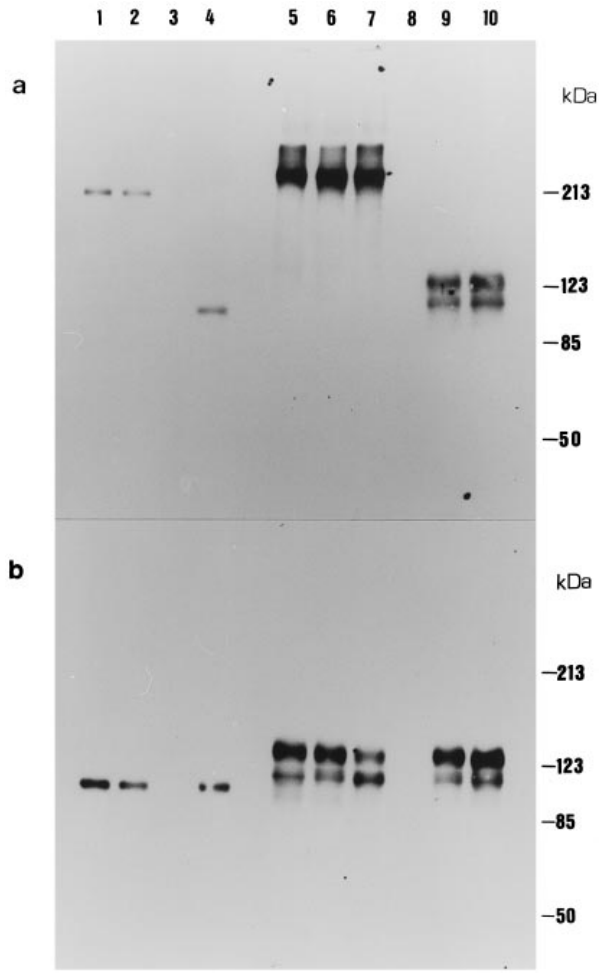

Figure 2 Immunoblot analyses of wild-type and cysteine mutants of NEP and ECE

Immunoblots of 15 μg portions of membrane fractions obtained from COS-1 cells transfected with wild-type NEP, wild-type ECE-1, mutant NEP and mutant ECE-1. The samples were subjected to SDS/PAGE (5–15 %-gradient gel) and analysed by immunoblotting using antibody 18B5 for NEP and AEC32-236 for ECE-1. (**a**, **b**) Electrophoresis carried out in the absence and presence of β-mercaptoethanol respectively. Lanes 1, E403C-NEP; lanes 2, double transfection of E403C-NEP and wild-type ECE-1; lanes 3, non-transfected COS-1 cells; lanes 4, wild-type NEP; lanes 5, wild-type ECE-1; lanes 6, double transfection of E403C-NEP and wild-type ECE-1; lanes 7, C376S-ECE-1; lanes 8, non-transfected COS-1 cells; lanes 9, C416E-ECE-1; lanes 10, C416S-ECE-1.

Expression and immunoblotting of NEP and ECE mutants

The various forms of NEP and ECE-1 were expressed in COS-1 cells, and the molecular forms present were analysed by immunoblotting. As seen in Figure 2(a), under non-reducing conditions

Table 1 Kinetic parameters of wild-type and E403C mutant NEP expressed in COS-1 cells

Membrane fractions were prepared from COS-1 cells transfected either with wild-type or E403C mutant rabbit NEP as described in the Experimental procedures section. The results were determined from five independent transfections and represent means ± S.E.M. PR, phosphoramidon; TH, thiorphan.

Substrate	Clones	K_m (μM)	V_{max} (nmol · min^{-1} · mg^{-1})	V_{max}/K_m (min^{-1} · mg^{-1} · ml)	IC_{50} (nM) PR	IC_{50} (nM) TH
[D-Ala^2,Leu^5]Enkephalin	Wild type	88.3 ± 3.1	20.5 ± 1.0	0.23 ± 0.01	3.3 ± 0.2	5.3 ± 0.5
	E403C	131.7 ± 5.3	19.7 ± 1.2	0.14 ± 0.01	10.7 ± 1.3	14.8 ± 1.5
Suc-Ala-Ala-Leu-NH-Np	Wild type	151.0 ± 13.3	234.8 ± 11.4	1.55 ± 0.16	4.3 ± 0.4	7.1 ± 0.8
	E403C	214.4 ± 22.0	140.0 ± 8.2	0.65 ± 0.08	20.8 ± 3.1	42.6 ± 4.8

E403C-NEP (lane 1) migrated as a dimer at approx. 200 kDa, whereas the wild-type was 92 kDa (lane 4). Wild-type human ECE-1β and the C376S mutant migrated at 260–300 kDa (lanes 5 and 7), whereas both C416E human ECE-1β or C416S ECE-1β were seen at 130 kDa (lanes 9 and 10). A minor additional band (at approx. 105 kDa) was seen consistently in the case of expressed ECE that has been observed previously and represents a proteolytic degradation product [34]. Under reducing conditions (Figure 2b), the wild-type and E403C-NEP mutant migrated identically at 92 kDa (lanes 1 and 4). The wild-type and mutant forms of ECE-1β all appear to be 130 kDa (lanes 5, 7, 9 and 10). Non-transfected COS-1 cells did not show the presence of either NEP or ECE-1 (lanes 3 and 8). This result clearly indicates that the E403C mutant of NEP creates a disulphide-linked homodimer, and Cys^{416}, rather than Cys^{376}, is established as the residue involved in intermolecular disulphide bonding in human ECE-1β. This is consistent with a previous report that the sole cysteine involved in dimerization of rat ECE-1α is the equivalent residue, Cys^{412} [15].

When E403C-NEP cDNA was co-transfected with wild-type ECE-1 cDNA and the expressed proteins subjected to immunoblotting, there was no evidence for formation of a NEP–ECE heterodimer, since no additional intermediate polypeptide band was seen when blotting with either the NEP antibody (Figure 1A, lane 2) or the anti-ECE-1 antibody (lane 6). These lanes appear identical to that of expressed wild-type cDNA, confirming that only homodimers can form. Even when the immunoblot was substantially overdeveloped, no intermediate band corresponding to a possible heterodimer was observed. Thus there is not sufficient similarity between ECE-1 and NEP to allow heterodimer formation. Immunocytochemical analysis of the wild type or the E403C mutant of NEP in transfected COS-1 cells showed no significant difference in cellular localization (results not shown), implying that covalent dimerization of NEP does not interfere with the efficient targeting of the protein to the cell surface.

The efficiency with which the E403C-NEP can form a homodimer can vary among different transfections from $> 95\%$ (Figure 1A) to 60% (results not shown). When only partial dimerization occurred, additional minor breakdown products of 130 and 45 kDa were occasionally seen in addition to the monomer and dimer. This suggests some constraint on dimerization leading to proteolysis between the membrane surface and the site of disulphide linkage in the extracellular domain.

Kinetic studies

Kinetic studies on mutant E403C-NEP were performed from five independent batches of transfections, in which the homodimer formed with negligible level of proteolysis (Figure 2). The homodimer was able to hydrolyse two different and typical NEP substrates, and the K_m was significantly increased ($P < 5\%$) by approx. 50% for both substrates (Table 1). No significant change in the V_{max} was noted. It has previously been reported that the natural ECE-1α dimer converts its substrate more efficiently than the mutant monomer [15]. In the case of NEP the converse appears to be true, presumably because of the constraint placed upon the enzyme by the forced dimerization. The binding of the inhibitors phosphoramidon and thiorphan to the E403C-NEP mutant was significantly less potent than to the wild-type enzyme ($P < 0.5\%$) but still well within the nanomolar range (Table 1). Dimerization of NEP does not, therefore, appear to confer upon it any ECE-like characteristics (e.g. inability to hydrolyse NEP substrates or μM affinity for the inhibitors). Other structural features yet to be determined within these proteins must therefore confer upon them their distinctive metabolic abilities.

Conclusions

NEP and ECE-1 share 40% sequence identity, indicating that these two proteins are likely to have the same fold. A striking difference between NEP and ECE-1, however, is that only the latter exists as a disulphide-linked dimer. Proteins with similar folds are likely to differ in their surface loops. In both NEP and ECE-1, the segment equivalent to that containing Cys^{412} in rat ECE-1α is predicted to form a loop between two helices. The fact that dimerization through a disulphide linkage can occur in the mutant NEP establishes unequivocally that both the secondary structure and the local tertiary structure at this site, which is very likely to be on the protein surface, are highly similar to that of ECE. Even a small deviation at this point would hinder dimerization.

Both mutant ECE monomers, and mutant NEP dimers, are able to hydrolyse substrates and bind inhibitors in an equivalent fashion to the respective wild types. Covalent dimer formation therefore cannot account for the highly restricted specificity of ECE. This implies that dimerization is remote from the active site, and that residues from one monomer are unlikely to form part of the active site in the other monomer. It is possible that the extracellular portions of both NEP and ECE-1 contain two domains: an N-terminal one containing the dimerization site, and a C-terminal, thermolysin-like region containing the active site.

In ECE-1, the dimerization site is surrounded by a cluster of positively charged residues (..KKTCLPR..) (Figure 1). The local fold of the polypeptide chain at this position must prevent positive charges in alternate monomers from repelling each other

and so preventing the formation of the covalent bond. It is very unlikely that this loop would form a non-covalent dimer interface if the cysteine residues were removed and, indeed, the C412S rat ECE-1α mutant has been shown to be monomeric on a native polyacrylamide gel [15]. In NEP, any positively charged residues are located further from Glu^{403} (Figure 1), and it is more likely that this segment could form part of a non-covalent dimer interface. Both rat and human NEP form non-covalent dimers. Thus, although no structural information is yet available on NEP or ECE-1, the present data establish that their sequence similarity is reflected in highly similar orientations of their polypeptide chains, allowing the novel conversion of wild type NEP to a fully functional covalent dimer.

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