# Characterization of a secretase activity which releases angiotensin-converting enzyme from the membrane

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Angiotensin-converting enzyme (ACE; EC 3.4.1.15.1) exists in both membrane-bound and soluble forms. Phase separation in Triton X-114 and a competitive e.l.i.s.a. have been employed to characterize the activity which post-translationally converts the amphipathic, membrane-bound form of ACE in pig kidney microvilli into a hydrophilic, soluble form. This secretase activity was enriched to a similar extent as other microvillar membrane proteins, was tightly membrane-associated, being resistant to extensive washing of the microvillar membranes with 0.5 M NaCl, and displayed a pH optimum of 8.4. The ACE secretase was not affected by inhibitors of serine-, thiol- or aspartic-proteases, nor by reducing agents or $\alpha_2$-macroglobulin. The metal chelators, EDTA and 1,10-phenanthroline, inhibited the secretase activity, with, in the case of EDTA, an inhibitor concentration of 2.5 mM causing 50% inhibition. In contrast, EGTA inhibited the secretase by a maximum of 15% at a concentration of 10 mM. The inhibition of EDTA was reactivated substantially (83%) by $Mg^{2+}$ ions, and partially (34% and 29%) by $Zn^{2+}$ and $Mn^{2+}$ ions respectively. This EDTA-sensitive secretase activity was also present in microsomal membranes prepared from pig lung and testis, and from human lung and placenta, but was absent from human kidney and human and pig intestinal brush-border membranes. The form of ACE released from the microvillar membrane by the secretase co-migrated on SDS/PAGE with ACE purified from pig plasma, thus the action and location of the secretase would be consistent with it possibly having a role in the post-translational proteolytic cleavage of membrane-bound ACE to generate the soluble form found in blood, amniotic fluid, seminal plasma and other body fluids.

## INTRODUCTION

A number of transmembrane proteins can also be found in soluble form as a result of a post-translational proteolytic processing event (reviewed in Ehlers and Riordan, 1991b). Proteins that fall into this category are functionally diverse, including receptors and receptor ligands, cell-adhesion molecules, leucocyte antigens, viral membrane proteins and ectoenzymes. Probably the most notable of this type of protein is the amyloid precursor protein, the normal post-translational cleavage of which by a putative secretase activity may be defective in Alzheimer's disease (Esch *et al.*, 1990; Selkoe, 1990; Wang et al., 1991). Although a significant number and variety of transmembrane proteins are now known to be released by proteolysis in an active form, little is known about the secretase activities responsible for these processes, and such proteolytic activities may prove to be novel targets for therapeutic intervention.

Angiotension-converting enzyme (ACE; peptidyl dipeptidase A; EC 3.4.15.1) is a widely distributed cell-surface peptidase which plays a key role in the control of blood pressure and fluid and electrolyte homoeostasis (Soffer, 1976). However, ACE does not always co-localize with other components of the renin–angiotensin system and the enzyme displays a broad substrate specificity, which has led to ACE being implicated in a number of other processes including immunity, reproduction and neuropeptide metabolism (reviewed in Ehlers and Riordan, 1989; Hooper, 1991). In mammals ACE exists as two distinct isoenzymes. The endothelial isoenzyme ($M_r$ 180000 in the pig) is present throughout the body and is composed of two highly similar domains, each of which bears a functional catalytic site (Soubrier et al., 1988; Ehlers and Riordan, 1991a; Wei et al., 1991b, 1992; Williams et al., 1992). The other isoenzyme ($M_r$ 110000 in the pig) is found exclusively in the testis and, with the exception of approx. 67 amino acids at the N-terminus, is identical to the C-terminal domain of endothelial ACE, thus containing only a single catalytic site (Lattion et al., 1989).

The observation that both the hydrophilic, trypsin-solubilized and amphipathic, detergent-solubilized forms of pig kidney ACE, which differ in $M_r$ by approx. 10000, possessed identical N-terminal sequences led us to propose that ACE was not anchored in the membrane by an uncleaved N-terminal signal sequence but was probably attached via its C-terminus (Hooper et al., 1987). The cDNA-predicted amino-acid sequence of endothelial ACE contains, apart from the cleavable N-terminal signal sequence, only one other region of high hydrophobicity which could possibly traverse the lipid bilayer and this is located 30 residues from the C-terminus (Soubrier et al., 1988). Expression in Chinese hamster ovary cells of a mutant cDNA encoding endothelial ACE in which the bases coding for the C-terminal 47 amino acids, including the putative transmembrane domain, had been deleted resulted in the secretion of a soluble form of ACE (Wei et al., 1991a). Similarly, the expression of mutants of testicular ACE lacking the C-terminal hydrophobic domain resulted in the production of a soluble, secreted form of this isoenzyme (Ehlers et al., 1991; Sen et al., 1991). A recent study has shown that ACE in human intestinal membranes possesses a small cytoplasmic domain that is susceptible to trypsin cleavage (Naim, 1992), and thus it would appear that the hydrophobic region at the C-terminus is responsible for the transmembrane anchoring of ACE.

Abbreviations used: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid); ACE, angiotensin-converting enzyme; Bz-Gly-His-Leu, benzyl-glycylhistidyl-leucine.

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Although ACE exists primarily as a membrane-bound enzyme, a soluble form is present under normal conditions in blood plasma, amniotic fluid, seminal plasma and other body fluids (reviewed in Erdös and Skidgel, 1987; Hooper, 1991). In certain diseases such as sarcoidosis, diabetes mellitus, Gaucher's disease, leprosy and hyperthyroidism the levels of soluble ACE in plasma are known to be altered, and, in the case of sarcoidosis, the abnormally high level of ACE is used as a diagnostic aid (Studdy et al., 1983; Erdös and Skidgel, 1987). The enzyme in plasma appears to be catalytically identical and immunologically very similar to membrane-bound endothelial ACE (Das et al., 1977; Lanzillo and Fanburg, 1977; Lanzillo et al., 1985). However, ACE purified from plasma is hydrophilic and is not recognized by a polyclonal antiserum raised to a synthetic peptide corresponding to the 20 cytoplasmic C-terminal amino acids of endothelial ACE (Wei et al., 1991a), indicating that it probably lacks the C-terminal hydrophobic anchoring domain. However, the mechanism of production of the soluble form of ACE in normal and disease situations is unknown. While studying the membrane anchorage of pig kidney ACE, we observed that the enzyme could be released from the membrane in a soluble form by an EDTA-sensitive secretase (Hooper et al., 1987). The released ACE was hydrophilic as assessed by phase separation in Triton X-114 and its failure to reconstitute into artificial lipid vesicles. In the present study we have further characterized the secretase activity which is capable of post-translationally cleaving membrane-bound ACE to generate a catalytically active, soluble form of the enzyme.

## EXPERIMENTAL

### Materials

Pig tissues were kindly provided by ASDA Farmstores, Lofthousegate, W. Yorkshire, U.K. and were obtained within 10 min of death. Adult male New Zealand white rabbits (2–3 kg) were used to raise polyclonal antibodies and were provided and maintained by the University of Leeds Animal Service. Biotinylated rabbit immunoglobulin from donkey and streptavidin-biotinylated horseradish peroxidase complex were purchased from Amersham International, Aylesbury, Bucks., U.K. 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), benzylglycylhistidylleucine (Bz-Gly-His-Leu), Triton X-114, benzamidine, iodoacetamide, *p*-chloromercuriphenylsulphonic acid and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Triton X-114 was precondensed before use (Bordier, 1981). EDTA and EGTA were purchased from BDH, Poole, Dorset, U.K. 1,10-Phenanthroline was purchased from Aldrich Chemicals, Gillingham, U.K. $\alpha_2$-Macroglobulin was purchased from Boehringer–Mannheim, Lewes, East Sussex, U.K. Lisinopril {MK 521,*N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline} and enalaprilat {MK 421, *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-alanyl-L-proline} were gifts from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. Zofenoprilat (SQ 26,703) and captopril (SQ 14,225) were gifts from Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, U.S.A. Thiorphan was a gift from Dr. B. Roques (Paris). All other materials were from sources noted previously. Protein was determined using bicinchoninic acid in a microtitre plate assay with BSA as standard.

### Purification of ACE

ACE was purified from pig kidney or plasma by a modification of the method described in Hooper et al., (1987) using lisinopril-2.8 nm-Sepharose as the affinity resin. The major difference was that bound enzyme was eluted with 50 mM $Na_2B_4O_7$/0.1 % (w/v) Triton X-100, pH 9.5 (Ehlers et al., 1986) instead of using free lisinopril.

### Enzyme assays

ACE enzymic activity was determined with Bz-Gly-His-Leu (5 mM) as substrate in 0.1 M Tris/HCl, 0.3 M NaCl, 10 $\mu$M $ZnCl_2$, pH 8.3. Reactions were terminated by heating at 100 °C for 5 min, and the substrate and reaction products resolved and quantified by reverse-phase h.p.l.c. as described by Hooper and Turner (1987). Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate as substrate, cathepsin C with L-histidyl-L-serine 2-naphthylamide as substrate, and NADPH-cytochrome *c* reductase and succinate dehydrogenase with 2,6-dichlorophenol indophenol as acceptor as described in Booth and Kenny (1974).

### E.l.i.s.a.

This was a modification of the methods described previously (Lanzillo and Fanburg, 1982; Dasarathy et al., 1990; Hooper et al., 1991). Affinity-purified pig kidney ACE was diluted in freshly prepared 0.05 M carbonate buffer, pH 9.6, and added to each well of a 96-well microtitre plate (100 ng of protein/well). The plates were rocked for 10 min at room temperature before incubation overnight at 4 °C. The wells were then washed twice with PBS (2 mM $NaH_2PO_4$/20 mM $Na_2HPO_4$/0.25 M NaCl, pH 7.4), filled with 1 % (w/v) BSA, and incubated for 3 h at room temperature. The wells were then washed four times with PBS, filled with PBS and stored at 4 °C. A polyclonal antibody raised to affinity-purified pig kidney ACE (Hooper and Turner, 1987) was diluted 1:30000 in 0.1 % (w/v) BSA in PBS and incubated with either known amounts of purified ACE (for a standard curve), or the phase-separated membrane samples for 6 h at 37 °C. The samples were centrifuged at 8800 *g* for 10 min and triplicate aliquots (0.1 ml) transferred to the coated microtitre plate wells and incubated overnight at 4 °C. The wells were then washed twice with PBS, twice with PBS containing 0.05 % (v/v) Tween 20, and finally twice with PBS. Biotinylated rabbit immunoglobulin [1:750 dilution in 0.1 % (w/v) BSA in PBS] was added to each well and incubated for 1 h at 37 °C. After washing as before, bound antibodies were detected with streptavidin-biotinylated horseradish peroxidase complex [1:1500 dilution in 0.1 % (w/v) BSA in PBS] and incubated for 1 at 37 °C. The wells were washed as before and the assay developed with 1 mM ABTS in 0.2 M citrate/sodium phosphate buffer, pH 4.3, containing 0.05 % (v/v) $H_2O_2$. After incubation at 23 °C in the dark, the reaction was terminated by the addition of 0.01 % (w/v) $NaN_3$ in 0.1 M citric acid and the absorbance was measured at 405 nm in an Anthos 2001 micro-titre plate reader. The e.l.i.s.a. detected purified pig kidney ACE with a sensitivity of 32 ng per ml.

### Membrane preparations

All operations were carried out at 4 °C. Microvilli were prepared from kidney cortex by the method of Booth and Kenny (1974), except that the 15000 *g* centrifugation steps were each extended from 12 min to 15 min, and from intestine by the method of Kessler et al. (1978). Crude microsomal membranes were prepared from lung, placenta and testis by homogenization in 10 vol. of 0.32 M sucrose/50 mM Hepes/NaOH, pH 7.4 and the homogenate centrifuged at 8000 *g* for 15 min. The supernatant was then centrifuged at 26000 *g* for 2 h and the resulting microsomal pellet resuspended in 10 mM Hepes/NaOH, pH 7.4.

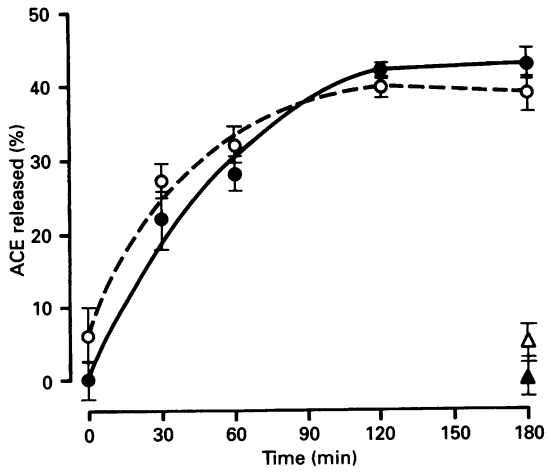


**Figure 1 Release of ACE from pig kidney microvillar membranes: correlation of enzyme activity and the e.l.i.s.a**

Pig kidney microvillar membranes (160 $\mu$g of protein) were incubated in 0.2 M Tris/maleate, pH 8.4 at 37 °C (○, ●) or 4 °C (△, ▲). After phase separation in Triton X-114, ACE was quantified in the detergent-rich and aqueous phases either by measuring enzyme activity with Bz-Gly-His-Leu (open symbols) or using the e.l.i.s.a. (closed symbols) as described in the Experimental section. The results are the mean (±S.E.M.) of triplicate determinations.

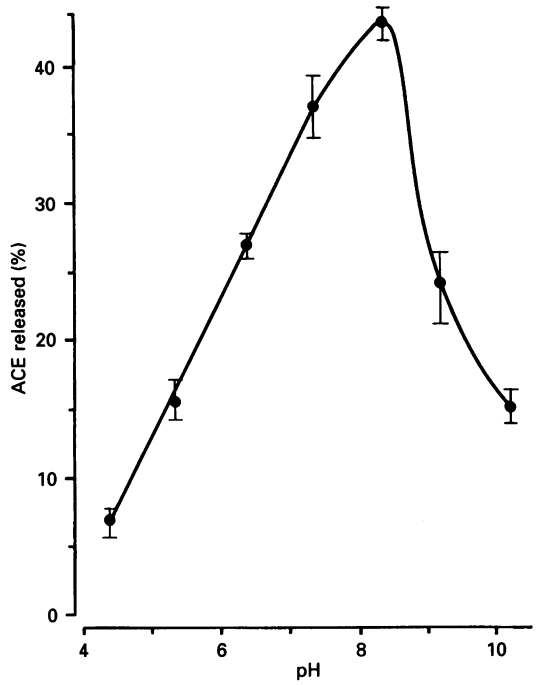


**Figure 2 Effect of pH on the release of ACE from pig kidney microvillar membranes**

Pig kidney microvillar membranes (160 $\mu$g of protein) were incubated in the appropriate buffer for 2 h at 37 °C. After phase separation in Triton X-114, ACE was quantified in the detergent-rich and aqueous phases using the e.l.i.s.a. as described in the Experimental section. The results are the mean (±S.E.M.) of triplicate determinations. Buffers used: 0.2 M sodium acetate, pH 4.4; 0.2 M. Tris/maleate, pH 5.4–8.4; 0.2 M glycine/NaOH, pH 9.2–10.2.

In the case of the placental membranes the resuspended microsomal pellet was washed further by centrifugation at 31000 ***g*** for 90 min.

### Release of ACE from membranes

Membrane samples were incubated in 0.2 M Tris/maleate, pH 8.4, at 37 °C. Controls were incubated at 4 °C. The reactions were terminated by placing the tubes in ice, before phase separation in Triton X-114 (Bordier, 1981). An equal volume of 10 mM Tris/HCl, 0.15 M NaCl, 2% (v/v) Triton X-114 (pH 7.4) was added to each sample and then incubated on ice for 5 min. The samples were then incubated at 30 °C for 3 min before centrifugation at 3000 ***g*** for 5 min. The resulting aqueous and detergent-rich phases were diluted appropriately and assayed either for enzymic activity with Bz-Gly-His-Leu as substrate or for enzyme protein by the e.l.i.s.a. The release of ACE is equivalent to the amount of ACE in the final aqueous phase as a percentage of the total amount of ACE in both the aqueous and detergent-rich phases. The final dilution of the detergent-rich phase for the e.l.i.s.a. was approx. 1:1000.

### Immunoelectrophoretic-blot analysis

SDS/PAGE was performed with a 7–17% (w/v) polyacrylamide gradient as described previously (Relton et al., 1983). Immunoelectrophoretic-blot analysis was carried out with Immobilon P [poly(vinylidene) difluoride] membranes and a $^{125}$I-labelled second antibody as detailed in Hooper and Turner (1987) and Hooper et al. (1990).

## RESULTS

### General properties of the process that releases ACE from pig kidney microvillar membranes

Incubation of pig kidney microvillar membranes at 37 °C resulted in the conversion of ACE from an amphipathic form to a hydrophilic form, as assessed by phase separation in Triton X-114 (Figure 1). This release of ACE from the membranes was quantified either by measuring enzymic activity with Bz-Gly-His-Leu as substrate, or by measuring the amount of enzyme protein with the competitive e.l.i.s.a. Both methods essentially gave the same result. At 37 °C the release of ACE from the membranes was relatively rapid, reaching a maximum of approx. 40% after 2 h. At 4 °C, maximally 5% of ACE was released (Figure 1). The effect of pH on the release of ACE from pig kidney

**Table 1 Enrichment of the activity that releases ACE in pig kidney microvillar membranes**

Enzymes were assayed as described in the Experimental section. Enzyme units are nmol/min, except for the secretase which are ng of ACE cleaved/min. The results are given as the mean (±S.E.M.) from three separate microvillar membrane preparations.

| Enzyme | Specific activity (units/mg of protein) | | Enrichment (fold) |
|---|---|---|---|
| | Homogenate | Microvillar membranes | |
| Secretase | 90.1±9.7 | 1103.7±40.2 | 12.2 |
| ACE | 54.5±6.8 | 502.8±113.4 | 9.2 |
| Alkaline phosphatase | 132.7±20.0 | 1094.1±259.2 | 8.0 |
| Cathepsin C | 3.43±0.42 | 1.17±0.32 | 0.3 |
| NADPH-cytochrome *c* reductase | 0.37±0.03 | 0.23±0.08 | 0.6 |
| Succinate dehydrogenase | 0.49±0.04 | 0.09±0.01 | 0.2 |

**Table 2 Determination of the membrane-association of the activity that releases ACE from pig kidney microvillar membranes**

Pig kidney microvillar membranes (160 $\mu$g of protein) were washed five times by resuspension in 10 mM Hepes, pH 7.4 in the absence or presence of 0.5 M NaCl followed by centrifugation at 31000 *g* for 90 min. The membranes were finally resuspended in 0.2 M Tris/maleate, pH 8.4, incubated for 2 h at 37 °C and the release of ACE quantified by measuring enzyme activity in the detergent-rich and aqueous phases following phase separation in Triton X-114 as described in the Experimental section. The results are the mean (±S.E.M.) of triplicate determinations.

| Treatment | ACE released (%) | Activity of ACE in final membrane preparation (nmol of Bz-Gly/min per ml) |
|---|---|---|
| Unwashed membranes | 42.6±1.1 | 129.0±2.8 |
| Washed membranes | | |
| no NaCl | 45.3±3.8 | 118.3±3.2 |
| +0.5 M NaCl | 42.6±1.5 | 114.8±5.5 |

**Table 3 Effect of protease inhibitors on the release of ACE from pig kidney microvillar membranes**

Pig kidney microvillar membranes (160 $\mu$g of protein) were preincubated in 0.2 M Tris/maleate (pH 8.4) in the absence or presence of the indicated inhibitors for 15 min on ice and then incubated for 2 h at 37 °C. After phase separation in Triton X-114, ACE was quantified in the detergent-rich and aqueous phases using the e.l.i.s.a. as described in the Experimental section. The results are the mean (±S.E.M.) of triplicate determinations.

| Class of protease | Inhibitor | Concentration | ACE released (%) |
|---|---|---|---|
| – | None | – | 100.0 |
| All classes | $\alpha_2$-Macroglobulin | 1 unit/mg | 95.5±6.0 |
| Metallo | EDTA | 5 mM | 2.2±4.4 |
| | EGTA | 5 mM | 85.9±2.9 |
| | 1,10-Phenanthroline | 5 mM | 0.4±0.3 |
| Serine | Aprotinin | 1 mM | 93.2±2.8 |
| | Benzamidine | 1 mM | 92.6±0.9 |
| | Phenylmethanesulphonylfluoride | 1 mM | 90.8±3.0 |
| Thiol | Iodoacetamide | 1 mM | 94.1±1.2 |
| | *p*-Chloromercuriphenylsulphonic acid | 1 mM | 99.0±2.4 |
| Aspartic | Antipain | 10 $\mu$g/ml | 93.6±2.8 |
| | Pepstatin A | 10 $\mu$g/ml | 97.9±0.0 |
| Disulphide | Dithiothreitol | 1 mM | 98.3±0.4 |
| | 2-Mercaptoethanol | 1 mM | 101.4±5.0 |
| ACE | Captopril | 1 $\mu$M | 91.7±2.4 |
| | Enalaprilat | 1 $\mu$M | 93.6±0.4 |
| | Zofenoprilat | 1 $\mu$M | 97.3±2.4 |
| Endopeptidase-24.11 | Phosphoramidon | 0.1 mM | 89.3±1.2 |
| | Thiorphan | 0.1 mM | 82.4±1.8 |

microvillar membranes was examined (Figure 2). The release process displayed a pH optimum of 8.4. The activity that releases ACE from the membrane was enriched in the microvillar membranes to a similar extent as both ACE itself and alkaline phosphatase, another microvillar membrane marker (Table 1). In contrast the mitochondrial marker, succinate dehydrogenase, the lysosomal marker, cathepsin C, and the endoplasmic reticulum marker, NADPH-cytochrome *c* reductase, were not enriched in the microvillar membrane preparation, in agreement with previous results (Booth and Kenny, 1974). The membrane association of the releasing activity was then investigated (Table 2). No decrease in the amount of ACE released from the membranes was observed after extensively washing the microvillar membranes either in the absence or presence of 0.5 M NaCl. Under the same conditions no significant decrease in the amount of ACE activity associated with the membranes was observed.

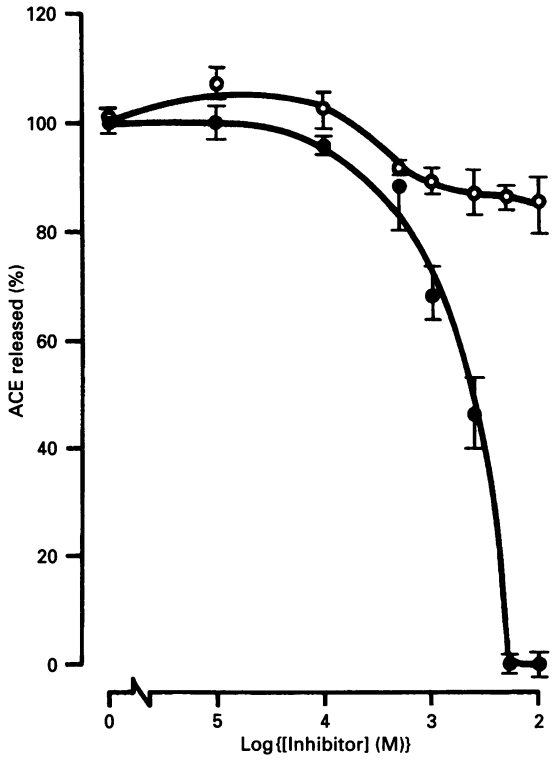


**Figure 3 Effect of EDTA and EGTA on the release of ACE from pig kidney microvillar membranes**

Pig kidney microvillar membranes (160 $\mu$g of protein) were preincubated in 0.2 M Tris/maleate (pH 8.4) in the absence or presence of the indicated inhibitor for 15 min on ice and then incubated for 2 h at 37 °C. After phase separation in Triton X-114, ACE was quantified in the detergent-rich and aqueous phases using the e.l.i.s.a. as described in the Experimental section. The results are the mean (±S.E.M.) of triplicate determinations. ●, EDTA; ○, EGTA.

## Effect of protease inhibitors on the release of ACE from pig kidney microvillar membranes

The effect of protease inhibitors on the release of ACE from pig kidney microvillar membranes was examined (Table 3). The release of ACE was not affected by $\alpha_2$-macroglobulin, nor by inhibitors of serine-, thiol- or aspartic-proteases, and the reducing agents dithiothreitol and 2-mercaptoethanol also had no inhibitory effect. However, the chelating agents EDTA and 1,10-phenanthroline (at 5 mM), caused significant (> 97%) inhibition of the release of ACE from the membranes. In contrast, EGTA (at 5 mM) caused maximally 15% inhibition of the release of ACE. The differential inhibition of the release of ACE by EDTA and EGTA was examined in more detail (Figure 3). EDTA caused 50% inhibition of the release process at a concentration of 2.5 mM, whereas EGTA caused maximally 15% inhibition at a concentration of 10 mM. The release of ACE from the membranes was not inhibited by the potent inhibitors of ACE, captopril, enalaprilat and zofenoprilat, nor by the inhibitors of endopeptidase-24.11 (EC 3.4.24.11), phosphoramidon and thiorphan (Table 3). The aminopeptidase inhibitors, amastatin, bestatin and puromycin also had no inhibitory effect on the release of ACE (results not shown).

**Table 4 Effect of bivalent cations on the release of ACE from pig kidney microvillar membranes**

Pig kidney microvillar membranes (160 $\mu$g of protein) were incubated in 0.2 M Tris/maleate (pH 8.4) in the absence or presence of EDTA (5 mM) and cations (2.5 mM or 7.5 mM respectively). The membranes were preincubated with EDTA for 5 min on ice before the addition of the cations. The samples were then incubated for 15 min on ice before incubation for 2 h at 37 °C. After phase separation in Triton X-114, ACE was quantified in the detergent-rich and aqueous phases using the e.l.i.s.a as described in the Experimental section. Results are the mean (±S.E.M.) of quadruplicate determinations.

| | ACE released (%) | |
|---|---|---|
| Bivalent cation | Cations (2.5 mM) only | EDTA (5 mM) + cation (7.5 mM) |
| None | 45.2±1.1 | 0.0±0.0 |
| $Mg^{2+}$ | 40.0±1.4 | 33.0±1.7 |
| $Ca^{2+}$ | 40.6±1.2 | 4.5±1.2 |
| $Mn^{2+}$ | 41.8±1.9 | 11.9±1.0 |
| $Co^{2+}$ | 28.0±5.3 | 2.4±0.1 |
| $Ni^{2+}$ | 35.2±6.0 | 0.8±0.4 |
| $Cu^{2+}$ | 38.4±3.3 | 2.2±0.5 |
| $Zn^{2+}$ | 42.4±4.7 | 14.2±2.1 |

**Table 5 Tissue distribution of the activity that releases ACE from the membrane**

Microvillar (duodenum, jejunum and kidney) or microsomal (lung, placenta and testis) membranes (160 $\mu$g of protein) were incubated in 0.2 M Tris/maleate (pH 8.4) in the absence or presence of EDTA (5 mM) for 2.5 h at 37 °C. After phase separation in Triton X-114, ACE was quantified in the detergent-rich and aqueous phases using the e.l.i.s.a. as described in the Experimental section. The results are given as the mean of duplicate determinations and where indicated are the mean (±S.E.M.) of duplicate determinations on three separate tissue samples.

| | | | Amount of ACE released (%) | |
|---|---|---|---|---|
| Species | Tissue | ACE specific activity (nmol of Bz-Gly/min per mg of protein) | In the absence of EDTA | In the presence of EDTA |
| Pig | Duodenum | 87.9±8.8 | 11.8±2.5 | 10.1 |
| | Jejunum | 140.0±10.3 | 14.0±1.4 | 12.3 |
| | Kidney | 262.5±12.4 | 44.7±2.6 | 8.4±2.6 |
| | Lung | 138.6±8.9 | 47.2±4.0 | 13.5±4.7 |
| | Testes | 48.6±5.5 | 39.9±0.8 | 12.6±3.0 |
| Human | Duodenum | 66.0 | 6.4 | 5.9 |
| | Jejunum | 125.5 | 7.0 | 6.1 |
| | Kidney | 116.2±14.1 | 8.8±2.1 | 9.2±3.3 |
| | Lung | 84.3 | 46.4 | 8.3 |
| | Placenta | 8.2 | 45.7 | 12.5 |

## Effect of bivalent cations on the release of ACE from pig kidney microvillar membranes

The ability of bivalent cations to reactive the release process following inhibition by EDTA was examined (Table 4). $Mg^{2+}$ ions reactivated the release of ACE to 82.5% of the uninhibited value while $Zn^{2+}$ and $Mn^{2+}$ ions reactivated the release by 33.5% and 28.5% respectively. The other bivalent cations, $Ca^{2+}$, $Co^{2+}$ and $Cu^{2+}$, reactivated the release process by a maximum of 11.1%. None of the cations alone caused > 15% inhibition of the release of ACE, except for $Ni^{2+}$ and $Ca^{2+}$ which caused 23% and 38% inhibition respectively.

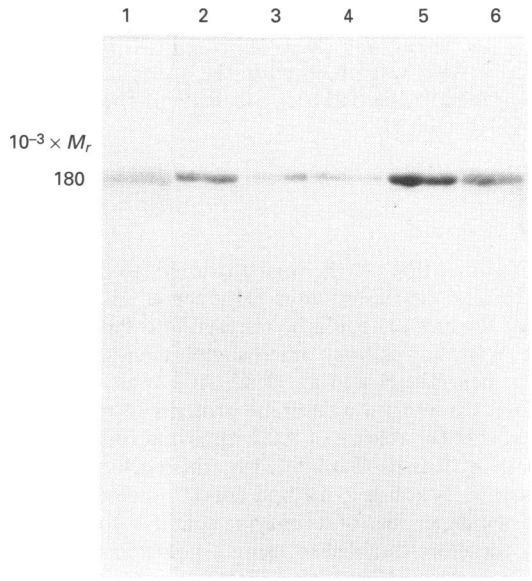


**Figure 4 Immunoelectrophoretic blot of the ACE released from pig microvillar membranes and ACE purified from plasma**

Pig kidney microvillar membranes (160 $\mu$g of protein) were incubated in 0.2 M Tris/maleate (pH 8.4) in the absence or presence of EDTA (5 mM) for 2 h at 37 °C. After phase separation in Triton X-114, the detergent-rich and aqueous phases were subjected to SDS/PAGE and immunoelectrophoretic-blot analysis as described in the Experimental section with a polyclonal antiserum raised to ACE purified from pig kidney (Hooper and Turner, 1987). Bound antiserum was detected with a $^{125}$I-labelled second antibody followed by autoradiography. Lane 1, ACE purified from pig plasma (2.5 $\mu$g of protein); lane 2, aqueous phase after incubation of microvillar membranes in the absence of EDTA; lane 3, detergent-rich phase after incubation of microvillar membranes in the absence of EDTA; lane 4, aqueous phase after incubation of microvillar membranes in the presence of EDTA; lane 5, detergent-rich phase after incubation of microvillar membranes in the presence of EDTA; lane 6, ACE purified from pig kidney after solubilization by incubating membranes at 37 °C (2.5 $\mu$g of protein).

## Tissue distribution of the activity that releases ACE from the membrane

Other pig and human tissues which are relatively rich in ACE were examined for the presence of the activity releasing ACE from the membrane (Table 5). ACE was also released from pig lung and testicular microsomal membranes to a similar extent as from pig kidney microvillar membranes, and in both cases this release was inhibited significantly (> 69%) by EDTA. In contrast to the pig, no significant EDTA-sensitive release of ACE was observed from human kidney microvillar membranes. However, ACE was released from both human lung and placental microsomal membranes, and in both cases the release was inhibited significantly (> 73%) by EDTA. Negligible EDTA-sensitive release of ACE was observed from either pig or human duodenal and jejunal brush-border membranes.

## Comparison of the form of ACE released from pig kidney microvillar membranes with ACE purified from plasma

Pig kidney microvillar membranes were incubated at 37 °C and the resultant aqueous and detergent-rich phases were subjected to SDS/PAGE and immunoelectrophoretic-blot analysis with a polyclonal antiserum raised to purified pig kidney ACE (Figure 4). The ACE released from the microvillar membranes (Figure 4, lane 2) was recognized by the antiserum and co-migrated with ACE purified from pig plasma (Figure 4, lane 1) and with ACE

purified from pig-kidney cortex following its solubilization by incubation of the membranes at 37 °C (Figure 4, lane 6). Less immunoreactive ACE was observed in the aqueous phase after the microvillar membranes had been incubated in the presence of EDTA (Figure 4, lane 4).

## DISCUSSION

While establishing that ACE was anchored to the plasma membrane through its C-terminus (Hooper et al., 1987), we observed that the enzyme could be released into the high-speed supernatant from pig kidney microsomal membranes upon incubation at neutral pH and at 37 °C. ACE was one of only several cell-surface integral membrane proteins so released. We also observed that the release of ACE appeared to be inhibited by EDTA. As ACE is itself inhibited by this chelating agent it was necessary to establish a method for the quantification of ACE that was independent of its enzyme activity. A competitive e.l.i.s.a. was therefore established using a polyclonal antiserum raised to affinity-purified pig kidney ACE (Hooper and Turner, 1987) to quantify enzyme protein. This antiserum also cross-reacts with the smaller testicular isoenzyme (Williams et al. 1992). We also chose to use phase separation in Triton X-114 (Bordier, 1981) to distinguish between the amphiphatic, membrane-bound form of ACE and the hydrophilic, soluble form rather than high-speed centrifugation as it is more rapid. After phase separation in Triton X-114, ACE in the detergent-rich and aqueous phases was accurately and reproducibly quantified by the e.l.i.s.a., the results correlating well with those obtained from measurements of enzyme activity (Figure 1).

The release of ACE from purified pig kidney microvillar membranes by the secretase activity was optimal at 37 °C (results not shown), and negligible at 4 °C (Figure 1), and displayed a pH optimum of 8.4 (Figure 2). The relative lack of contamination of the microvillar enzyme preparation by lysosomal, mitochondrial and endoplasmic reticulum marker enzymes and the co-enrichment of the secretase activity with the microvillar membrane markers alkaline phosphatase and ACE itself (Table 1), would indicate that the presence of the secretase activity is almost certainly not due to contamination of the microvillar membranes by other membrane fractions but that the secretase activity is probably a component of the microvillar membrane. To assess whether the secretase activity was a soluble or peripheral membrane protein, the microvillar membranes were further washed extensively with 0.5 M NaCl (Table 1). No reduction was observed in the amount of ACE released, indicating that the secretase activity that is responsible for the release of ACE is tightly membrane associated, possibly an integral membrane protein.

To determine the class of protease that the ACE secretase belongs to, we examined the effect of class-specific protease inhibitors on the release of ACE from pig kidney microvillar membranes (Table 3). The general protease inhibitor $\alpha_2$-macroglobulin and inhibitors of serine-, thiol- and aspartic-proteases had no effect on the release of ACE. In contrast, the metal chelators, EDTA and 1,10-phenanthroline, caused significant inhibition of the secretase activity. The inhibition by EDTA could be reversed by $Mg^{2+}$ ions, and to a lesser extent by $Zn^{2+}$ and $Mn^{2+}$ ions (Table 4). The lack of significant inhibition by EGTA (Figure 3) and the observation that $Mg^{2+}$ ions were the most effective at reversing the inhibition by EDTA, indicate that the secretase activity is possibly a $Mg^{2+}$-dependent metalloprotease. The lack of inhibition of the secretase by a range of ACE inhibitors indicates that the release is not due to an autocatalytic event, and the failure of either phosphoramidon or thiorphan to cause significant inhibition indicates that endopeptidase-24.11 also is not responsible.

A survey of other tissues which are relatively rich in membrane-bound ACE (Table 5) revealed that, in addition to pig kidney, ACE in both pig and human lung, human placenta and pig testis could be released in a soluble form by an EDTA-sensitive activity at pH 8.4 and 37 °C. In contrast, no significant release of ACE was observed from human kidney or human and pig intestinal microvillar membranes. Membrane-bound endothelial ACE in the lungs is a probable source under normal circumstances of most of the soluble form of ACE in plasma, by virtue of both the high activity of ACE in, and the enormous passage of blood through, this organ (Erdös and Skidgel, 1987). As the form of ACE released from the pig kidney microvillar membranes by the EDTA-sensitive secretase co-migrated on SDS/PAGE with ACE purified from plasma (Figure 4), the presence of the secretase activity in lung microsomal membranes would possibly be consistent with it having a role in the normal physiological generation of the soluble form of ACE in plasma. The presence of the secretase activity in microsomal membranes from testis and placenta and in microvillar membranes from kidney would suggest that the enzyme may have a role in the generation from membrane-bound ACE of the soluble form of ACE found in seminal plasma, amniotic fluid and urine respectively.

Much interest has been focused recently on the family of matrix-degrading metalloproteinases, e.g. collagenase, stromelysin, etc (reviewed in Matrisan, 1990; Woessner, 1991). These proteases degrade extracellular matrix and basement-membrane components and may play important roles in normal processes and in pathological conditions. The matrix-degrading metalloproteinases are characteristically soluble secreted proteins, contain $Zn^{2+}$, and are inhibited by chelating agents and by specific-tissue inhibitors of metalloproteinases. Although the secretase activity characterized in the present study is sensitive to certain chelating agents, it differs from the matrix-degrading metalloproteinases in being tightly membrane-associated, possibly $Mg^{2+}$-dependent, and insensitive to inhibition by $\alpha_2$-macroglobulin.

Several other candidate secretase activities have been identified (reviewed in Ehlers and Riordan, 1991b); however, only a few are metalloproteases and only one has been characterized in sufficient detail to indicate that it may be similar to the activity described in the present study. A $Mg^{2+}$-dependent, EDTA-sensitive protease has been detected in human placental chorionic villi and rat kidney homogenates which appears to be responsible for the release in a soluble active form of the transmembrane folate receptor (Antony et al., 1989; Elwood et al., 1991; Verma and Antony, 1991). However, a recent report (Verma et al., 1992) demonstrated the lack of this $Mg^{2+}$-dependent protease in extensively washed placental membranes, suggesting that the protease is either soluble or a loosely attached peripheral membrane protein, which contrasts with the integral membrane nature of the ACE secretase described in the present study.

In conclusion, we have developed a reproducible, sensitive and rapid assay based on phase separation in Triton X-114 and a competitive e.l.i.s.a. to characterize the process that releases hydrophobic membrane-bound ACE into a soluble, hydrophilic form. This secretase activity is enriched in pig kidney microvillar membranes, is tightly membrane-associated, has a neutral pH optimum, and on the basis of inhibitor and bivalent cation studies appears to be a $Mg^{2+}$-dependent metalloprotease. The secretase is also present in microsomal membranes prepared from pig lung and testis, and human lung and placenta, and is thus a candidate for the mechanism that generates the soluble

form of ACE in plasma and other body fluids from membrane-bound ACE by a post-translational proteolytic cleavage.

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