

Angiotensin-converting enzyme secretase is inhibited by zinc metalloprotease inhibitors and requires its substrate to be inserted in a lipid bilayer

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Mammalian angiotensin-converting enzyme (ACE; EC 3.4.15.1) is one of several proteins that exist in both membrane-bound and soluble forms as a result of a post-translational proteolytic processing event. For ACE we have previously identified a metalloprotease (secretase) responsible for this proteolytic cleavage. The effect of a range of structurally related zinc metalloprotease inhibitors on the activity of the secretase has been examined. Batimastat (BB94) was the most potent inhibitor of the secretase in pig kidney microvillar membranes, displaying an IC_{50} of 0.47 μ M, whereas TAPI-2 was slightly less potent (IC_{50} 18 μ M). Removal of the thienothiomethyl substituent adjacent to the hydroxamic acid moiety or the substitution of the P2' substituent decreased the inhibitory potency of batimastat towards the secretase. Several other non-hydroxamate-based collagenase inhibitors were without inhibitory effect on the secretase, indicating that ACE secretase is a novel zinc metalloprotease that is related to, but distinct from, the matrix metalloproteases. The full-length amphipathic form of ACE was labelled selectively

with 3-trifluoromethyl-3-(*m*-[¹²⁵I]iodophenyl)diazirine in the membrane-spanning hydrophobic region. Although trypsin was able to cleave the hydrophobic anchoring domain from the bulk of the protein, there was no cleavage of full-length ACE by a Triton X-100-solubilized pig kidney secretase preparation when the substrate was in detergent solution. In contrast, the Triton X-100-solubilized secretase preparation released ACE from pig intestinal microvillar membranes, which lack endogenous secretase activity, and cleaved the purified amphipathic form of ACE when it was incorporated into artificial lipid vesicles. Thus the secretase has an absolute requirement for its substrate to be inserted in a lipid bilayer, a factor that might have implications for the development of cell-free assays for other membrane protein secretases. ACE secretase could be solubilized from the membrane with Triton X-100 and CHAPS, but not with *n*-octyl β -D-glucopyranoside. Furthermore trypsin could release the secretase from the membrane, implying that like its substrate, ACE, it too is a stalked integral membrane protein.

INTRODUCTION

Numerous proteins exist in both membrane-bound and soluble forms as a result of a post-translational proteolytic processing event (reviewed in [1–3]). Such proteins include the Alzheimer's amyloid precursor protein, tumour necrosis factor α and its receptors, transforming growth factor α , the interleukin 6 receptor, L-selectin and angiotensin-converting enzyme (ACE; EC 3.4.15.1). The proteases (secretases, sheddases or convertases) responsible for the release of such proteins cleave the membrane-bound form in a membrane-proximal stalk region on the extracellular side of the single membrane-spanning domain. The biological function of the proteolytic release of membrane proteins varies. In some cases it might be a process for rapidly down-regulating the protein from the surface of the cell, in others it might be to generate a soluble form of the protein that has properties either identical with or subtly different from those of the membrane-bound form.

ACE is a type I integral membrane glycoprotein that plays a key role in the control of blood pressure and fluid and electrolyte homeostasis. In mammals ACE exists as two distinct isoenzymes that are derived from a single gene by transcription from one of two alternative promoters (reviewed in [4–6]). The somatic or endothelial isoenzyme (molecular mass 180 kDa) is present throughout the body (e.g. kidney, lungs and placenta) and is composed of two highly similar domains, each of which bears a

functional catalytic site. 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 [5,7]). The enzyme in plasma seems to be catalytically identical with, and immunologically very similar to, membrane-bound somatic ACE [8], except that it lacks the hydrophobic anchoring domain [9]. While studying the mode of membrane anchorage of pig kidney ACE, we observed that the enzyme could be solubilized from the membrane in a time- and temperature-dependent manner by a post-translational proteolytic cleavage [10]. The released form of ACE was hydrophilic, as determined by temperature-induced phase separation in Triton X-114 and by its failure to reconstitute into artificial lipid vesicles. Using an assay system in which both the substrate (ACE) and its proteolytic processing enzyme (secretase) were co-localized within the same isolated membrane fraction, we further characterized the properties of the secretase [11]. ACE secretase was shown to be a metalloprotease that co-localized with ACE in a number of tissues including pig kidney, lung and testis, and human lung and placenta, although the activity was absent from human kidney and human and pig small-intestinal brush-border membranes [11]. Within pig kidney the secretase was enriched in the highly purified brush-border membranes to a similar extent as ACE and alkaline phosphatase, and was shown to be an integral plasma membrane protein [11] as confirmed subsequently by others [12–14].

Abbreviations used: ACE, angiotensin-converting enzyme; [¹²⁵I]-TID, 3-trifluoromethyl-3-(*m*-[¹²⁵I]iodophenyl)diazirine.

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In the present study we have examined the effect of a number of zinc metalloprotease inhibitors on the activity of ACE secretase. The structure-activity relationship of the inhibitors reveals interesting features of the active site of the secretase. We have also developed a reconstituted cell-free assay for ACE secretase that has been used to monitor the ability of detergents and trypsin to solubilize the enzyme from the membrane, revealing that the secretase has an absolute requirement for its substrate to be inserted in a lipid bilayer. These observations should have general applicability to the study of membrane protein secretases.

EXPERIMENTAL

Materials

Batimastat (BB94) and compounds 1–9 (see Table 1 below) were synthesized at SmithKline Beecham Pharmaceuticals (Harlow, Essex, U.K.). TAPI-2 was a gift from Dr. R Black (Immunex, Seattle, WA, U.S.A.). Lisinopril {MK 521; *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline} was a gift from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). Lisinopril–2.8nm-Sepharose affinity resin was prepared as described previously [15]. 3-Trifluoromethyl-3-(*m*-[¹²⁵I]iodophenyl)-diazirine ([¹²⁵I]-TID; approx. 10 Ci/mmol) was purchased from Amersham (Little Chalfont, Bucks., U.K.). Triton X-114 was precondensed before use [16].

Purification of the amphipathic and hydrophilic forms of ACE

The amphipathic full-length form of ACE was purified from pig kidney cortex after solubilization with Triton X-100 in the presence of 10 mM EDTA by affinity chromatography on lisinopril–2.8nm-Sepharose as described previously [10]. The trypsin-solubilized hydrophilic form of ACE was purified after incubation of the pig kidney cortex membranes with trypsin as described previously [10].

Quantification of ACE

ACE enzymic activity was determined with benzoyl-Gly-His-Leu (5 mM) as substrate in 0.1 M Tris/HCl (pH 8.3)/0.3 M NaCl/10 μ M ZnCl₂. Reactions were terminated by heating at 100 °C for 4 min; the substrate and reaction products were resolved and quantified by reverse-phase HPLC as described previously [15]. ACE protein was determined by a competitive ELISA with a polyclonal antiserum raised against purified pig kidney ACE as described previously [11]. Protein was quantified with bicinchoninic acid in a microtitre plate assay with BSA as standard [17].

Photolabelling of ACE with [¹²⁵I]-TID

Aliquots of affinity-purified ACE (0.25–0.5 mg of protein) were made up to 0.1% (w/v) Triton X-100 and then dialysed extensively against 20 mM Tris/HCl (pH 7.5)/0.3 M KCl/0.1 mM ZnCl₂/0.1% Triton X-100. In a quartz cuvette equilibrated at 0 °C, [¹²⁵I]-TID (50–100 μ Ci) was added to the dialysed ACE and the sample was photolysed for 20 min with a 50 W Leitz mercury lamp with the beam directed through a filter of saturated copper sulphate [18]. The labelled sample was then applied to a lisinopril–2.8nm-Sepharose affinity column (1 ml bed volume) equilibrated in 20 mM Tris/HCl (pH 7.5)/0.3 M KCl/0.1 mM ZnCl₂/0.1% Triton X-100. Labelled Triton X-100 was removed by extensive washing of the column with the equilibration buffer before elution of the bound ACE with free

lisinopril. The lisinopril was removed and the enzyme re-activated by dialysis as described previously [15]. [¹²⁵I]-TID-labelled amphipathic ACE (4 μ g of protein) was incubated with various amounts of trypsin in 10 mM Hepes/NaOH, pH 7.0 for 10 min at 37 °C. The samples were then immediately either analysed by SDS/PAGE followed by autoradiography or subjected to temperature-induced phase separation in Triton X-114 [16] and the resulting detergent-rich and aqueous phases were assayed for enzyme activity and radioactivity.

Detergent and trypsin solubilization of ACE secretase

Pig kidney cortex (10 g) was homogenized in 10 vol. of 0.33 M sucrose/10 mM Tris/HCl (pH 7.5) at 4 °C. The homogenate was centrifuged at 8000 *g* for 15 min and the resulting supernatant was then centrifuged at 26000 *g* for 2 h. The microsomal membrane pellet was resuspended in 10 mM Hepes/NaOH (pH 7.5)/0.3 M KCl. The membranes were solubilized with one of: (1) Triton X-100 (20%, v/v) added to a final detergent-to-protein ratio of 7:1, (2) CHAPS added to give a final concentration of 20 mM, (3) *n*-octyl β -D-glucopyranoside added to give a final concentration of 60 mM and incubated with stirring for 1 h at 4 °C, or (4) trypsin added to a ratio of 1:10 (trypsin to protein, w/w) and then incubated at 37 °C for 1 h. At the end of the incubation the trypsin activity was irreversibly inhibited by the addition of 1 mM di-isopropyl fluorophosphate. The solubilized samples were then centrifuged at 31000 *g* for 90 min, and the resulting supernatant containing the solubilized secretase was incubated overnight at 4 °C with lisinopril–2.8 nm-Sepharose to remove the endogenous ACE [10]. The unbound run-through fraction was then used as the source of the detergent- or trypsin-solubilized ACE secretase.

Co-localized secretase assay with kidney microvillar membranes

Pig kidney cortex microvillar membranes were prepared as described previously [19]. The membranes were preincubated in 0.1 M borate buffer, pH 8.3, for 20 min at 4 °C in the absence or presence of inhibitors, and then incubated at 37 °C for 4 h. After the incubation, the samples were subjected to temperature-induced phase separation in Triton X-114 [16], and the resulting detergent-rich and aqueous phases assayed for ACE enzymic activity with benzoyl-Gly-His-Leu as substrate. The release of ACE (i.e. the secretase activity) 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.

Reconstituted secretase assay with intestinal microvillar membranes as substrate

Microvillar membranes were prepared from pig small intestine by the method of Kessler et al. [20]. The Triton X-100-solubilized kidney ACE secretase preparation (50 μ g of protein) was incubated with the intestinal microvillar membranes (200 μ g of protein) for 2 h at 37 °C in 0.2 M Tris/maleate buffer, pH 8.4. After the incubation, the reaction was stopped by placing the samples on ice and then immediately subjecting them to temperature-induced phase separation in Triton X-114 [16]. ACE present in the resulting detergent-rich and aqueous phases was quantified by measurement of enzyme protein with a competitive ELISA [11]. ACE secretase activity is measured by the release of ACE as determined by 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.

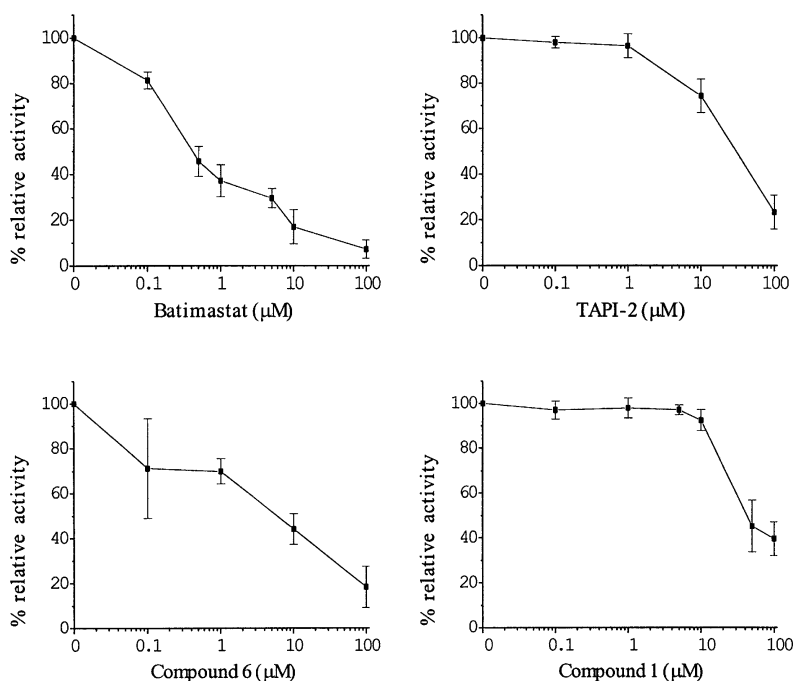


Figure 1 Inhibition of ACE secretase by hydroxamic acid-based metalloprotease inhibitors

Pig kidney microvillar membranes (76.2 μg of protein) were incubated in the absence or presence of the indicated compounds for 20 min at 4 °C followed by incubation for 4 h at 37 °C. At the end of the incubation the samples were subjected to temperature-induced phase separation in Triton X-114 and the resulting aqueous and detergent phases were assayed for ACE activity as described in the Experimental section. Each point is the mean for triplicate incubations with inhibitor.

Reconstituted secretase assay with the purified amphipathic form of ACE incorporated in lipid vesicles as substrate

The purified amphipathic full-length form of ACE was reconstituted into dimyristoyl phosphatidylcholine vesicles as described previously [10]. The detergent- or trypsin-solubilized secretase preparation was incubated with the lipid vesicles containing the amphipathic form of ACE (5 μg of protein) at 37 °C in 0.1 M borate buffer, pH 8.3. After the incubation the samples were immediately subjected to temperature-induced phase separation in Triton X-114, and ACE present in the resulting detergent-rich and aqueous phases was either quantified by measurement of enzyme activity with benzoyl-Gly-His-Leu as substrate or subjected to immunoelectrophoretic blot analysis. ACE secretase activity is measured by the release of ACE as determined by 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.

SDS/PAGE and immunoelectrophoretic blot analysis

SDS/PAGE was performed with a 7–17% (w/v) polyacrylamide gradient as described previously [21]. Immunoelectrophoretic blot analysis was performed with PVDF (Immobilon P) membranes and an antibody raised against affinity-purified pig kidney ACE as described previously [15]. Bound antibody was detected with peroxidase-conjugated secondary antibodies in conjunction with the enhanced chemiluminescence detection method (Amersham).

Collagenase assay

The collagenase assay was performed by the method of Cawston

and Barrett [22], in which various concentrations of the inhibitor were incubated with 13 ng of human recombinant fibroblast collagenase and 0.01 μCi of [³H]acetylated type 1 bovine collagen for 18 h at 37 °C in 0.05 M Tris/HCl, pH 7.6. Unhydrolysed collagen was separated from proteolysis products by centrifugation. The amount of radioactivity in the supernatant was determined by scintillation counting and expressed as a percentage of the value obtained for a control sample in the absence of inhibitor. IC₅₀ values were determined from the line of best fit of the concentration–response curves.

RESULTS

Effect of zinc metalloprotease inhibitors on the activity of ACE secretase

The effect of batimastat and a range of structurally similar hydroxamic acids on the activity of ACE secretase was monitored through their effect on the release of ACE from pig kidney microvillar membranes in the co-localized assay (Figure 1 and Table 1). In this assay, batimastat was a potent secretase inhibitor (IC₅₀ 0.47 ± 0.07 μM), whereas TAPI-2 and compounds 1, 2 and 6 were significantly less effective (IC₅₀ 7.5–80 μM). In contrast, the related compounds 3–5 all showed minimal secretase inhibitory activity at concentrations of 100 μM or less. Other classes of metalloprotease inhibitor, such as the thiol ester (compound 7), the aminophosphonate (compound 8) and the phosphinate (compound 9) were also ineffective in this assay. With the exception of compound 1 (35% inhibition) and TAPI-2 (21% inhibition), no compound inhibited the activity of ACE at a concentration of 0.1 mM by more than 20% (results not shown).

Presence in the amphipathic form of ACE of a hydrophobic anchoring domain that is susceptible to release by trypsin

The amphipathic form of ACE purified from pig kidney cortex after solubilization with Triton X-100 in the presence of EDTA was photolabelled with the hydrophobic reagent [125 I]-TID and then analysed by SDS/PAGE (Figure 2). Autoradiography of the SDS gel revealed that a single polypeptide band of 180 kDa had been radiolabelled that corresponded to the only polypeptide band visible on the Coomassie-stained gel (Figure 2, lane 1). In contrast, the hydrophilic form of ACE released from the membrane by trypsin was not radiolabelled with [125 I]-TID (Figure 2, lane 7), consistent with this form of the protein lacking the hydrophobic membrane-anchoring domain. On incubation of the [125 I]-TID-labelled amphipathic form of ACE with increasing amounts of trypsin, there was a dose-dependent cleavage of the 125 I label from the 180 kDa polypeptide with the concomitant appearance of a smaller fragment of approx. 6.9 kDa (Figure 2, lanes 2–6). There was no corresponding decrease in the amount of the 180 kDa polypeptide detected by Coomassie Blue staining of the SDS gel, indicating that only a small fragment of the protein had been removed and consistent with the lack of size difference on SDS/PAGE previously observed between the amphipathic and hydrophilic forms of ACE [10]. Temperature-induced phase separation in Triton X-114 of the 125 I-labelled amphipathic form of ACE revealed that both the enzyme activity and the radioactivity partitioned to a similar extent (50–60%) into the detergent-rich phase (Figure 3). On digestion with increasing amounts of trypsin the enzyme activity partitioned increasingly into the aqueous phase, whereas the radioactivity partitioned increasingly into the detergent-rich phase (Figure 3).

Secretase does not cleave the amphipathic form of ACE in solution

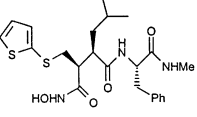
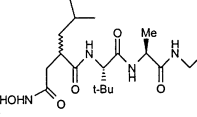
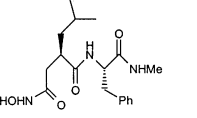
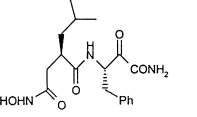
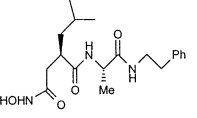
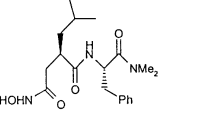
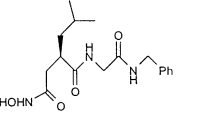
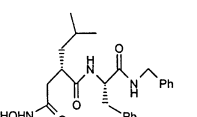
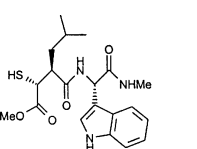
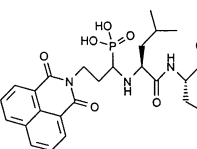
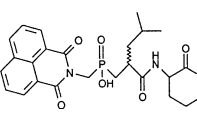
The 125 I-labelled amphipathic form of ACE (5 μ g of protein) was incubated with pig kidney microvillar membranes (0.23 mg of protein) in the presence of 0.1% Triton X-100 in 0.2 M HEPES/NaOH, pH 8.5 (final volume 50 μ l) for 2 h at 37 °C to determine whether the secretase within the membranes would cleave the radiolabelled hydrophobic anchor from the bulk of the protein. However, there was no measurable increase in the partitioning of the radioactivity into the detergent-rich phase (55.7% compared with 58.6% for the 125 I-labelled amphipathic form of ACE on its own). In a further experiment, pig kidney cortex microsomal membranes were solubilized with Triton X-100 and the resulting solubilized material was applied to a lisinopril–2.8nm-Sepharose affinity column to remove the endogenous ACE. This Triton-solubilized secretase preparation (0.24 mg of protein) was then incubated for 2 h at either 4 °C or 37 °C in 10 mM HEPES/NaOH, pH 7.4 (final volume 50 μ l), with the affinity-purified amphipathic form of ACE (1 μ g of protein). After temperature-induced phase separation in Triton X-114, no increase in the amount of ACE present in the aqueous phase was observed after incubation at 37 °C compared with 4 °C (45.5% compared with 57.2%).

Secretase cleaves ACE inserted in a lipid bilayer

Pig intestinal microvillar membranes, which contain ACE but lack detectable EDTA-sensitive secretase activity [11], were investigated as a possible source of substrate for a solubilized ACE secretase preparation (Table 2). Negligible release of ACE occurred when the intestinal microvillar membranes were incubated at either 4 or 37 °C in the absence of a source of secretase, which is consistent with our previous results (Table 2) [11].

Table 1 Structures and inhibitory effects of compounds on ACE secretase and collagenase

Results are means \pm S.E.M. for three determinations or the means of duplicate determinations from inhibition curves as shown in Figure 1.

Compound	Structure	ACE secretase I_{50} (μ M)	Collagenase I_{50} (μ M)
Batimastat		0.47 \pm 0.07	0.005
TAPI-2		18.33 \pm 1.76	–
1		38.3 \pm 6.12	0.056
2		80.3	3.0
3		100	0.40
4		100	0.22
5		100	1.1
6		7.50 \pm 2.55	10
7		100	0.012*
8		100	0.053†
9		100	0.065‡

*Data from [24]; †data from [25]; ‡data from [35].

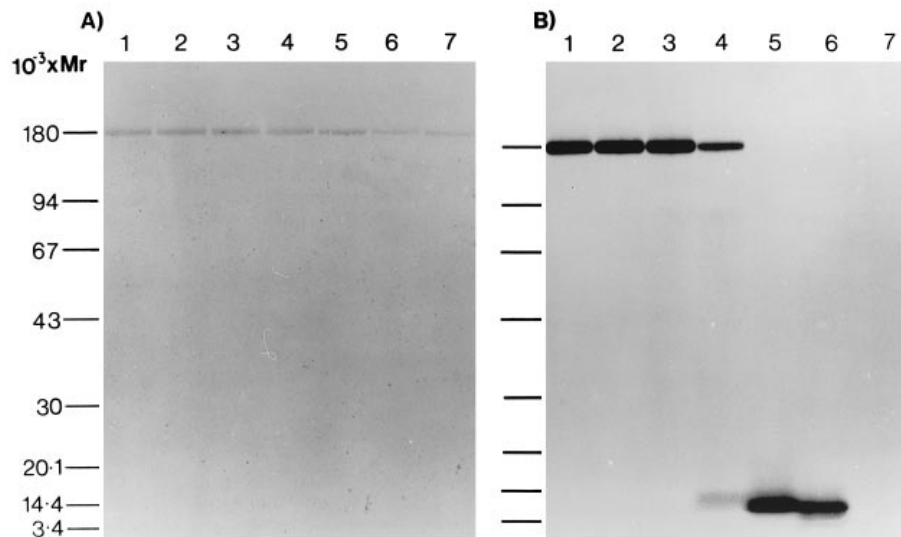


Figure 2 Cleavage of the hydrophobic anchoring domain of ACE with trypsin

The amphipathic form of ACE and the hydrophilic form of ACE after release from the membrane with trypsin were photolabelled with [125 I]-TID as described in the Experimental section. The [125 I]-TID-labelled amphipathic form of ACE (lanes 1–6; 4 μ g of protein per lane) was incubated with increasing amounts of trypsin for 10 min at 37 °C and the samples were then analysed by SDS/PAGE followed by autoradiography. (A) SDS gel stained with Coomassie Brilliant Blue; (B) corresponding autoradiograph. Lane 1, untreated amphipathic form of ACE; lane 2, as lane 1 after incubation with 1 ng of trypsin; lane 3, as lane 1 after incubation with 10 ng of trypsin; lane 4, as lane 1 after incubation with 100 ng trypsin; lane 5, as lane 1 after incubation with 1 μ g of trypsin; lane 6, as lane 1 after incubation with 10 μ g of trypsin; lane 7, trypsin-solubilized ACE labelled with [125 I]-TID (5 μ g of protein).

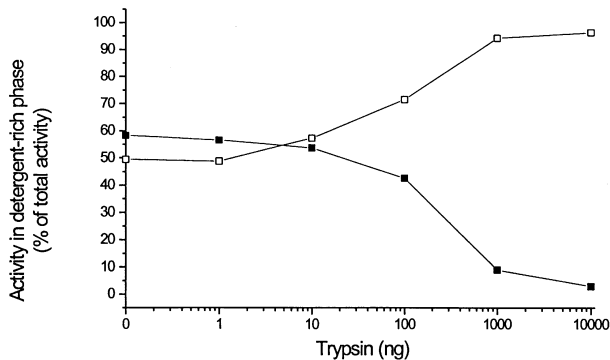


Figure 3 Phase separation in Triton X-114 of the [125 I]-TID-labelled amphipathic form of ACE

125 I-labelled amphipathic ACE (2 μ g) was incubated with trypsin for 10 min at 37 °C and the samples were then subjected to phase separation in Triton X-114 as described in the Experimental section. The resulting detergent-rich and aqueous phases were assayed for radioactivity and enzyme activity. Results are the means of duplicate determinations with each concentration of trypsin. Symbols: ■, enzyme activity; □, radioactivity.

However, on addition to the intestinal microvillar membranes of the Triton-solubilized pig kidney ACE secretase preparation, significant (47%) release of ACE from the membrane was observed after incubation for 2 h at 37 °C (Table 2). Negligible release was observed when either a source of ACE secretase was used that had been preheated to 95 °C or the incubation was performed at 4 °C (Table 2). The release of ACE from the intestinal microvillar membranes was blocked when the Triton-solubilized kidney ACE secretase preparation was preincubated with EDTA (Table 2), indicating that a metalloprotease was responsible. The addition of trypsin to the intestinal microvillar membranes caused a significant (88%) release of ACE. The

Table 2 Use of pig intestinal microvillar membranes as substrate for the assay of the secretase

Pig intestinal microvillar membranes (0.2 mg of protein) were incubated in the absence or presence of either the Triton X-100-solubilized pig kidney ACE secretase preparation or trypsin for 2 h at the temperature indicated. The intestinal microvillar membranes were also incubated with the solubilized ACE secretase preparation that had been either preheated at 95 °C for 5 min or incubated with EDTA (5 mM). After incubation the samples were subjected to phase separation in Triton X-114; the ACE released was quantified by ELISA as described in the Experimental section. The results are means \pm S.E.M. for triplicate determinations.

Enzyme	Incubation temp. (°C)	ACE released (% of total)
None	4	11.4 \pm 2.6
None	37	12.3 \pm 1.8
ACE secretase	4	14.6 \pm 2.9
ACE secretase	37	46.7 \pm 4.5
ACE secretase + EDTA	37	13.9 \pm 3.0
Heat-inactivated ACE secretase	37	13.5 \pm 1.4
Trypsin	37	87.9 \pm 3.4

affinity-purified amphipathic form of ACE was reconstituted into artificial lipid vesicles as described previously [10] and the Triton-solubilized secretase preparation was then incubated with this reconstituted form of ACE (Figure 4). After temperature-induced phase separation in Triton X-114 a substantial amount of ACE was recovered in the aqueous phase as determined by immunoelectrophoretic blot analysis. The size of the hydrophilic form of ACE released was essentially the same as that of the membrane-bound form, indicating that the secretase had cleaved within the stalk region, releasing the bulk of the protein from the relatively small membrane-anchoring domain. Incubation of the Triton X-100-solubilized ACE secretase preparation in the presence of batimastat significantly (54.1% at 10 μ M) inhibited the release of ACE from the lipid vesicles.

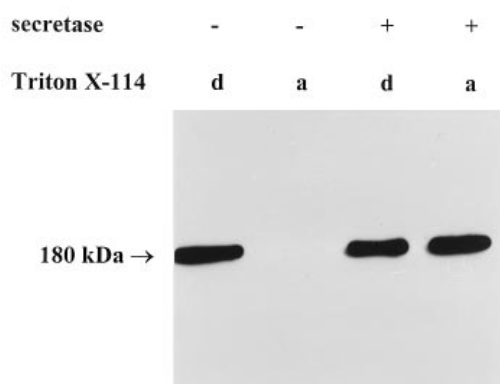


Figure 4 Use of the amphipathic form of ACE incorporated into lipid vesicles as substrate for the secretase

The amphipathic form of ACE (5 μ g) was incorporated into lipid vesicles and then incubated in the absence or presence of a Triton X-100-solubilized pig kidney secretase preparation (127 μ g of protein) for 4 h at 37 °C. At the end of the incubation the samples were subjected to temperature-induced phase separation in Triton X-114 and the resultant aqueous and detergent-rich phases were subjected to immunoelectrophoretic blot analysis with a polyclonal antiserum against pig kidney ACE as described in the Experimental section. Abbreviations: d, detergent-rich phase; a, aqueous phase.

Table 3 Solubilization of the secretase from the membrane

Pig kidney microsomal membranes were incubated in the absence or presence of either detergent or trypsin as described in the Experimental section. After centrifugation at 31000 *g* for 90 min, the supernatant was assayed for ACE activity before incubation with lisinopril–2.8nm-Sepharose. The amount of ACE solubilized was measured by the ACE activity recovered in the solubilized supernatant as a percentage of the total activity in the microsomal membranes before solubilization. After incubation with lisinopril–2.8nm-Sepharose, the secretase activity in the unbound fraction was determined by using the purified amphipathic form of ACE reconstituted into lipid vesicles as substrate. The results are means of duplicate determinations and are representative of two such experiments.

Detergent or protease	Solubilization of ACE secretase		Solubilization of ACE (% of total activity)
	(ng of ACE/min per mg)	(ng of ACE/min per ml)	
None	6.8	13.5	30.2
Triton X-100	26.0	165.7	105.4
CHAPS	28.3	159.4	84.2
Octyl glucoside	2.6	19.8	95.3
Trypsin	31.4	67.7	94.0

Solubilization of ACE secretase from the membrane

The ability of trypsin and a range of detergents to solubilize ACE secretase from pig kidney microsomal membranes was investigated (Table 3). After solubilization, the endogenous ACE was removed by chromatography on lisinopril–2.8nm-Sepharose before incubation with the amphipathic form of ACE reconstituted in lipid vesicles. For comparison, the ability of these reagents to solubilize ACE itself was assessed. Although ACE was solubilized effectively by all of the detergents examined (Table 3), the secretase was only solubilized effectively by Triton X-100 and CHAPS. *n*-Octyl β -D-glucopyranoside failed to solubilize the secretase from the pig kidney microsomal membranes. Although trypsin effectively solubilized 94% of the ACE activity, only 41% of the apparent secretase activity solubilized by Triton X-100 was solubilized by trypsin. Prolonged incubation with trypsin led to no further increase in the amount of secretase released from the microsomal membranes, and incubation with bacterial phosphatidylinositol-specific phospholipase C also failed to release the secretase (results not shown).

DISCUSSION

On the basis of its inhibition by chelating agents and subsequent re-activation with bivalent metal ions, we have proposed that ACE secretase is a metalloprotease [10,11]. In the present study we have provided the first detailed structure–activity relationship for inhibition of the ACE secretase by examining the effect of representative members of the matrix metalloprotease inhibitors, particularly those of the hydroxamic acid class. The most potent inhibitors of ACE secretase from these various classes, all of which are known inhibitors of collagenase [23–26], were the hydroxamates batimastat, TAPI-2 and compound 6, which elicited IC_{50} values in the low micromolar range. The TAPI-2 result is consistent with the recent finding that this agent can inhibit the release of the testicular isoform of rabbit ACE from a transfected mouse epithelial cell line [14]. The reduced activity shown by compound 1 in particular is especially interesting, because it differs from batimastat only in the absence of the thienothiomethyl substituent adjacent to the hydroxamic acid moiety. A similar decrease in potency has been observed on collagenase and is likely to reflect the importance of this group in orientating the inhibitor backbone [27]. The further decrease in potency with compounds 2 and 4 is also consistent with the finding that primary and tertiary C-terminal amides are less effective inhibitors of collagenase than simple secondary amides, although it is interesting to note that the other two secondary amides (compounds 3 and 5) do not have secretase inhibitory activity. The potency of compound 6, which has the ‘unnatural’ configuration at P1’ (see [28] for nomenclature), is particularly interesting because such compounds are known to lose significant inhibitory activity against matrix metalloproteases. Indeed, compound 6 is more than three orders of magnitude less potent than batimastat against collagenase, yet is only one-fifth as potent as an inhibitor of ACE secretase. Thus although batimastat and many related compounds are non-selective inhibitors of matrix metalloproteases [29], our results imply that marked differences exist between the recognition features essential for the inhibition of matrix metalloproteases and ACE secretase, and reinforce the earlier suggestion that the latter is a unique, albeit related, zinc metalloenzyme [11]. Further support for the uniqueness of ACE secretase is derived from the poor activity shown by compounds 7–9, which are representative examples of non-hydroxamate-based collagenase inhibitors [24–26].

Although the primary recognition features of the ACE secretase have yet to be established, the site of cleavage in the human somatic isoform of ACE is reported to be between Arg-1137 and Leu-1138 [30]. Moreover, more recent studies with a truncated N-terminal domain mutant have identified a second cleavage site closer to the transmembrane region between Arg-1227 and Val-1228 [13]. From the presence of a hydrophobic P1’ amino acid at both of these cleavage sites it is tempting to speculate that ACE secretase has a hydrophobic S1’ pocket, a suggestion that is supported by the presence of an isobutyl moiety at P1’ in batimastat and the other inhibitors described here. In addition to ACE, a number of other membrane proteins, including tumour necrosis factor α , the interleukin 6 receptor, the tumour necrosis factor receptors I and II, Fas ligand, transforming growth factor α and L-selectin, are now known to be released from the lipid bilayer in a soluble form by the action of metallosecretases that are blocked by hydroxamic acid-based compounds with IC_{50} values in the low micromolar range (reviewed in [3]), suggesting a close similarity to, or identity with, ACE secretase. However, in no case has such an extensive structure–activity relationship been reported as in the present study for the ACE secretase.

To assay the ACE secretase once it had been solubilized from

the membrane, we attempted to use an exogenous source of the substrate. Although the hydrophobic anchoring domain of the purified amphipathic form of pig kidney ACE can readily be cleaved with trypsin, we found no evidence for cleavage of the hydrophobic domain from the bulk of the protein on incubation of this form of ACE with either kidney microvillar membranes or a detergent-solubilized secretase preparation. Using as a source of substrate pig intestinal microvillar membranes that contain significant amounts of ACE but lack detectable EDTA-sensitive secretase activity [11], we were able to detect EDTA-sensitive secretase activity in the detergent-solubilized kidney membranes. We then incorporated the purified amphipathic form of ACE into lipid vesicles [10] and used these as substrate for the secretase. Using this reconstituted cell-free assay system we were readily able to quantify the amount of secretase activity in the solubilized preparation. Thus it seems that the secretase requires its substrate to be membrane-associated in order to act on it. It seems unlikely that this is because of a requirement for a particular cofactor normally present in the membrane, because cleavage still occurred in the reconstituted system when only ACE and phosphatidylcholine were present in the vesicles. More probably this reflects a conformational requirement that exists when ACE is embedded in a membrane but is absent when the protein is free in solution. One possibility is that in the absence of a lipid bilayer to insert into, the hydrophobic transmembrane domain of ACE binds to other hydrophobic regions on the surface of the same or neighbouring ACE molecules, thereby occluding or altering the conformation of the stalk region and preventing secretase access. Thus it seems that ACE secretase has an absolute requirement for its substrate to be inserted in a lipid bilayer. Interestingly, a similar conclusion has been reached indirectly for both the α - and β -secretases involved in processing the amyloid precursor protein [31,32], the metallosecretase responsible for cleaving Kit ligand [33] and the protease involved in cleaving colony-stimulating factor [34], and it might hold true for other membrane proteins that are cleaved by secretases. This observation has a significant bearing on the means by which such membrane protein secretases can be assayed and effectively rules out the use of short soluble synthetic peptides as substitute substrates.

The fact that the ACE secretase could be released from the membrane by trypsin and still retain activity shows clearly that there is not a requirement for the secretase itself to be inserted in a membrane to be active. In addition, release by trypsin indicates that the secretase might have a similar topology in the membrane to that of its substrate ACE; that is, a membrane-anchoring domain, possibly a membrane-spanning polypeptide, and a relatively large extracellular domain containing the active site that is separated from the membrane-anchoring domain by a stalk region that is itself susceptible to cleavage by (exogenous) proteases. Although significant ACE secretase activity was solubilized with Triton X-100 and CHAPS, negligible activity was solubilized with *n*-octyl β -D-glucopyranoside. This pattern of detergent solubility parallels that observed for a candidate amyloid precursor protein α -secretase [31]. Indeed, a comparison of the properties of the α -secretase, which were obtained by using a co-localized assay system, with those of ACE secretase reveals that they share many properties. In addition to their similar detergent solubilization profiles, neither enzyme was removed by washing the membranes with a high-salt solution. Both enzymes were inhibited by EDTA and 1,10-phenanthroline and not by inhibitors of serine or aspartic proteases, α_2 -macroglobulin or

phosphoramidon. Furthermore, both enzymes cleave their respective substrates between a basic and a hydrophobic residue (Lys-Leu and Arg-Leu) and are stimulated by phorbol esters. It remains to be determined whether these two enzymes are in fact the same or are two closely related members of a larger family of integral membrane zinc metalloproteases.

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