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Pig kidney angiotensin converting enzyme

Purification and characterization of amphipathic and hydrophilic forms of the enzyme establishes *C*-terminal anchorage to the plasma membrane

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Angiotensin converting enzyme from pig kidney was isolated by affinity chromatography after solubilization from the membrane by one of four different procedures. Solubilization with Triton X-100, trypsin or by an endogenous activity in microvillar membranes all generated hydrophilic forms of the enzyme as assessed by phase separation in Triton X-114 and failure to incorporate into liposomes. Only when solubilization and purification was effected by Triton X-100 in the presence of EDTA (10 mM) could an amphipathic form of the enzyme (membrane- or m-form) be generated. The m-form of angiotensin converting enzyme (ACE) appeared slightly larger (M_r approx. 180000) than the hydrophilic forms (M_r approx. 175000) after SDS/polyacrylamide-gel electrophoresis, and the m-form incorporated into liposomes, consistent with retention of the membrane anchor. The m-form of ACE showed an *N*-terminal sequence identical with that of preparations of enzyme isolated after solubilization with detergent alone (d-form), with trypsin (t-form) or by the endogenous mechanism (e-form). These data imply that ACE is anchored to the plasma membrane via its *C*-terminus, in contrast with the *N*-terminal anchorage of endopeptidase-24.11. No release of ACE from the membrane could be detected with a variety of phospholipases, including bacterial phosphatidylinositol-specific phospholipases C, although an endogenous EDTA-sensitive membrane-associated hydrolase was capable of releasing a soluble, hydrophilic, form of the enzyme.

INTRODUCTION

The peptidases of the kidney microvillar membrane exist as 'stalked' proteins with their catalytic sites exposed at the external surface of the plasma membrane (Kenny & Maroux, 1982). One of the best characterized of these ectoenzymes is endopeptidase-24.11 (EC 3.4.24.11), which is anchored in the plasma membrane through a hydrophobic sequence of amino acids near its *N*-terminus, which probably represents an uncleaved signal-peptide sequence (Fulcher *et al.*, 1986; Devault *et al.*, 1987; Malfroy *et al.*, 1987). It has been tacitly assumed that the family of microvillar membrane peptidases are all synthesized and assembled in the plasma membrane in a similar fashion. Recently, however, we have shown that renal dipeptidase (EC 3.4.13.11) can be released from microvillar membrane preparations by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) (Hooper *et al.*, 1987). This would indicate that the dipeptidase is anchored to the plasma membrane via a *C*-terminal glycolipid moiety (Low, 1987). The mode of anchorage of other membrane peptidases therefore requires reconsideration.

Angiotensin converting enzyme (ACE; peptidyl dipeptidase A; EC 3.4.15.1) has been isolated and characterized from numerous tissues and species (reviewed in Patchett & Cordes, 1985). The membrane-bound form of ACE is a glycoprotein of M_r 180000 in pig kidney (Hooper & Turner, 1987). Closely related soluble forms of the enzyme are found in plasma and cerebrospinal fluid and appear to arise by release from membrane surfaces. The mode of attachment of ACE to the membrane and the mechanism of its endogenous release could therefore have a bearing on the decreased levels of ACE observed in certain pathological situations (Beckman *et al.*, 1984; Zubenko *et al.*, 1985). Here we demonstrate that the amphipathic and hydrophilic forms of pig kidney ACE have identical *N*-terminal sequences, showing no homology with the hydrophobic *N*-terminal sequence of the amphipathic form of endopeptidase-24.11. This implies that ACE is anchored via its *C*-terminus, involving either a hydrophobic sequence of amino acids or, possibly, a covalently attached glycolipid moiety. Although no release of ACE from the membrane was detected by using several PI-PLCs, we detected an endogenous EDTA-sensitive hydrolase activity in pig kidney membrane preparations that was capable of releasing from the membrane a soluble, hydrophilic, form of ACE.

EXPERIMENTAL

Materials

Pig kidneys were obtained from ASDA Farm Stores, Lofthousegate, W. Yorks., U.K. Captopril (SQ14225, D-3-mercapto-2-methylpropanoyl-L-proline) was a gift from Squibb Institute for Medical Research (Princeton, NJ, U.S.A.). Lisinopril {MK521, *N*-[(*S*)-1-carboxy-3-

Abbreviations used: ACE, angiotensin converting enzyme; Bz-Gly, benzoylglycyl; DMPC, dimyristoylphosphatidylcholine; PI-PLC, phosphatidylinositol-specific phospholipase C.

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phenylpropyl]-L-lysyl-L-proline}, enalaprilat {MK422, *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-alanyl-L-proline}, L155212 (compound 11a; Wyvratt *et al.*, 1983) and L155360 (compound 3b; Parsons *et al.*, 1983) were gifts from Dr. A. A. Patchett, Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). [^{3}H]-Dioleoyl phosphatidylcholine was synthesized and supplied by the Department of Biochemistry, University of Cambridge, Cambridge, U.K. PI-PLC from *Staphylococcus aureus* and from *Bacillus thuringiensis* were purified as described previously (Malik & Low, 1986; Hooper *et al.*, 1987) and were gifts from Dr. M. G. Low. Both preparations were more than 90% pure as judged by SDS/polyacrylamide-gel electrophoresis. *B. cereus* phospholipase C type III, *Clostridium perfringens* phospholipase C type IX or XII, phospholipase A_2 (bee venom), phospholipase D (cabbage) type IV, *Streptomyces chromofuscus* phospholipase D type VI, trypsin type III-S, Triton X-114, dimyristoyl L-α-phosphatidylcholine and myristic acid were from Sigma. Units of phospholipase activity are μmol/min, except those for phospholipase D, which are μmol/h. Other materials were from sources previously noted. Triton X-114 was precondensed before use (Bordier, 1981). Lisinopril–1.4 nm–Sepharose and lisinopril–2.8 nm–Sepharose were prepared as described by Bull *et al.* (1985) and Hooper & Turner (1987) respectively.

Affinity purification of ACE from pig kidney

Pig kidney cortex (approx. 200 g) was homogenized in 2 litres of 0.33 M-sucrose/50 mM-Hepes, pH 7.5. The resultant homogenate was centrifuged at 8000 *g* for 15 min and the supernatant then centrifuged at 26000 *g* for 2 h. The microsomal pellet was resuspended in 5 mM-Hepes, pH 7.5, and ACE was solubilized from the membranes by addition of either trypsin or Triton X-100 (see the Results section). After solubilization, the suspension was centrifuged at 31000 *g* for 1.5 h and the supernatant then dialysed against 8 vol. of 10 mM-Hepes/0.3 M KCl/100 μM-$ZnCl_2$, pH 7.5.

When solubilization of ACE by Triton X-100 was performed in the presence of EDTA, both the homogenization buffer and resuspension buffer contained 10 mM-EDTA. Also, after resuspending the microsomal pellet, the suspension was stirred for 1 h at room temperature and centrifuged at 31000 *g* for 1.5 h before resuspending again and then solubilizing with Triton X-100. Although EDTA inhibits ACE, virtually complete re-activation of the enzyme could be obtained by dialysing extensively against buffer containing 100 μM-$ZnCl_2$ (Bull *et al.*, 1985).

The dialysed extract was then applied to either a lisinopril–1.4 nm–Sepharose or lisinopril–2.8 nm–Sepharose affinity column as described by Bull *et al.* (1985) or Hooper & Turner (1987) respectively. The only difference in the procedure was that an increase of pH (from 8.5 to 9.0) in the elution buffer containing free lisinopril was utilized for the 2.8 nm spacer arm. All operations were carried out at 4 °C, and when solubilization of ACE was carried out using Triton X-100, all the subsequent buffers contained 0.1% (w/v) Triton X-100. The affinity columns were routinely washed with 30–50 ml of the following buffers: (i) 10 mM-glycine/0.3 M-KCl/5 mM-EDTA, pH 9.5; (ii) 10 mM-glycine/0.3 M-KCl/100 μM-$ZnCl_2$/1.0 M-NaSCN, pH 9.5; (iii) 10 mM-Tris/0.3 M-KCl/100 μM-$ZnCl_2$/10 μM-lisinopril/0.1%(w/v)TritonX-100, pH 9.0; (iv) 5 mM-Hepes/0.5 M-NaCl/0.5 mM-EDTA/2% (w/v) Triton X-100, pH 7.5; and (v), when stored for long periods, 5 mM-Hepes/150 mM-KCl/0.1 mM-EDTA/0.1% (w/v) Triton X-100/0.1% (w/v) NaN_3, pH 7.5; this was followed by re-equilibration.

Subcellular fractionation of pig kidney cortex and solubilization of membrane proteins

All operations were performed at 4 °C unless stated otherwise. A 10% (w/v) homogenate of pig kidney cortex in 0.33 M-sucrose/50 mM-Hepes, pH 7.5, was centrifuged at 8000 *g* for 15 min. The supernatant was decanted off and further centrifuged at 26000 *g* for 2 h. The microsomal pellet was resuspended in 10 mM-Hepes, pH 7.0, and centrifuged at 31000 *g* for 1.5 h. The pellets were resuspended in the appropriate buffer, and a portion of a phospholipase preparation added where applicable. After solubilizing protein at 37 °C, the incubation mixtures were centrifuged at 31000 *g* for 1.5 h. Enzyme activities were determined in the total incubation mixture after solubilization but before centrifugation, and in the supernatant after centrifugation at 31000 *g*; the solubilized activity was expressed as a percentage of the total (100%) activity in the original incubation mixture.

SDS/polyacrylamide-gel electrophoresis and immunoelectrophoretic blot analysis

SDS/polyacrylamide-gel electrophoresis was performed by using the system of Laemmli (1970) with a 7–17%-(w/v)-polyacrylamide gradient as described previously (Relton *et al.*, 1983). Immunoelectrophoretic ('Western') blot analysis was carried out as described by Towbin *et al.* (1979) and as detailed by Hooper & Turner (1987).

Reconstitution of proteins into liposomes

This was carried out by the procedure of Scotto & Zakim (1985). Myristate (1.5 mg/ml) in ethanol was added to a glass tube and the solvent removed under a stream of N_2, leaving a film of myristate on the vessel wall. Dimyristoyl phosphatidylcholine (DMPC, 35 mg) was dispersed in 40 ml of 10 mM-Tris/100 mM-KCl/1 mM-ascorbate, pH 8.0, and added to the tube containing the myristate. [^{3}H]Dioleoyl phosphatidylcholine was also added, and the solution sonicated at 30 °C to form vesicles. Protein (typically 5 μg) was mixed with the DMPC vesicles (200 μg) at 30 °C in 10 mM-Tris/100 mM-KCl/1 mM-ascorbate, pH 8.0. The mixture was incubated at 18 °C for 10 min to allow the protein to incorporate into the DMPC vesicles and then warmed to 30 °C for 10 min before centrifugation. The lipid vesicles and proteins were separated from each other by centrifugation through 4 ml of 20% (w/v) sucrose/10 mM-Tris, pH 7.5, at 30 °C in a swing-out rotor at 100000 *g* for 18 h. After centrifugation, the tubes were fractionated from the bottom, and fractions (approx. 400 μl each) were collected. Each fraction was assayed for the appropriate enzyme activity and then the radioactivity in the remainder of the fraction was determined by liquid-scintillation spectrometry.

Solid-phase sequencing of pig kidney ACE

This was carried out by a modification of the method used for endopeptidase-24.11 (Fulcher *et al.*, 1986). Samples of pig kidney ACE (0.2–1.5 mg) were dialysed exhaustively against water and freeze-dried. The freeze-

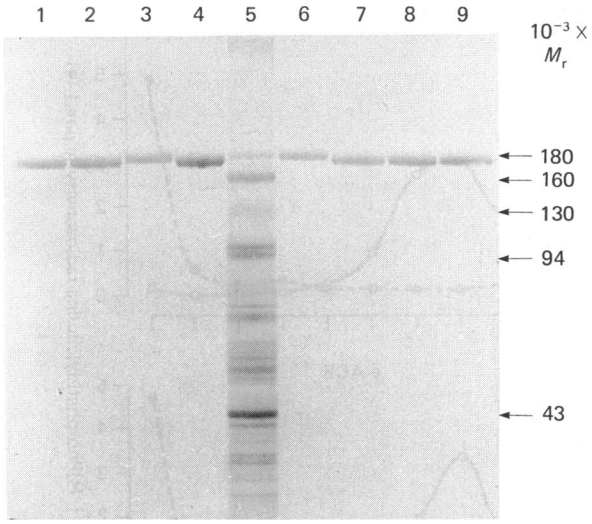

Fig. 1. SDS/polyacrylamide-gel electrophoresis of pig kidney ACE

Samples were prepared and analysed as described in the Experimental section. Lanes 1 and 7, pig kidney ACE solubilized with trypsin (t-form) (6 and 5 μg respectively); lanes 2 and 9, pig kidney ACE solubilized with Triton X-100 (d-form) (6 and 5 μg respectively); lanes 3 and 6, pig kidney ACE solubilized with Triton X-100 in the presence of EDTA (m-form) (5 and 4 μg respectively); lanes 4 and 8, pig kidney ACE solubilized by an endogenous mechanism (e-form) (4 and 3 μg respectively); lane 5, pig kidney microvillar membrane preparation prepared as described by Booth & Kenny (1974) (100 μg of protein). The gel was stained with Coomassie Brilliant Blue.

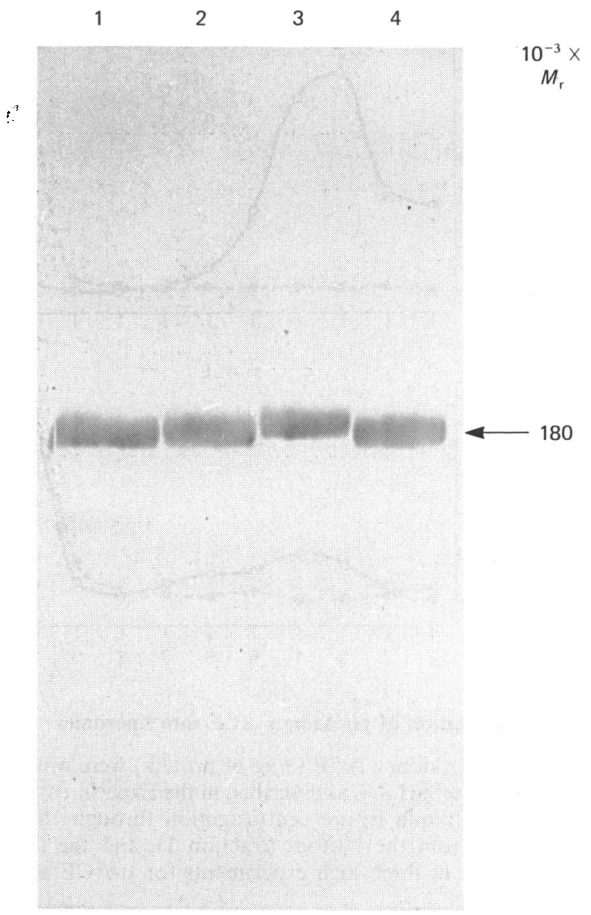

Fig. 2. 'Western' blot of pig kidney ACE

Samples were prepared and immunoelectrophoretic (Western) blot analysis was performed as described in the Experimental section. After electrophoretic transfer to nitrocellulose sheets, the tracks were blotted with antiserum to pig kidney ACE. Lane 1, pig kidney ACE solubilized with trypsin (t-form) (5 μg of protein); lane 2, pig kidney ACE solubilized with Triton X-100 (d-form) (5 μg of protein); lane 3, pig kidney ACE solubilized by Triton X-100 in the presence of EDTA (m-form) (5 μg of protein); lane 4, pig kidney ACE solubilized by an endogenous mechanism (e-form) (4 μg of protein).

dried peptide was dissolved in 50 μl of 0.2 M-$NaHCO_3$, containing 0.25% (w/v) SDS, and added to 15 mg of *p*-phenylene di-isothiocyanate–glass (17 nm pore size, 200–400 mesh), prepared by a modification of the method of Wachter *et al.* (1973). The glass was incubated at 56 °C for 60 min, then washed with water, and methanol containing 0.5% (v/v) *n*-propylamine was added to remove non-covalently bound material. Approx. 5 mg of the glass-coupled peptide was then sequenced by automated solid-phase Edman degradation (Laursen, 1971) using the microsequencing facility of the Sequencing Unit, Department of Biochemistry, University of Leeds. Anilinothiazolinone amino acids were converted, in 0.35 ml of 35% (v/v) trifluoroacetic acid for 7 min at 70 °C, into corresponding phenylthiohydantoin amino acids, which were identified by microbore reverse-phase h.p.l.c. on a Brownlee Spheri-5 C_{18} column using a method adapted from Zimmerman *et al.* (1977). The phenylthiohydantoin residues were quantified at 269 nm by using a Hewlett–Packard HP79994A Analytical Workstation. Serine and threonine residues were confirmed by the detection of their dehydro derivatives at 313 nm.

Enzyme assays

ACE activity was assayed either by h.p.l.c. or by a fluorimetric method modified from that described by Yang & Neff (1972), with Bz-Gly-His-Leu as substrate as described previously (Hooper & Turner, 1987). Alkaline phosphatase was assayed by the spectrophotometric method of Bessey *et al.* (1946), with *p*-nitrophenyl phosphate as substrate. Endopeptidase-24.11 and aminopeptidase-N were assayed as described previously (Matsas *et al.*, 1985). Renal dipeptidase was assayed by an h.p.l.c. method, with Gly-D-Phe as substrate (Hooper *et al.*, 1987). Protein was determined by the method of Lowry *et al.* (1951), or by a modification of the method of Bensadoun & Weinstein (1976).

RESULTS

Solubilization and purification of pig kidney ACE

Pig kidney ACE was solubilized from the membrane by four different procedures: (i) by incubating the membranes with trypsin [1:10 (w/w) trypsin/protein ratio] at 37 °C for 1 h, resulting in the trypsin-form (t-ACE); (ii) by addition of Triton X-100 (7:1 detergent/protein ratio) and stirring at room temperature for 1 h, resulting in the detergent-form (d-ACE); (iii) by incu-

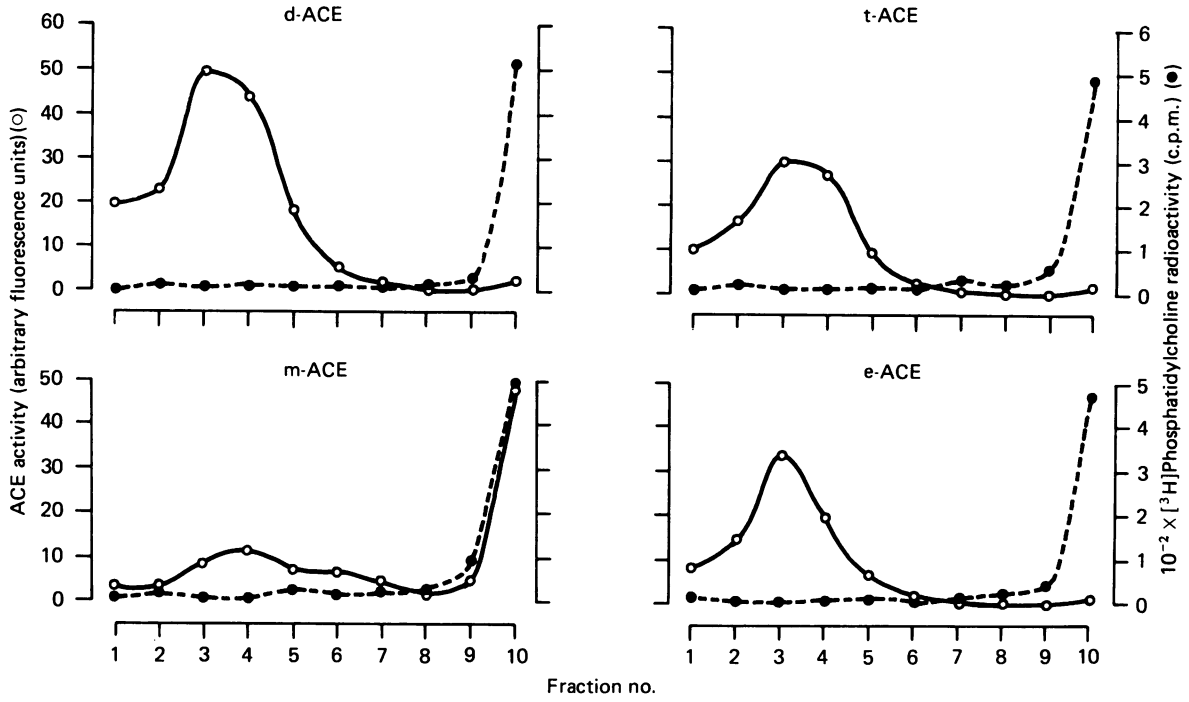

Fig. 3. Reconstitution of pig kidney ACE into liposomes

Samples of pig kidney ACE (5 μg of protein) were mixed with DMPC vesicles (200 μg) at 30 °C in 10 mM-Tris/100 mM-KCl/1 mM-ascorbate, pH 8.0, as described in the Experimental section. The mixture was treated at 18 °C for 10 min, and then warmed to 30 °C for 10 min before centrifugation through 20% sucrose/10 mM-Tris, pH 7.5, at 100000 *g* for 18 h. The tubes were fractionated from the bottom (fraction 1), and the fractions assayed for enzyme activity and radioactivity. The Figure is representative of three such experiments for d-ACE and t-ACE, seven for e-ACE and nine for m-ACE.

bating the membranes at room temperature for 1 h, resulting in the endogenous-form (e-ACE); or (iv) by incubating the membranes in the presence of 10 mM-EDTA for 1 h at room temperature, pelleting and washing the membranes, resuspending them in buffer containing 10 mM EDTA and adding Triton X-100 (7:1 detergent/protein ratio) and stirring at 4 °C or at room temperature for 1 h, resulting in the membrane-form (m-ACE).

After solubilization by each of the above methods, ACE was purified in a single step by either lisinopril–1.4 nm–Sepharose or lisinopril–2.8 nm–Sepharose as described in the Experimental section. At least two separate purifications were carried out for each solubilization method. Typically, a 700–1000-fold enrichment of activity was obtained, with the yield varying from 500 μg (using lisinopril–1.4 nm–Sepharose) to 10–15 mg (using lisinopril–2.8 nm–Sepharose), starting from 200 g of pig kidney cortex.

When the purified forms of pig kidney ACE were subjected to SDS/polyacrylamide-gel electrophoresis, only one band, of approx. M_r 180000, was observed in each case. The t-, d- and e-forms migrated identically, whereas the m-form appeared slightly larger (Fig. 1) and co-migrated with the polypeptide band corresponding to ACE in pig kidney microvillar membrane preparations (Fig. 1 and Hooper & Turner, 1987). The difference in M_r between the m-form and the other three forms was 5000 ± 500 ($n = 4$). All four forms of pig kidney ACE were also recognized by a polyclonal antiserum (Fig. 2), again with the observable size difference between m-ACE and the other forms.

Captopril, enalaprilat, lisinopril, L155 212 and L155 360, all at 1 μM, virtually completely inhibited (> 95%) the hydrolysis of Bz-Gly-His-Leu by all four purified forms of pig kidney ACE.

Incorporation of pig kidney ACE into liposomes

The ability of the various forms of pig kidney ACE to reconstitute into liposomes was examined. Triton X-100-solubilized immunoaffinity-purified pig kidney endopeptidase-24.11 was employed throughout to demonstrate reconstitution into the liposomes (Kenny *et al.*, 1983). Under conditions in which endopeptidase-24.11 reconstituted into the liposomes, the d-, t- and e-forms of pig kidney ACE failed to incorporate, whereas the m-form reconstituted to a similar extent as endopeptidase-24.11 (Fig. 3).

Phase separation of pig kidney ACE in Triton X-114

Samples of the four forms of pig kidney ACE were analysed by phase separation in Triton X-114 as described by Bordier (1981), and the detergent-rich and detergent-poor phases assayed for enzyme activity (Table 1). The d-, t- and e-forms of pig kidney ACE partitioned predominantly (> 93%) into the detergent-poor phase on treatment with Triton X-114, whereas the m-ACE partitioned predominantly (> 64%) into the detergent-rich phase. Under identical conditions, pig kidney endopeptidase-24.11, solubilized with Triton X-100, partitioned predominantly (84%) into the detergent-rich phase, and papain-solubilized pig kidney amino-

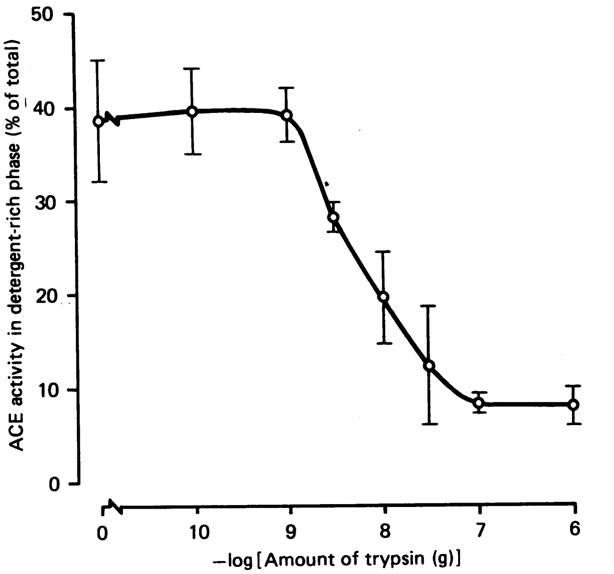

Fig. 4. Conversion of amphipathic membrane-ACE to a hydrophilic form by trypsin

Samples of purified pig kidney membrane-ACE (2 μg of protein) were incubated with increasing amounts of trypsin for 10 min at 37 °C in 10 mM-Hepes, pH 7.0, in a final volume of 50 μl. Immediately after the incubation, 150 μl of 10 mM-Tris/150 mM-NaCl/1.0% Triton X-114, pH 7.4, was added and the samples subjected to phase separation as described in Table 1. The results are means ($\pm$S.E.M.) for four incubations followed by phase separations with each amount of trypsin. The detergent-rich and detergent-poor phases were assayed in duplicate for enzyme activity, and the activity recovered in the detergent-rich phase is expressed as percentage of the total activity.

peptidase-N partitioned predominantly (96%) into the detergent-poor phase.

Treatment of membrane-ACE with trypsin

The purified m-form of pig kidney ACE was treated with increasing amounts of trypsin and immediately subjected to phase separation in Triton X-114 (Fig. 4). On increasing the amount of trypsin, the m-ACE partitioned increasingly into the detergent-poor phase until, at a trypsin/protein ratio of 1:20, only 8% of the activity remained in the detergent-rich phase.

Similarly, if the m-ACE were reconstituted into liposomes and then treated with trypsin (1:50 trypsin/protein ratio) the ACE activity failed to co-migrate with the liposomes after centrifugation through sucrose, whereas in parallel experiments m-ACE untreated with trypsin co-migrated with the liposomes (cf. Fig. 3).

Table 1. Triton X-114 phase separation of pig kidney ACE

Samples of purified pig kidney ACE (2 μg of protein) were made up to 200 μl with 10 mM-Tris/150 mM-NaCl/1.0% Triton X-114, pH 7.4, and subjected to phase separation at 30 °C for 3 min as described by Bordier (1981). The detergent-rich and detergent-poor phases were separated through a sucrose cushion by centrifugation at 3000 g and assayed in duplicate for enzyme activities. The results are means ($\pm$S.E.M.) for four phase separations. Activity recovered in the detergent-rich phase is expressed as percentage of the total activity.

Form of ACE	Enzyme activity in detergent-rich phase (% of total activity)
d-	6.2±4.0
t-	3.8±1.1
e-	2.6±2.6
m-	64.5±8.2

N-Terminal sequence of pig kidney ACE

Affinity-purified samples of each form of pig kidney ACE were sequenced as described in the Experimental section (Fig. 5). The d- and m-forms of pig kidney ACE had identical *N*-terminal sequences up to residue 24. Less sequence was obtained for the other forms of ACE, but the t-form was identical up to residue 9 and the e-form up to residue 6.

Release of pig kidney membrane enzymes by bacterial PI-PLC

When pig kidney membranes were incubated with purified preparations of PI-PLC from *S. aureus* or *B. thuringiensis*, no specific release of ACE was observed (Fig. 6). Under these conditions, alkaline phosphatase and renal dipeptidase were rapidly released, in agreement with previous results (Low & Finean, 1977; Takesue *et al.*, 1986; Hooper *et al.*, 1987). Negligible (< 5%) specific release of ACE was observed with *B. cereus* phospholipase C, *C. perfringens* phospholipase C,

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                  5                  10                  15                  20                  25
Leu-Asp-Ser-Ala-Leu-Gln-Pro-Gly-Cys-Phe-Thr-Ala-Asp-Glu-Ala-Gly-Ala-Glu-Asp-Phe-Ala-Gln-Ser-Phe-Leu-

——————————————————————————————— m-form ———————————————————————————————
——————— t-form ———————
———— e-form ————
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Fig. 5. *N*-Terminal sequence of pig kidney ACE

Samples of purified pig kidney ACE were coupled to glass and then sequenced by automated solid-phase Edman degradation as described in the Experimental section. The sequence is that of the d-form of pig kidney ACE. The extents of the sequences of the t-, e- and m-forms of pig kidney ACE are indicated. No differences in amino acid sequence among any of the four forms were observed.

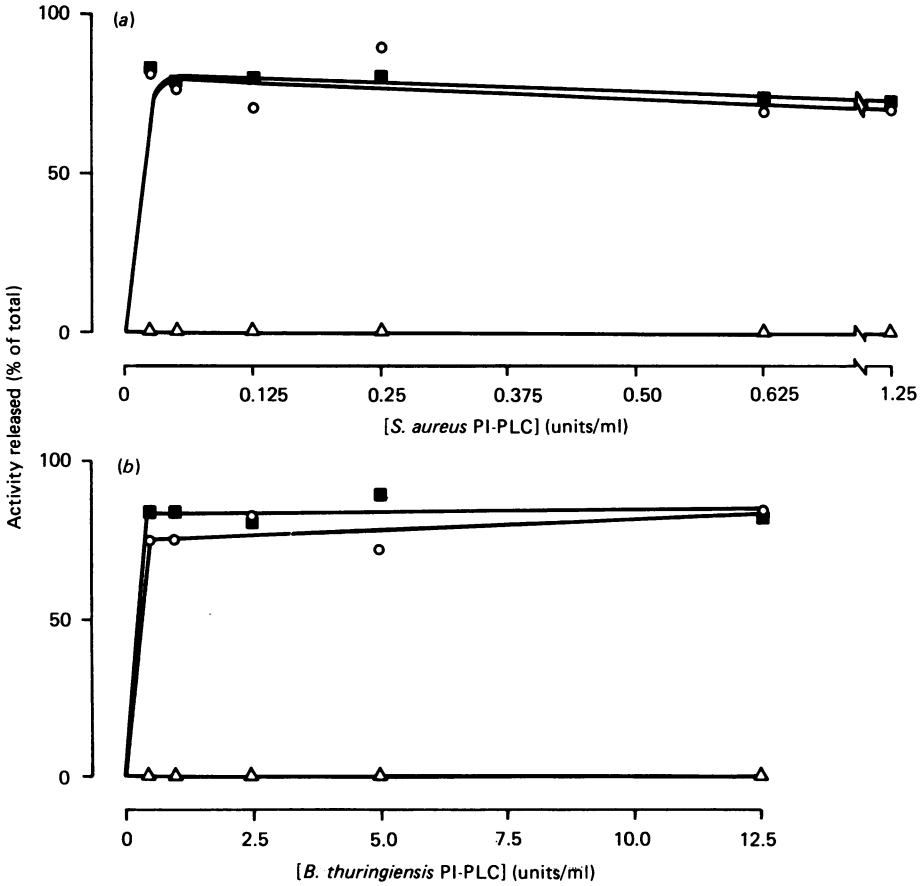

Fig. 6. Release of membrane enzymes from a pig kidney membrane fraction by bacterial PI-PLCs

Pig kidney membrane fraction (approx. 13 mg of protein/ml) was incubated in the presence of: (*a*) 10 mM-Hepes, pH 7.0, and *S. aureus* PI-PLC for 60 min at 37 °C. Data from a typical experiment are shown, one of four giving similar results. The highest concentration of PI-PLC used in this experiment was 2500-fold greater than that required to cause maximal release of alkaline phosphatase. (*b*) 10 mM-Hepes, pH 7.0, and *B. thuringiensis* PI-PLC for 60 min at 37 °C. Data from a typical experiment are shown, one of two giving similar results. In these experiments, no changes in total activity as a result of incubation either in the presence or absence of phospholipase were observed. ■, Alkaline phosphatase; ○, renal dipeptidase; △, ACE, endopeptidase-24.11, aminopeptidase N and protein.

phospholipase A_2 or phospholipase D (results not shown).

Treatment of membrane-ACE with phospholipases

Purified samples of m-ACE were incubated with the various phospholipases and then subjected to phase separation in Triton X-114 (Table 2). None of the phospholipases tested had any effect on the partitioning of the m-form of pig kidney ACE in Triton X-114.

The endogenous releasing mechanism for pig kidney ACE

When pig kidney membranes were incubated in 10 mM-Hepes, pH 7.0, at 37 °C, a fairly rapid and selective release of ACE from the membrane was observed (Fig. 7). Approx. 35% of the total ACE activity could be released in 3 h at 37 °C. No such release (maximum 2%) was observed for endopeptidase-24.11, aminopeptidase N, alkaline phosphatase and renal dipeptidase, and only 7% of the total protein was released. This release converted ACE from an amphipathic form into a hydrophilic form, as shown by subjecting the samples to phase separation in Triton X-114 (results not shown). The endogenous release of ACE was inhibited at 4 °C, 8.5% of the activity being released after 3 h as compared with 35.0% at 37 °C (Fig. 7). When pig kidney membranes were incubated at 37 °C in the presence of EDTA (5 mM final concn.), and the samples dialysed against excess $ZnCl_2$ before being assayed, the endogenous release of ACE was inhibited by 58.3% ± 9.3% ($n = 4$). Neither captopril (100 μM), an inhibitor of ACE, nor phosphoramidon (100 μM), an inhibitor of endopeptidase-24.11, inhibited the endogenous release.

DISCUSSION

ACE is known to exist primarily in a membrane bound form and, therefore, must be solubilized from the membrane before purification. The two most usual methods for releasing ACE from the membrane are treatment either with a mild detergent, such as Triton X-100, or with trypsin (Nishimura *et al.*, 1977; Stewart *et al.*, 1981; Weare *et al.*, 1982). In the present study, ACE was solubilized from pig kidney membranes by both Triton X-100 (d-form) and trypsin (t-form). Surprisingly, no difference in M_r value between these two forms of

Table 2. Triton X-114 phase separation of membrane-ACE after incubation with phospholipases and trypsin

Pig kidney m-ACE (2 μg of protein) was incubated with various phospholipases or trypsin in the appropriate buffer for 10 min at 37 °C in a total volume of 50 μl, and then immediately subjected to phase separation as described in Table 1. Duplicate phase separations were performed at two different concentrations of each phospholipase. The detergent-rich and detergent-poor phases were assayed in duplicate for enzyme activity and the results expressed as the mean. Buffers used were: a, 10 mM-Hepes, pH 7.0; b, 10 mM-sodium phosphate/0.1 M-NaCl, pH 7.0; c, 10 mM-Tris/10 mM-$CaCl_2$, pH 7.0; d, 10 mM-sodium acetate/50 mM-$CaCl_2$/30 mM-NaCl, pH 6.2. The lower of the two concentrations of *S. aureus* and *B. thuringiensis* PI-PLC used were equivalent to the concentration releasing 83.3 and 91.6% of alkaline phosphatase from pig kidney membranes respectively. Further abbreviations: PLA_2 and PLD, phospholipases A_2 and D respectively

Buffer	Enzyme	Enzyme activity in detergent-rich phase (% of total activity)
a	None	32.7
	S. aureus PI-PLC (0.215 munit)	37.9
	S. aureus PI-PLC (21.5 munits)	47.1
	B. thuringiensis PI-PLC (4.3 munits)	35.3
	B. thuringiensis PI-PLC (430 munits)	33.9
	Trypsin (0.1 μg)	8.2
b	None	45.8
	B. cereus PLC (5 munits)	49.9
	B. cereus PLC (500 munits)	43.3
c	None	49.8
	C. perfringens PLC (5 munits)	49.8
	C. perfringens PLC (500 munits)	46.6
	Bee venom PLA_2 (50 munits)	45.0
	Bee venom PLA_2 (5 units)	48.2
d	None	48.1
	Cabbage PLD (50 munits)	46.8
	Cabbage PLD (5 units)	51.8
	S. chromofuscus PLD (50 munits)	49.0
	S. chromofuscus PLD (5 units)	53.8

ACE was detectable on SDS/polyacrylamide-gel electrophoresis (Figs. 1 and 2). Furthermore, when subjected to phase separation in Triton X-114, both forms were found to partition almost exclusively into the detergent-depleted phase (Table 1), and neither form reconstituted into liposomes (Fig. 3). Therefore, on solubilization either by trypsin or by Triton X-100, it would appear that the hydrophobic membrane anchor of pig kidney ACE has been removed, producing an enzyme with hydrophilic properties.

When ACE was solubilized from pig kidney membranes by Triton X-100 in the presence of EDTA and subsequently purified (m-form), the pure protein appeared as a larger form, on SDS/polyacrylamide-gel electrophoresis, than either the t- or d-forms (Fig. 1). The difference in M_r of 5000 corresponds to that observed between trypsin-solubilized and membrane-bound human kidney ACE (Erdös & Gafford, 1983), although Lanzillo *et al.* (1985) failed to detect such a difference. The m-form of pig kidney ACE partitioned predominantly into the detergent-rich phase on phase separation in Triton X-114 (Table 1) and reconstituted into liposomes (Fig. 3), indicating that this form of ACE has amphipathic properties and retains the hydrophobic membrane anchor. Amphipathic m-ACE was converted into a hydrophilic form by treatment with trypsin, as shown by subjecting the enzyme to phase separation in Triton X-114 (Fig. 4) and by reconstitution into liposomes. These results indicate that the hydrophobic portion of m-ACE, which is responsible for its incorporation into liposomes, is readily cleaved from the bulk of the enzyme by trypsin, consistent with the ability of trypsin to solubilize membrane-bound ACE.

A number of ectoenzymes are known to be anchored in the plasma membrane by a sequence of hydrophobic amino acids near the *N*-terminus, including sucrase-isomaltase (Hunziker *et al.*, 1986), endopeptidase-24.11 (Fulcher *et al.*, 1986; Devault *et al.*, 1987; Malfroy *et al.*, 1987) and aminopeptidase N (Kenny & Maroux, 1982). Thus the *N*-terminal sequences of d-, t- and m-ACE were obtained (Fig. 5). The sequence of d-ACE shows 76% sequence homology with that of ACE from bovine lung (St. Clair *et al.*, 1986) and 62% homology with that from rabbit lung (Iwata *et al.*, 1983) over the first 21 residues. However, it was unclear from the work of those authors whether the hydrophilic or amphipathic forms of ACE had been isolated. We detected no sequence variability at the *N*-terminus between the amphipathic m-form of ACE and the hydrophilic d- and t-forms of ACE. Also, there is no sequence of hydrophobic amino acids starting within the first 15–20 residues as observed in *N*-terminally anchored membrane proteins (Fulcher *et al.*, 1986). From these results we conclude that pig kidney ACE is not anchored in the membrane through an *N*-terminal sequence of hydrophobic amino acids, but rather through its *C*-terminus. These data would be consistent with the observations that bovine lung ACE appears to be synthesized without a transient *N*-terminal signal sequence (St. Clair *et al.*, 1986) and that a *C*-terminal fragment of rabbit lung ACE aggregates readily, implying hydrophobic properties (Iwata *et al.*, 1983).

It is becoming evident that a number of plasma-membrane proteins are anchored in the lipid bilayer via a *C*-terminal glycolipid moiety containing covalently attached phosphatidylinositol [reviewed by Low *et al.* (1986) and Low (1987)]. A number of these proteins are ectoenzymes (alkaline phosphatase, acetylcholinesterase, 5′-nucleotidase, renal dipeptidase, trehalase) and, therefore, the possibility of ACE being anchored in the membrane by a similar glycosyl-phosphatidylinositol structure must be considered. The release of ACE from pig kidney membranes by bacterial PI-PLCs, a useful indicator of a phosphatidylinositol membrane anchor, was examined. ACE was not released from pig kidney membranes by *S. aureus* or *B. thuringiensis* PI-PLCs even at concentrations much greater than those required to release alkaline phosphatase and renal dipeptidase (Fig. 6). Aminopeptidase N and endopeptidase-24.11 were not released, as previously reported (Hooper *et al.*, 1987). A variety of phospholipases, including *S. aureus* and *B. thuringiensis* PI-PLCs, had negligible effects on the partitioning of purified m-ACE in Triton X-114 (Table 2), indicating that, even in the purified form, the hydrophobic anchor of m-ACE is resistant to hydrolysis

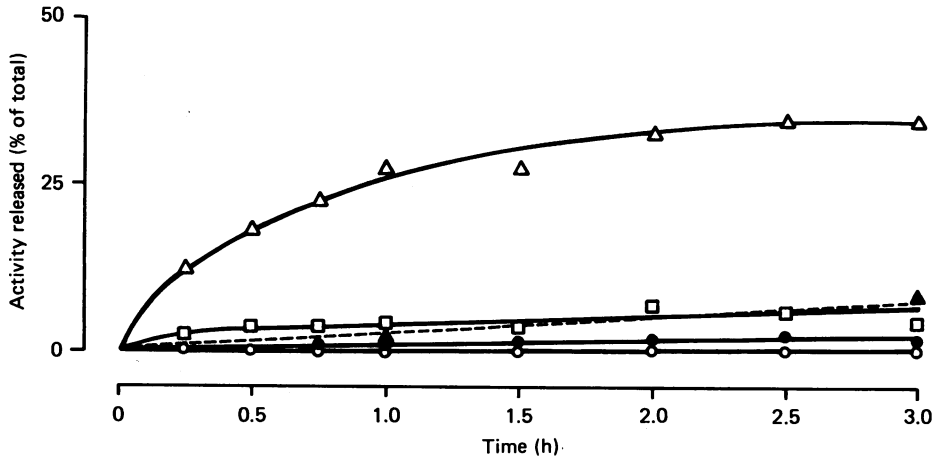

Fig. 7. Release of ACE from pig kidney membranes by an endogenous mechanism

Pig kidney membrane fraction (approx. 9 mg of protein/ml) was incubated in the presence of 10 mM-Hepes, pH 7.0, at 37 °C and 4 °C. Duplicate incubations were performed at each time interval and the results are means. No changes in total (100%) activity of the original incubation mixture as a result of the incubations were observed. △, ACE at 37 °C, ▲, ACE at 4 °C; □, protein at 37 °C; ●, aminopeptidase N at 37 °C; ○, alkaline phosphatase, endopeptidase-24.11 and renal dipeptidase at 37 °C.

by phospholipases. Although neither membrane-associated ACE nor the purified m-form appear to be susceptible to bacterial PI-PLC, the presence of a phosphatidylinositol membrane anchor cannot be ruled out, as several other proteins which are resistant to PI-PLC release are known to be anchored in this way (Futerman *et al.*, 1985; Davitz *et al.*, 1986; Low, 1987). Alternatively, the enzyme may be anchored by a hydrophobic *C*-terminal sequence of amino acids.

A fourth form of pig kidney ACE was solubilized merely by incubating the membranes at 37 °C; after affinity purification this was termed the e-form. An endogenous mechanism for releasing ACE has been previously observed on homogenization of lung tissue (Igic *et al.*, 1972; Nakajima *et al.*, 1973; Patchett & Cordes, 1985). The e-form of pig kidney ACE had properties identical with those of the d- and t-forms with respect to its M_r on SDS/polyacrylamide-gel electrophoresis (Figs. 1 and 2), hydrophilic properties in Triton X-114 (Table 1), failure to reconstitute into liposomes (Fig. 3) and its *N*-terminal sequence (Fig. 5). The time course for the endogenous release of a soluble form of ACE from pig kidney membranes (Fig. 7) showed that it was rapid at 37 °C and that it was relatively specific for ACE. Four other membrane enzymes, alkaline phosphatase, renal dipeptidase, aminopeptidase N and endopeptidase-24.11, were not released to any extent. The presence of EDTA appeared to inhibit the endogenous release of ACE from pig kidney membranes, and only when EDTA was present could solubilization of ACE by Triton X-100 result in the retention of the hydrophobic anchor (m-form). Low & Zilversmit (1980) observed that the decrease in M_r upon butanol solubilization of alkaline phosphatase from pig kidney microsomes was also inhibited by EDTA. The endogenous mechanism responsible for the release of a soluble form of ACE may resemble the endogenous activity present in human placenta, which converts alkaline phosphatase from a membrane-bound to a soluble form (Malik & Low, 1986). We cannot, at this stage, exclude the possibility that the endogenous activity originates from blood rather than from the microvillar membrane itself. Whether the endogenous mechanism for releasing ACE in a soluble form from pig kidney membranes is responsible for the production of soluble ACE found in plasma and other body fluids awaits further study.

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