**Angiotensin-converting enzyme of the human small intestine**

**Subunit and quaternary structure, biosynthesis and membrane association**

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Angiotensin-converting enzyme (ACE) was isolated from detergent-derived extracts of human intestinal brush-border membranes (BBMs) by immunoprecipitation using a monoclonal antibody. Analysis of the immunoprecipitates by SDS/PAGE revealed a polypeptide of apparent Mr, 184000 under reducing and non-reducing conditions, indicating that ACE does not contain intermolecular disulphide bridges. The quaternary structure of ACE was examined using cross-linking experiments with dithiobis(succinimidylpropionate) (DSP) and density gradient centrifugation on sucrose gradients. Both approaches demonstrated that ACE is assembled in the membrane as a monomer. By contrast, the control glycoprotein aminopeptidase N (APN) exists as a dimer. Biosynthetic labelling experiments in intestinal tissue explants demonstrated that the 184000-Mr protein is generated from a single-polypeptide, mannose-rich precursor of ACE (Mr, 175000) by modification of the carbohydrate side-chains in the Golgi apparatus. The mode of association of the mature form of the enzyme with BBMs was investigated by hydrophobic labelling of right-side-out brush-border vesicles with the photocleavable carbene-generating reagent [125I]-labelled 3-(trifluoromethyl)-3-(m-formylamino)phenyl)diazirine ([125I]-labelled TID), followed by treatment with trypsin at dilutions that do not cause substantial degradation of ACE. These studies demonstrated that ACE is associated with the membrane via a hydrophobic segment. Furthermore, treatment of [35S]-labelled inside-out membrane vesicles with trypsin revealed that ACE possesses a cytoplasmic tail, and therefore has a transmembrane orientation.

**INTRODUCTION**

Among the most important functions of angiotensin I-converting enzyme (ACE) (kininase II; EC 3.4.15.1) is its role in the regulation of blood pressure; ACE hydrolyses angiotensin I to the potent vasoconstrictor angiotensin II, and converts the vasodilator bradykinin into an inactive peptide (Yang et al., 1970; Erdös, 1975; Skeggs et al., 1976; Soffer, 1981; Patchett & Cordes, 1985). ACE has been identified in many tissues, for example lung, kidney, areas of the brain, and gut epithelium, with the lung being the site of the conversion of angiotensin I into angiotensin II (Ng & Vane, 1967). By virtue of this wide tissue distribution, ACE appears to have several important physiological functions other than its participation in blood pressure regulation.

ACE is a glycoprotein that consists of a single subunit of an apparent Mr, varying between 130000 and 180000 (Erdös, 1975; Soffer, 1976). The difference in the Mr's is presumably due to variations in the glycosylation pattern of the enzyme or to differences in the methods used in the estimation of Mr.

In most of the tissues investigated striking similarities were discerned in the physiochemical, immunological and catalytic properties of the enzyme (Soffer, 1981). This is further supported by cloning of cDNAs encoding ACE from different species and tissues. Mouse kidney ACE, for example, displays approx. 83% identity to human endothelial ACE in both nucleic-acid and amino-acid sequences, with the catalytic regions of both enzymes being the most highly conserved (Soubrier et al., 1988; Bernstein et al., 1989). An interesting feature of the ACE protein is its organization in two large homologous domains, each comprising a potential catalytic site (Soubrier et al., 1988; Bernstein et al., 1989). Each of these regions is almost half the size of the whole polypeptide. In light of these observations it has been suggested that the ACE gene has arisen by duplication of a common precursor gene.

Testicular ACE, on the other hand, is characterized by a shorter polypeptide of approx. 100000-Mr, (El Dorry et al., 1982a,b; Lanzillo et al., 1985; Erdös & Skidgel, 1987) and a different pattern of expression, since it first appears at puberty (Cushman & Cheung, 1971a). Cloning of a cDNA encoding this form from the human and rabbit testis (Ehlers et al., 1989; Kumar et al., 1989; Lattion et al., 1989) indicated that its amino-acid sequence is approximately half that of the endothelial or kidney enzyme and shows striking sequence similarities (almost 90%); (Kumar et al., 1989) with the C-terminal half of the endothelial enzyme (and probably also with the kidney enzyme). In contrast with the endothelial enzyme, however, testicular ACE contains one active catalytic centre. The fact that the testicular enzyme is as active as the endothelial protein suggests that one of the two catalytic sites of the endothelial enzyme, the counterpart of the testicular site, is responsible for the conversion of angiotensin I into angiotensin II. In accordance with these findings the consensus opinion has emerged that endothelial (or lung) ACE has resulted from gene duplication, whereby testicular ACE corresponds to an ancestral form of the non-duplicated gene.

ACE is found as a membrane-bound enzyme and as a circulating molecule in body fluids (Das et al., 1977). The membrane-bound forms, e.g. in endothelial cells, testis or kidney, are thought to be anchored in the membrane via a hydrophobic segment near the C-terminus (Hooper et al., 1987; Soubrier et al., 1988; Bernstein et al., 1989; Kumar et al., 1989).

The mechanisms that lead to the synthesis and expression of the membrane-bound and secreted forms are unknown. Based

Abbreviations used: ACE, angiotensin I-converting enzyme; APN, aminopeptidase N; BBM, brush-border membrane; DMS, dimethylsuberimidate; DSP, dithiobis(succinimidylpropionate); DSS, disuccinimidylsulinate; DTBP, dimethyl-3,3'-dithiobispropionimidate; IBLM, intracellular/basolateral membrane; [125I]-labelled TID, [125I]-labelled 3-(trifluoromethyl)-3-(m-formylamino)phenyl)diazirine; PBS, phosphate-buffered saline.
on the observations that secreted forms of ACE could be released merely by incubating the membranes at 37 °C (Hooper et al., 1987) or following tissue homogenization (Iig et al., 1972; Nakajima et al., 1973; Patchett & Cordes, 1985), an endogenous mechanism has been proposed for the generation of secreted ACE from membrane-bound ACE forms.

ACE has been also identified in intestinal epithelial cells. Here it was found associated with the microvilli in high concentration (Ward et al., 1980; Defendini et al., 1983) and is assumed to play a role in fluid and sodium absorption (Crocker & Mundy, 1970) or to participate in the terminal steps of digestion of dipeptides in the intestinal lumen (Yoshioka et al., 1987). However, several aspects of its structure, function and biosynthesis remain unknown. As an essential step towards a better understanding of the role and function of ACE in the intestinal brush-border membrane (BBM) and towards unravelling the molecular mechanisms underlying its secretion I investigated, in this paper, the subunit and quaternary structure of ACE in the human intestinal brush border, analysed its association with the membrane, and identified its precursor and mature forms in biosynthetically labelled intestinal tissue.

**EXPERIMENTAL**

**Materials**

Organ tissue-culture dishes (60 mm x 15 mm style with a centre well) were obtained from Falcon, Division Becton, Dickinson and Co. (Basel, Switzerland). RPMI 1640, methionine-free RPMI 1640, streptomycin, penicillin and foetal calf serum were purchased from Gibco. [35S]Methionine ( > 800 Ci/mmol; translation grade) and Enlightening were purchased from New England Nuclear, Dreieich, Germany; [181I]-labelled 3-(trifluoromethyl)-3-(m(formylamino)phenyl)diazirine ([181I]-labelled TID) (> 10 Ci/mmol) was obtained from Amersham. Pepstatin, leupeptin, benzamidine, antipain, antipain, Mr standards for SDS/PAGE, β-amylose and aperoferritin were purchased from Sigma Chemical Co. Acrylamide, NN'-methylenebisacrylamide, SDS, tris(hydroxymethyl)aminomethane, NN'/N'-tetramethylenediamine (TEMED), ammonium persulfate, 2-mercaptoethanol, dithiothreitol, and Triton X-100 were obtained from BioRad laboratories. Dodecyl-β-maltoside was from Calbiochem. Endo-β-N-acetylglucosaminidase H (endo H) (from a recombinant Streptomyces lividus strain carrying the Streptomyces pilcatus gene), and phenylmethanesulphonyl fluoride (PMSF) were obtained from Boehringer-Mannheim. Protein A-Sepharose and wheat-germ-agglutinin-Sepharose 4B were purchased from Pharmacia Fine Chemicals. The Protein A-Sepharose beads were washed alternately with 0.1 m-sodium phosphate, pH 8.1, and 0.1 m-sodium citrate, pH 4.0, three times with each buffer and three times with phosphate-buffered saline (PBS), pH 7.4, and finally suspended in an equal volume of PBS (denoted Protein A-Sepharose suspension). Dithiobis(succinimidylpropionate) (DSP), dimethylsuberimidate (DMS), dimethyl-3,3′-dithiobispropionimidate (DTBP) and disuccinimidylsuberate (DSS) were from Pierce Chemical Company, Rockford, IL, U.S.A. All other reagents were of superior analytical grade.

**Biological and immunological materials**

Human small intestinal mucosa (approx. 5–10 mg wet wt.) were obtained from patients biopsied for diagnostic purposes. They were examined by light microscopy and showed normal intestinal morphology with villi structures and expressed normal levels of sucrase and lactase (sucrase 28–80 i.u./g and lactase 16–49 i.u./g, determined according to Asp et al., 1975). Monoclonal antibodies against ACE (product of hybridoma HBB 3/264/18), and aminopeptidase N (ApN) (HBB 3/153/63) were gifts from Dr. Hans-Peter Hauri (Biocenter, Basel, Switzerland) (Hauri et al., 1985) and were used in the form of ascites prepared from hybridoma-bearing pristane-primed Balb/c mice. The specificities of the antibodies were assessed by enzyme-activity measurements of immunosolated antigens (Hauri et al., 1985) and were confirmed in our laboratory by following the same experimental protocol. Thus the antigenic material precipitated by the hybridoma HBB 3/264/18 revealed ACE activity as assessed by the method of Cushman & Cheung (1971b) using N-α-hippuryl-L-histidyl-L-leucine as substrate. Enzymic activities corresponding to other BBM disaccharidases, e.g. sucrase, isoamylase, lactase-phlorizin hydrolase, or BBM peptidases, e.g. ApN, dipeptidyl peptidase IV, were not detected. Likewise, the antigenic material precipitated by 3/153/63 was found to hydrolyse only substrates for ApN.

For immunoprecipitation experiments, 2–3 μl of the anti-ACE antibody (or anti-ApN) was added to 60 μl of Protein A-Sepharose suspension and 1 ml of 0.1 M-phosphate buffer, pH 8.0, and incubated for 1 h at 4 °C. The beads (denoted immunobeads) were washed with phosphate buffer and further used for immunoprecipitation with the corresponding antigen.

**Biosynthetic labelling of biopsy specimens**

Intestinal biopsy specimens were biosynthetically labelled, essentially as described by Naim et al. (1987, 1988a). In brief, each biopsy specimen was placed on stainless-steel grids in organ culture dishes (Browning & Trier, 1969) and labelled with 1 ml of an incubation medium containing methionine-free RPMI 1640, streptomycin (100 μg/ml), penicillin (100 units/ml), 10% (v/v) dialysed fetal calf serum (FCS) and 150 μCi of [35S]methionine. Labelling was performed for both 30 min and 4 h. After labelling, the biopsy specimens were cooled to 4 °C, washed three times in cold PBS and homogenized with a Teflon–glass homogenizer in 1 ml of buffer A [25 mm-Tris/HCl (pH 8.1), 50 mm-NaCl, 1 mm-PMSF, 1 μg of pepstatin, 5 μg of leupeptin, 17.4 μg of benzamidine, 5 μg of antipain and 1 μg of aprotinin]. The homogenates were either further processed directly for immunoprecipitation or kept frozen at −20 °C until used.

**Preparation of right-side-out and inside-out brush-border vesicles**

Right-side-out BBM were purified by lectin affinity chromatography using wheat germ agglutinin. Briefly, 2 ml of BBM [1 mg/ml; FII fraction (Sterchi & Woodley, 1980)] was incubated with 2 ml of the wheat-germ-agglutinin-Sepharose beads for 2 h at 4 °C. The beads were washed four times with 25 mm-Tris/HCl, pH 7.5/50 mm-NaCl (buffer B) and eluted for 1 h at 4 °C with 2 ml of buffer B supplemented with 2.5% (w/v) N-acetylglucosamine. The eluates were centrifuged (25000 g, 30 min) and the pellet resuspended in 1 ml of buffer B. Hydrophobic labelling of these vesicles with 100 μCi of [181I]-labelled TID was performed according to Lüscher et al. (1985). The labelled membranes were spun at 100000 g for 20 min, resuspended in 2 ml of buffer B (see above) and then divided into two equal parts. One part was solubilized in 0.5% Triton-100 and 0.5% sodium deoxycholate (final concentrations) and immunoprecipitated with anti-ACE antibodies. The other part was treated with trypsin [1:10 (w/w) trypsin/protein] at 37 °C for 1 h. The reaction was stopped by the addition of a 4-fold excess, over trypsin, of soybean trypsin inhibitor. The membranes were then centrifuged at 100000 g for 1 h at 4 °C and the supernatant was immunoprecipitated with anti-AE antibodies.

Inside-out membrane vesicles were purified as follows. Four biopsy samples (about 20–40 mg wet wt.) were labeled for 6 h
with [³⁵S]methionine and homogenized in buffer A. BBM were purified by subcellular fractionation using CaCl₂ (Naim et al., 1988a) and the pellet homogenized in buffer B. These homogenates contain both inside-out and right-side-out vesicles. The homogenates were then depleted of the right-side-out vesicles by absorption on a wheat-germ-agglutinin-conjugated Sepharose 4B column. After the first run the column was eluted with 2.5% (w/v) N-acetylglucosamine. The inside-out vesicles, which were recovered as unbound material in the column flow-through, were passed once again through the column to ensure complete depletion of right-side-out vesicles. Finally, half of the inside-out vesicles was treated with 50 μg of trypsin at 37 °C for 1 h and the other half was incubated under the same conditions without trypsin. Trypsin-treated and non-treated vesicles were centrifuged at 100 000 g for 1 h at 4 °C, the pellets were solubilized and then immunoprecipitated with anti-ACE antibodies.

**Sucrose-gradient centrifugation**

Biopsy samples were biosynthetically labelled for 6 h with [³⁵S]methionine, homogenized and subcellularly fractionated, using CaCl₂, into BBMs and intracellular/basolateral membranes (IBLMs) according to Schmitz et al. (1973). The BBM fraction, corresponding to four biopsy samples, was solubilized in 500 μl of 6 mM-dodecyl-β-maltoside, 50 mM-Tris/HCl, 100 mM-NaCl, pH 7.5, and a mixture of protease inhibitors (see above) for 1 h at 4 °C. After centrifugation at 100 000 g for 1 h at 4 °C the supernatant was loaded on to a sucrose gradient that had been made on the top of 600 μl of 50% (w/v) sucrose cushion and consisted of 10–30% (w/v) sucrose, 50 mM-Tris/HCl, 100 mM-NaCl, 6 mM-dodecyl-β-maltoside, pH 7.5. The gradient was centrifuged at 105 000 g for 16 h at 4 °C and divided into 16 equal parts (approx. 640 μl/fraction), with fraction 1 representing the bottom of the gradient. The fractions were diluted with an equal volume of water and the pH was adjusted to 8.0 before immunoprecipitation with a mixture of anti-ACE and anti-ApN antibodies. Finally, the immunoprecipitates were analysed by SDS/PAGE on 5% gels.

**Other procedures**

Preparation of BBM vesicles [fraction II (FI)] from small-intestinal mucosa was performed according to the CaCl₂-fractionation method of Schmitz et al. (1973) and the modification by Sterchi & Woodley (1980). Subcellular fractionation of biosynthetically labelled biopsy specimens into BBMs and IBLMs was according to Naim et al. (1988a). Chemical cross-linking of BBMs using DSP in dimethyl sulphoxide was performed as described by Naim et al. (1988b). Cross-linking experiments were also performed using several other homobifunctional reagents such as DMS, DTBP and DSS. The results with these reagents were similar to those obtained with DSP and will therefore not be reported here. Immunoprecipitations were essentially performed according to Naim et al. (1987). Digestion of [³⁵S]labelled immunoprecipitates with endo H was performed as previously described (Naim et al., 1987, and references therein); the endo H concentration used was 3 mi.u. Digested proteins were recovered by precipitation with trichloroacetic acid, 15% (w/v) final concentration and the pellets were washed twice with ice-cold acetone and kept at −20 °C.

One-dimensional electrophoresis was conducted in 4% or 6% polyacrylamide slab gels containing 0.1% SDS by the method of Laemmli (1970).

**RESULTS AND DISCUSSION**

**Molecular forms of human intestinal ACE**

To assess the subunit structure of ACE in the intestinal BBM, we isolated ACE from detergent-derived extracts of BBMs by immunoprecipitation with a monoclonal antibody conjugated to Protein A-Sepharose. The immunoprecipitates were further analysed by SDS/PAGE.

Fig. 1 shows that under completely denaturing conditions [4% (w/v) SDS, 100 mM-dithiothreitol] a single polypeptide of apparent Mr 184 000 was identified (lane 1) (the band at the bottom of the gel of Mr 50 000 is the heavy chain of the immunoglobulin molecule). In the absence of reducing agents a polypeptide of identical apparent Mr is revealed (lane 2) (the band of apparent Mr 150 000 is the immunoglobulin molecule and the faint bands indicated by closed circles represent non-specific binding since they are also, together with the IgG molecule, found in the control (lane 3)). These observations indicated that ACE does not contain intermolecular disulphide bonds and consists of a single subunit under completely denaturing conditions.

To identify precursor and mature forms of ACE and assess its pattern of glycosylation, biopsy samples were biosynthetically labelled with [³⁵S]methionine for 30 min or 4 h and homogenized. The detergent-derived extracts were immunoprecipitated with anti-ACE antibodies. The precursor and mature forms of ACE were identified by probing for their endo H sensitivity. Within 30 min of labelling ACE was detected as a polypeptide of apparent Mr 174 000 (Fig. 2, lane 1) which was sensitive to endo H treatment (Fig. 2, lane 2), indicating that it represents the mannose-rich precursor of ACE. After 4 h of labelling a band of apparent Mr 184 000 was revealed (Fig. 2, lane 3). This band was practically resistant to treatment with endo H (Fig. 2, lane 4) indicating that it carries complex types of oligosaccharides and has been processed in the Golgi apparatus. Together with the identification of the 184 000-Mr protein in BBMs (see Fig. 1) the results demonstrate that ACE is synthesized as a single-chain.
Normal human intestinal biopsy samples were biosynthetically labelled for 30 min (lanes 1 and 2) and 4 h (lanes 3 and 4) with [35S]methionine and homogenized. Detergent extracts of the homogenates were immunoprecipitated with anti-ACE and analysed by SDS/PAGE on 5% gel after endo H treatment (lanes 2 and 4) or without treatment (lanes 1 and 3). Shown is a fluorogram of the gel.

**(a)** Detergent extracts of the homobifunctional lysine-specific reagent DSP were solubilized with Triton X-100 and ACE and a control brush-border peptidase, ApN, were immunoprecipitated and analysed by SDS/PAGE. **Fig. 3(a)** demonstrates that no difference in the electrophoretic pattern of ACE could be detected in the presence (lane 2) or absence (lane 1) of the cross-linking reagent. By contrast, significant changes in the band pattern of the control glycoprotein, ApN, were observed on cross-linking with DSP (Fig. 3b). In the absence of the cross-linker ApN is characterized by one major band of apparent Mr 160000 and a faint band of apparent Mr 265000 (Fig. 3b, lane 1). On cross-linking the staining intensity of the 265000-Mr protein increases significantly, and a faint, high-molecular-mass band additionally appears (indicated by the arrow) with a concomitant decrease in the staining intensity of the 160000-Mr protein (Fig. 3b, lane 2). We tested several other cross-linking reagents also, such as DMS, DTBP and DSS. These were effective in cross-linking ApN but failed to cross-link ACE (results not shown). These results indicate the existence of homodimers of ApN and suggest that ACE exists as a monomeric protein in the BBM. However, we cannot exclude the possibility that the failure to find cross-linked products of ACE is caused by the absence of correctly positioned residues for the cross-linking reaction. Moreover, among the possible disadvantages of chemical cross-linking is that it is not necessarily a quantitative and efficient approach. Because of these limitations I chose sucrose-density-gradient centrifugation as an alternative approach by which to probe the oligomeric state of ACE in BBMs. For this purpose BBMs were purified from biosynthetically labelled biopsy samples and solubilized with dodecyl-β-maltoside. The detergent extracts were subjected to centrifugation in a 10–30% gradient of sucrose. The gradient was then fractionated into 16 equal aliquots, with fraction 1 representing the top of the gradient (i.e. approx. 10% (w/v) sucrose). The fractions were immunoprecipitated with anti-ACE antibodies as well as with antibodies against the control glycoprotein ApN and the precipitates were analysed by SDS/PAGE. Fig. 4 shows that ACE was found exclusively in fraction 7 (Fig. 4, lane 4). The control glycoprotein, ApN, which has an apparent Mr of 160000 and therefore is slightly smaller than ACE, appeared mainly in fraction 9 and to a lesser extent in fraction 10 (Fig. 4, lanes 6 and 7), i.e. in a denser phase than ACE, thus demonstrating that ACE and ApN have different quaternary structures.

**Fig. 2. Biosynthesis of ACE in organ culture of human intestinal biopsy specimens**

Normal human intestinal biopsy samples were biosynthetically labelled for 30 min (lanes 1 and 2) and 4 h (lanes 3 and 4) with [35S]methionine and homogenized. Detergent extracts of the homogenates were immunoprecipitated with anti-ACE and analysed by SDS/PAGE on 5% gel after endo H treatment (lanes 2 and 4) or without treatment (lanes 1 and 3). Shown is a fluorogram of the gel.

**Fig. 3. Immunoprecipitation of (a) ACE and (b) ApN from cross-linked BBMs**

(a) BBMs were cross-linked with DSP (lane 2) or not cross-linked (lane 1). Detergent extracts of both probes were immunoprecipitated with anti-ACE and analysed by SDS/PAGE on 4% slab gels under non-reducing conditions. A Coomassie Blue stained gel is shown. (b) ApN was immunoprecipitated as in (a), from cross-linked (lane 2) or non-cross-linked (lane 1) BBMs and analysed by SDS/PAGE on 4% slab gels followed by Coomassie Blue staining.

precursor that acquires complex glycans in the Golgi and is further transported to the BBM.

**ACE is present in the BBM as a monomeric protein**

The quaternary structure of ACE was probed by chemical cross-linking to examine whether non-covalently associated homodimeric structures of ACE do exist. BBM vesicles were cross-linked with the homobifunctional lysine-specific reagent DSP to retain their native membrane organization. After cross-linking, the membranes were solubilized with Triton X-100 and ACE and a control brush-border peptidase, ApN, were immunoprecipitated and analysed by SDS/PAGE. **Fig. 3(a)** demonstrates that no difference in the electrophoretic pattern of ACE could be detected in the presence (lane 2) or absence (lane 1) of the cross-linking reagent. By contrast, significant changes in the band pattern of the control glycoprotein, ApN, were observed on cross-linking with DSP (Fig. 3b). In the absence of the cross-linker ApN is characterized by one major band of apparent Mr 160000 and a faint band of apparent Mr 265000 (Fig. 3b, lane 1). On cross-linking the staining intensity of the 265000-Mr protein increases significantly, and a faint, high-molecular-mass band additionally appears (indicated by the arrow) with a concomitant decrease in the staining intensity of the 160000-Mr protein (Fig. 3b, lane 2). We tested several other cross-linking reagents also, such as DMS, DTBP and DSS. These were effective in cross-linking ApN but failed to cross-link ACE (results not shown). These results indicate the existence of homodimers of ApN and suggest that ACE exists as a monomeric protein in the BBM. However, we cannot exclude the possibility that the failure to find cross-linked products of ACE is caused by the absence of correctly positioned residues for the cross-linking reaction. Moreover, among the possible disadvantages of chemical cross-linking is that it is not necessarily a quantitative and efficient approach. Because of these limitations I chose sucrose-density-gradient centrifugation as an alternative approach by which to probe the oligomeric state of ACE in BBMs. For this purpose BBMs were purified from biosynthetically labelled biopsy samples and solubilized with dodecyl-β-maltoside. The detergent extracts were subjected to centrifugation in a 10–30% gradient of sucrose. The gradient was then fractionated into 16 equal aliquots, with fraction 1 representing the top of the gradient (i.e. approx. 10% (w/v) sucrose). The fractions were immunoprecipitated with anti-ACE antibodies as well as with antibodies against the control glycoprotein ApN and the precipitates were analysed by SDS/PAGE. Fig. 4 shows that ACE was found exclusively in fraction 7 (Fig. 4, lane 4). The control glycoprotein, ApN, which has an apparent Mr of 160000 and therefore is slightly smaller than ACE, appeared mainly in fraction 9 and to a lesser extent in fraction 10 (Fig. 4, lanes 6 and 7), i.e. in a denser phase than ACE, thus demonstrating that ACE and ApN have different quaternary structures.
The gradient was calibrated using the proteins \( \beta \)-amylase (\( M_T \), 200000) and apoferritin (\( M_T \), 450000). \( \beta \)-Amylase was revealed to be mainly in fraction 7, while apoferritin was found in fraction 12. The fact that ApN appears in fraction 9 of the gradient, i.e. between \( \beta \)-amylase and apoferritin, is compatible with a homodimeric structure of the protein (\( 2 \times 160000 = 320000-M_T \)). The possibility that ApN is also assembled in the membrane as a trimeric (\( 3 \times 160000 = 480000-M_T \)) or tetrameric (\( 4 \times 160000 = 640000-M_T \)) protein could be excluded since these forms should be found in gradient fractions that have a comparable density to apoferritin (trimeric ApN) or an even higher density than apoferritin (tetrameric ApN), and this was not the case. Collectively, the cross-linking and the gradient-centrifugation experiments demonstrate that ApN is a dimeric protein. On the other hand, the density-gradient-centrifugation experiments show that ACE is assembled in the BBM as a monomer since: (i) dimeric ACE (\( 2 \times 184000 = 368000-M_T \)) should be recovered in gradient fractions of density similar to dimeric ApN (320000-M_T), i.e. fraction 9, or even of slightly higher density, i.e. fraction 10; and (ii) ACE is revealed in the same fraction as the control protein \( \beta \)-amylase (\( M_T \), 200000).

Mode of association of ACE with the membrane

The mode of attachment of intestinal ACE to the membrane was examined by performing hydrophobic labelling of intact BBM vesicles using \( ^{125}\)I-TID. TID is a photolabel that exclusively reacts with the hydrophobic core of membrane proteins (Spiess et al., 1982). Right-side-out vesicles were enriched from BBMs by chromatography using wheat-germ-agglutinin–Sepharose beads and were subsequently labelled with TID. Fig. 5(a) shows that ACE was immunoprecipitated from detergent-derived extracts of TID-labelled vesicles (lane 2), indicating the presence of a hydrophobic segment in ACE that is accessible to the label. To examine whether the label is specifically confined to a hydrophobic domain in the molecule, labelled vesicles were treated with trypsin, centrifuged and the supernatant immunoprecipitated with anti-ACE antibodies. As shown in Fig. 5(a), this treatment resulted in the failure of ACE antibodies to immunoprecipitate radioactively labelled ACE bands from the supernatant of trypsin-treated vesicles (lane 1). By contrast, Coomassie Blue staining of the same gel revealed strongly stained bands in the supernatant of trypsin-treated vesicles (Fig. 5b, lane 1; note that lane 1 in Fig. 5b is the Coomassie Blue-stained lane 1 in Fig. 5a) and in the pellet of non-treated samples (Fig. 5b, lane 2). Moreover, the size of trypsin-treated ACE was slightly smaller (about 5000-M_T) than the non-treated sample. Therefore the failure to detect radioactively labelled ACE in the supernatant of trypsin-treated samples (Fig. 5a, lane 1) is not the consequence of a substantial degradation of ACE by trypsin. The results are indicative of the existence of a domain in the ACE molecule that is accessible to hydrophobic probes. Since a major part of the molecule (\( M_T \), 179000) was cleaved from the membrane by trypsin treatment, released into the supernatant as a water-soluble molecule and not labelled by the hydrophobic label, I conclude that the label is confined to a hydrophobic domain in ACE that is responsible for its anchorage in the membrane. Furthermore I wanted to determine whether ACE possesses a cytoplasmic tail and has therefore a transmembrane orientation. One approach is to separate inside-out membrane vesicles from total BBMs and to subject them to treatment with trypsin. A removal of a putative cytoplasmic tail by trypsin would be consistent with a reduction in the apparent \( M_T \) of ACE, which could be electrophoretically detected. For this purpose biopsy samples were labelled with \( ^{125}\)I-methionine for 6 h. The biopsy homogenates were subcellularly fractionated into IBLMs and BBMs. Immunoprecipitation of part of these membrane fractions with anti-ACE antibodies revealed two bands in the IBLM fraction having apparent \( M_T \)s of 174000 and 184000 (Fig. 6a, lane 2) and one predominant band in the BBM fraction of apparent \( M_T \), 184000 (Fig. 6a, lane 1). The BBMs containing the 184000-M_T, proteins were passed through a lectin column to separate right-side-out from inside-out vesicles. The right-side-out vesicles are those retained by the lectin column due to the surface expression of glycosylated proteins, while inside-out vesicles expose existing cytoplasmic tails of glycoproteins and are therefore not retained by the column and instead are recovered in the column flow-through fractions. The inside-out vesicles were then digested with trypsin and subjected to immunoprecipitation with anti-ACE antibodies. Fig. 6(b) shows that trypsin-treated inside-out vesicles contain an ACE band that has a slightly reduced apparent \( M_T \) (lane 2) in comparison with ACE isolated from non-trypsin-treated vesicles (lane 1). The results therefore provide evidence for the existence of a cytoplasmic portion of ACE that is cleaved off during trypsin treatment. The reduction in the apparent \( M_T \) by approx. 3000 is consistent with the proposed length of the cytoplasmic tail of ACE deduced from cDNA cloning of the endothelial enzyme (Soubrier et al., 1988).

In view of the fact that ACE possesses a membrane-anchoring domain and a cytoplasmic tail, we conclude that membrane-bound intestinal ACE has a transmembraneous orientation. The difference in \( M_T \) of about 5000 between ACE and hydrophilic ACE that was generated by trypsin treatment of right-side-out vesicles probably represents the size of the cytoplasmic tail together with the hydrophobic stretch that spans the lipid bilayer. Hooper et al. (1987) have also observed that the membrane-bound form of pig kidney ACE has an \( M_T \) approx. 5000 greater than that of trypsin-treated or hydrophilic ACE forms.

Concluding remarks

In this paper I investigated the biosynthesis, subunit and

Fig. 5. Association of ACE with the membrane

(a) Right-side-out brush border vesicles were labelled with the hydrophobic reagent \( ^{125}\)I-TID, and divided into two equal parts. One part was solubilized and immunoprecipitated with anti-ACE antibodies (lane 2) and the other was treated with trypsin [1:10 (w/w) trypsin/protein ratio] at 37°C for 1 h, centrifuged and the supernatant was immunoprecipitated with anti-ACE antibodies (lane 1). The precipitates were analysed by SDS/PAGE on 5% gels and autoradiography. (b) Coomassie Blue staining of the gel in (a). Note that the bands at the same level around the centre of the gel represent the antibody molecule. The fact that these bands run at the same level while trypsin-treated ACE (lane 1) is slightly smaller than ACE found in non-treated vesicles (lane 2) confirms that the shift in the apparent \( M_T \), of the ACE molecule is indeed due to the cleavage of a 5000-M_T fragment.
quaternary structure and membrane association of ACE in the human small intestine.

I could demonstrate that ACE is synthesized as a single-chain polypeptide precursor that matures to the final BBM form (M, 184,000). ACE does not contain intermolecular disulphide bridges and is assembled in the BBM as a monomeric glycoprotein, in contrast with ApN which exists as a homodimeric glycoprotein. Finally, ACE is associated with the BBM via a hydrophobic segment that spans the membrane at least once and has a transmembrane orientation. My results lend strong support to cloning data which have proposed that membrane-bound ACE in various species and tissues has a hydrophobic segment near its C-terminus that might function as a membrane anchor (Soubrier et al., 1988; Bernstein et al., 1989; Ehlers et al., 1989; Kumar et al., 1989) and support the notion that ACE is not attached to the membrane by a glycoporpholipid anchor (Hooper et al., 1987).

I do not know whether ACE is also found in a monomeric form in other tissues and species. Most of the brush-border components so far characterized are known to be assembled in the BBM as homo- or hetero- or homoheterodimers (Cowell et al., 1986; Naim et al., 1988b; Daniels, 1990; Jascur et al., 1991; for reviews see Kenny & Maroux, 1982; Semenza, 1986). In many cases, dimerization or oligomerization of these proteins have been shown to be important for intracellular transport, since it occurs before cell surface insertion (Danielsen, 1990; Jascur et al., 1991). This is consistent with current concepts of biosynthesis and processing of membrane and secretory proteins that have assigned a crucial role to oligomerization in intracellular protein transport (Gething et al., 1986; Kreis & Lodish, 1986; Rose & Doms, 1988; Hurtley & Helenius, 1989). In this respect it would be important to determine whether the processing and intracellular transport of ACE are affected or hampered by its monomeric structure or whether, by virtue of the two large homologous domains (Soubrier et al., 1988; Bernstein et al., 1989; Chen & Riordan, 1990), monomeric ACE forms pseudodimers that are transport competent.

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Structural features of human intestinal ACE


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