Proteolytic Release of Human Angiotensin-converting Enzyme
LOCALIZATION OF THE CLEAVAGE SITE*

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Angiotensin-converting enzyme (EC 3.4.15.1, ACE) is a transmembrane protein with a short carboxyl-terminal cytoplasmic domain, a 17-amino acid hydrophobic anchor domain, and a large N-terminal extracellular region containing two catalytically homologous domains. An active soluble form of ACE circulates in human plasma and is produced in culture medium of Chinese hamster ovary (CHO) cells transfected with the full-length human ACE cDNA. The mechanism of ACE release in CHO cells involves a post-translational proteolytic cleavage occurring in the carboxyl-terminal region. The carboxyl terminus of the secreted recombinant ACE, AGQR, was established by carboxyl-terminal microsequencing and corresponds to a cleavage site between Arg-1137 and Leu-1138. Two independent studies confirmed this proposed cleavage site: amino acid analysis of a carboxyl-terminal peptide derived from soluble ACE and immunocharacterization of membrane-bound and soluble ACE with antibodies raised against three peptides located along the carboxyl-terminal ACE sequence. In order to assess the importance of Arg-1137, this amino acid was mutated to a glutamine residue. This mutation did not prevent the secretion of ACE, suggesting that the solubilizing enzyme can accommodate this change or can use an alternative cleavage site. Finally, the production of soluble ACE in CHO cells appears to be proportional to the level of cellular ACE, implying that the solubilizing enzyme is not a limiting factor. In addition, the carboxyl-terminal sequence of the human plasma ACE was identified as AGQR, thus supporting the fact that a similar mechanism could operate in human vascular cells.

Angiotensin-converting enzyme (ACE) is a class III zinc protease (1) that catalyzes the hydrolysis of dipeptides from the carboxyl terminus of polypeptides. ACE participates in the control of blood pressure by converting the inactive decapeptide angiotensin II (2). It also inactivates bradykinin, a potent vasodilator peptide, by two sequential dipeptide hydrolytic reactions (3). ACE displays peptidyl tripeptidase and endopeptidase activity on some peptides, such as substance P and luteinizing hormone-releasing hormone (4). ACE is an ectoenzyme found in most mammalian tissues bound to the external surface of the plasma membrane of endothelial, epithelial, neural, and neuroepithelial cells.

There are two ACE isoforms derived from a single gene, by transcription from two alternate promoters (5, 6). Somatic ACE is a glycoprotein composed of a single polypeptide chain with an apparent mass of 170 kDa containing two large homologous domains, each domain bearing an active catalytic site (7); germinal ACE is synthesized as a lower molecular mass form of 110 kDa containing only the active carboxyl-terminal domain of the endothelial ACE (8). The complete amino acid sequences of human endothelial (9), mouse kidney (10), bovine endothelial (11), and rabbit pulmonary ACE (12) have been deduced from their corresponding cDNAs. The cDNA for human (8, 13) and rabbit (14) testicular ACE have also been isolated and sequenced. In all species, both isoforms contain a hydrophobic transmembrane domain of 17 amino acids, located at positions 1230–1247 and 886–702 in the human somatic and testicular forms respectively. Both forms possess an intracellular hydrophilic carboxyl-terminal sequence of 30 amino acids (15). Therefore, ACE is an integral membrane protein anchored to the plasma membrane by its hydrophobic carboxyl-terminal segment (16).

The membrane-bound enzyme can be fully solubilized, in vitro, by detergents or by limited proteolytic cleavage (16). In vivo, a soluble form of the enzyme exists in plasma and other body fluids (17). The source of circulating ACE in plasma has not been firmly established but is thought to be derived from vascular endothelial sources, such as pulmonary capillaries. Several hypotheses can be proposed to explain the relationship between plasma ACE and its membrane-bound form. Alternative mRNA splicing of a common transcript can be ruled out, since a single mRNA species was detected in endothelial cells (9). In addition, Wei et al. (15) showed that the full-length endothelial recombinant ACE cDNA, expressed in CHO cell lines, is secreted into the culture medium from the membrane form by a post-translational proteolytic cleavage occurring in the carboxyl-terminal region. Human plasma ACE also lacks the carboxyl terminus and has a molecular size similar to that of membrane-bound ACE (15). Therefore, the most likely hypothesis is that human plasma ACE is derived from endothelial cells by post-translational processing near the anchoring domain.

The aim of the present study was to investigate the site of cleavage of membrane-bound ACE into soluble ACE using somatic recombinant ACE expressed in CHO cells as a model and to compare it with the carboxyl terminus of the human...
plasma enzyme. To do this, we established a micromethod for sequencing the carboxyl-terminal end of ACE secreted by recombinant CHO cells and plasma ACE. Both soluble enzymes exhibited the same carboxyl terminus with a cleavage site located between Arg-1137 and Leu-1138 in the human somatic ACE sequence. This was clarified by immunoreactivity of membrane-bound and soluble ACE using antibodies raised against specific carboxyl-terminal regions of the enzyme. In order to test the importance of Arg-1137 in the secretory process, this residue was mutated into a glutamine residue. This mutation did not prevent the secretion of ACE in recombinant CHO cells, suggesting that either the solubilizing enzyme can accommodate this change or that alternative sites of cleavage can be used.

**MATERIALS AND METHODS**

*Angiotensin-converting Enzyme Purification—Human kidney ACE* was purified from membranes prepared from renal cortex homogenate after solubilization by CHAPS and dialysis. The protein was purified by chromatography on phenyl-Sepharose followed by lissopiril affinity chromatography, as previously described (9).

Human plasma ACE was purified from frozen human serum by essentially the same procedure. However, as plasma ACE did not bind to the phenyl-Sepharose gel, the filtrate was applied to a lissopiril affinity column prepared using the method of Hooper and Turner (18). The inclusion of a 2.8-nm spacer arm between the ligand and matrix in place of the 1.4-nm spacer arm increased the binding capacity of the column for the human plasma enzyme (19). The purification was completed by Ultrogel AAc 44 (IBI) filtration.

Membrane-bound human recombinant ACE was purified from CHO transfectants coexpressing the cDNA encoding the wild-type endothelial human ACE (15). CHO cells collected after extensive washing with cold phosphate-buffered saline were homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM MgCl₂ and 0.25 mM EDTA. The preparation was then sequentially centrifuged at 600 × g (10 min), 10,000 × g (10 min), and 105,000 × g (60 min). The membrane pellets were resuspended in 150 mM potassium phosphate buffer, pH 7.5, containing 0.5% CHAPS. After stirring for 15 h to solubilize the membrane-bound ACE, the suspension was centrifuged at 105,000 × g (60 min) and the resultant supernatant purified by phenyl-Sepharose and lissopiril affinity chromatography.

Secreted recombinant ACE was obtained from the culture medium of transfected CHO cells grown for 2 days in serum-free medium. After concentration by tangential flow ultrafiltration (Millipore, Milli-Q filtration system with polysulfone membrane of 100-kDa cutoff), the enzyme was purified, using the method previously described for human plasma ACE.

The enzymatic activity of the different forms of ACE was determined by the method described by Cushman and Cheung (22) using p-benzoyl-L-tyrosyl-l-histidyl-l-leucine (Hip-His-Leu; Bachem, Switzerland) as a substrate. The detection and quantification was performed by a micromethod using HPLC for the separation of hippuric acid (23). Kinetic parameters for the hydrolysis of Hip-His-Leu were determined from Lineweaver-Burk plots, as already described (7). The micromethod of Lowry et al. (24) was used for protein quantitation.

**Microsequence Carboxyl-terminal Analysis—Carboxypeptidases** were used to sequentially release amino acids from the ACE carboxyl terminus on a microscale (25, 26).

Two carboxypeptidases of broad specificity were selected as follows: carboxypeptidase Y from Yeast (Sigma and Boehringer Mannheim) and carboxypeptidase P from Penicillium janthinellum (Sigma and Boehringer Mannheim). These enzymes release all amino acids from the carboxyl terminus of polypeptides, and the relative rates of release of these amino acids provide the carboxyl-terminal sequence. Both carboxypeptidases were first tested on native, denatured, and alkylated samples of myoglobin, ribonuclease, or lysozyme to optimize the enzymatic reactions. Thereafter, 200–400 pmol of native ACE (membrane-bound or soluble forms) were incubated at 37 °C with either carboxypeptidase Y in 40 μl of 50 mM ammonium acetate buffer, pH 5.5, or carboxypeptidase P in 40 μl of 50 mM ammonium acetate buffer, pH 4. Ten-μl aliquots were sampled at 0, 30, 60, and 90 min and the amino acids released in each aliquot identified and quantified by micro-amino acid analysis after precolum derivatization with phenylisothiocyanate to produce the phenylthiocarbamyl amino acids. A Waters picoscale cationic reverse-phase HPLC column (50 cm × 0.46 mm) and a Waters picoscale UV detector (27). All phenylthiocarbamyl amino acids, in particular, phenylthiocarbamyl asparagine and phenylthiocarbamyl glutamine, could be resolved clearly using this system. In all cases, a carboxypeptidase Y or P and a substrate blank were subjected to the same incubation conditions. L-α-amino-n-butyric acid was used as an internal standard.

For each ACE preparation, several time-course hydrolysis reactions were performed with different enzyme/substrate (E/S) ratios. As control, a synthetic peptide of known structure corresponding to the membrane-bound and to the putative soluble ACE carboxyl-terminal sequence was analyzed with similar conditions. Three synthetic peptides were studied as follows: peptide YSLHRHSHGPQFGSEVa20, peptide ELRHS corresponding to sequence 1258-1277 of the human endothelial ACE was synthesized and purified by Dr. J. A. Heberts (Centre National de la Recherche Scientifique and Institut National de la Recherche en Biotechnologie, Montpellier, France). An extra tyrosine residue was added to the N-terminal position to facilitate isolation of this form when it is not present in the ACE sequence. Two other peptides, CDDYYKEAGQR and YVSVFLGDLDAQQR, corresponding to putative carboxyl-terminal membrane-accessible residues of sequence 1252-1277, respectively, were obtained from Neosystem (Strasbourg, France).

The carboxyl-terminal sequence obtained was verified by identification of a carboxyl-terminal peptide isolated from an ACE endopeptidyl digest by chromatography on anhydrotrypsin-agarose gel (28). Isolation of carboxyl-terminal peptides from proteolytic digests of soluble ACE was performed using immobilized anhydrotrypsin, which catalytically inert derivative of trypsin in which the Ser-195 residue of the active site has been chemically converted to a dehydroalanine residue (29). The anhydrotrypsin-agarose gel (Takaara Biomedical) selectively binds peptides possessing a carboxyl-terminal arginine, lysine, or carboxyaminoacidyleucine. The usual procedure for determination of carboxyl-terminal peptide by the anhydrotrypsin method is to cleave the protein with trypsin in which case all peptides except the carboxyl-terminal peptide bind. For soluble ACE, the carboxyl-terminal residue was suspected to be arginine, and the reverse procedure was used as follows: 300–600 pmol of 50 μl of the soluble recombinant ACE were digested for 24 h at 37 °C in 100 μl of 50 mM ammonium acetate buffer, pH 4, by 2% W/W Staphylococcus aureus protease (Boehringer Mannheim), which cleaves after glutamic acid residues. The hydrolysate was then centrifuged on an ultrafree-Mc membrane PLGC 10,000 PMNL (400 μl) (Millipore), and the filtrate was applied to the microcolumn containing anhydrotrypsin column (0.5 ml), which had been previously equilibrated with 50 mM ammonium acetate buffer, pH 5, 20 mM CaCl₂. The nonretained fraction contained peptides possessing a carboxyl-terminal glutamic acid residue. The adsorbed fraction containing the carboxyl-terminal arginine or lysine peptides was eluted by 1 M HCl and reverse-digested with trypsin, which correspond to the two putative proteolytic cleavage sites (see Fig. 3).

The enzymatic activity of the different forms of ACE was deter-
min at a flow rate of 0.2 ml/min at 38 °C. The carboxy-terminal peptide collected was subjected to quantitative amino acid analysis after acid hydrolysis at 110 °C for 24 h in a Waters gas-phase hydrolysis vessel and was identified using the Picotag system.

Construction of Expression Plasmids for the Mutants ACE1137, ACE1227, and ACE3788—A 1.5-kilobase cDNA fragment (base pairs 2527-4024 of the ACE cDNA) was isolated from the plasmid pACE2 comprising the full-length ACE cDNA (9) by digestion with SphI and SauI. This was then subcloned into the M13 vector, as previously described (15). A mutation was introduced into the putative Arg-1137 cleavage site identified by carboxy-terminal sequencing using an oligonucleotide-directed mutagenesis system (Amersham, France), with the method of Taylor et al. (30). The following three mutants were constructed: Arg-1137 (the identified cleavage site), Arg-1227 (a possible alternative site of processing), and a double mutant (Arg-1137, Arg-1227). These arginine residues (CGC) were mutated to glutamine (CAA or CAG) at nucleotide sequence positions 3518 and 3566, respectively. The mutant DNAs were obtained and designated hereafter ACE1137G, ACE1227G, and ACE3788G. Glutamine was chosen on the assumption of the presence of a trypsin-like sensitive cleavage site and also because glutamine substitution should not modify the protein conformation. The three mutants were screened by sequencing, and the entire 1.5-kilobase fragment of each mutant cDNA was sequenced to ensure that no other mutation had occurred. Mutated SphI/SauI 1.5-kilobase fragments were isolated from M13 and used to replace the corresponding part of ACE cDNA in the wild-type ACE cDNA (pcACE) expression plasmid, to generate pcACE1137G, pcACE1227G, and pcACE3788G. The expression plasmids were characterized by restriction mapping and sequencing of the mutated region. The mutants were confirmed by digestion of pure, soluble ACE samples with time during incubation with carboxypeptidase P was

TABLE I

| Carboxy-terminal sequencing of recombinant soluble ACE and of plasma ACE |
|-----------------------------|-----------------------------|
| Elution profile of a carboxypeptidase P time-course hydrolysis of the recombinant secreted enzyme and of the human plasma ACE is shown in Table I. Similar profiles were obtained for the two sources of soluble ACE. Several hydrolysis experiments with different E/S ratios were performed on the secreted recombinant form. Using the same preparation of soluble recombinant ACE, three kinetic runs with E/S ratios of 1/40, 1/10, and 1/4 were performed, and the release in 60 min from a constant quantity of ACE digested by a variable amount of carboxypeptidase P (E/S = 1/200, 1/100, 1/50, 1/25) was studied. The results obtained were confirmed by digestion of pure, soluble ACE samples from two different CHO cells cultures. Interpretation of all the results (n = 9) verified the presence of the sequence AGQR. Carboxypeptidase Y cleaved soluble ACE and plasma ACE very slowly. This was to be expected from the proposed carboxy-terminal sequence since carboxypeptidase Y is strongly affected by the basic nature of the carboxy-terminal arginine.

The sequence AGQR corresponding to the carboxy-terminal part of the two soluble proteins was localized at Arg-1137 of the endothelial human ACE sequence, which would then correspond to the proteolytic cleavage site (see Fig. 3). Nevertheless, a somewhat similar sequence is located near Arg-1137, DAQQAR. To prove that the carboxy-terminal sequence of the soluble ACE form was indeed AGQR, two synthetic peptides corresponding to the two putative proteolytic cleavage sites were hydrolyzed by the two carboxypeptidases. The carboxypeptidase hydrolysis reactions were carried out using the same E/S ratio for easier comparison of the sequential rate and amount of amino acid release. Fig. 1 shows that the actual carboxy-terminal sequences of the two peptides determined by carboxypeptidase P time-course hydrolysis were in agreement with those expected. The peptide containing Arg-1137 showed a sequential release of amino acids sequentially generated the same amino acids as follows: serine, histidine, and arginine (data not shown). The rate of appearance of the amino acids released during the hydrolysis procedure of both ACE molecules provided sufficient evidence to deduce the carboxy-terminal sequence, ELRHS, which is in agreement to that found in the cDNA sequence encoding the human endothelial ACE. To further validate the carboxy-terminal sequencing method, hydrolysis of the synthetic peptide corresponding to the ACE carboxy terminus was performed with carboxypeptidases Y and P. The results obtained with both carboxypeptidases were consistent with the presence of a carboxy-terminal sequence, ELRHS (data not shown).

Carboxy-terminal Microsequencing of Recombinant Soluble and Human Plasma ACE—A representative carboxypeptidase P time-course hydrolysis of the recombinant secreted enzyme and of the human plasma ACE is shown in Table I. Similar profiles were obtained for the two sources of soluble ACE. Several hydrolysis experiments with different E/S ratios were performed on the secreted recombinant form. Using the same preparation of soluble recombinant ACE, three kinetic runs with E/S ratios of 1/40, 1/10, and 1/4 were performed, and the release in 60 min from a constant quantity of ACE digested by a variable amount of carboxypeptidase P (E/S = 1/200, 1/100, 1/50, 1/25) was studied. The results obtained were confirmed by digestion of pure, soluble ACE samples from two different CHO cells cultures. Interpretation of all the results (n = 9) verified the presence of the sequence AGQR. Carboxypeptidase Y cleaved soluble ACE and plasma ACE very slowly. This was to be expected from the proposed carboxy-terminal sequence since carboxypeptidase Y is strongly affected by the basic nature of the carboxy-terminal arginine.

| Carboxy-terminal Microsequencing of Human Kidney and Recombinant Membrane-bound ACE — The carboxy-terminal sequencing method was tested first on human kidney ACE and recombinant membrane-bound ACE digested by carboxypeptidases P and Y. Kidney ACE was hydrolyzed in an inhibitor (lisinopril) complex to prevent any possible carboxy-terminal autocatalysis. The yield of amino acids released with time during incubation with carboxypeptidase P was higher than that obtained with carboxypeptidase Y. Both carboxypeptidases sequentially generated the same amino acids as follows: serine, histidine, and arginine (data not shown). The rate of appearance of the amino acids released during the hydrolysis procedure of both ACE molecules provided sufficient evidence to deduce the carboxy-terminal sequence, ELRHS, which is in agreement to that found in the cDNA sequence encoding the human endothelial ACE. To further validate the carboxy-terminal sequencing method, hydrolysis of the synthetic peptide corresponding to the ACE carboxy terminus was performed with carboxypeptidases Y and P. The results obtained with both carboxypeptidases were consistent with the presence of a carboxy-terminal sequence, ELRHS (data not shown).

| Carboxy-terminal Sequencing of Human Kidney and Recombinant Membrane-bound ACE — The carboxy-terminal sequencing method was tested first on human kidney ACE and recombinant membrane-bound ACE digested by carboxypeptidases P and Y. Kidney ACE was hydrolyzed in an inhibitor (lisinopril) complex to prevent any possible carboxy-terminal autocatalysis. The yield of amino acids released with time during incubation with carboxypeptidase P was higher than that obtained with carboxypeptidase Y. Both carboxypeptidases sequentially generated the same amino acids as follows: serine, histidine, and arginine (data not shown). The rate of appearance of the amino acids released during the hydrolysis procedure of both ACE molecules provided sufficient evidence to deduce the carboxy-terminal sequence, ELRHS, which is in agreement to that found in the cDNA sequence encoding the human endothelial ACE. To further validate the carboxy-terminal sequencing method, hydrolysis of the synthetic peptide corresponding to the ACE carboxy terminus was performed with carboxypeptidases Y and P. The results obtained with both carboxypeptidases were consistent with the presence of a carboxy-terminal sequence, ELRHS (data not shown).

<table>
<thead>
<tr>
<th>Results</th>
<th>Amino acid</th>
<th>Arginine</th>
<th>Glutamine</th>
<th>Glycine</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant ACE</td>
<td>0.15</td>
<td>0.38</td>
<td>0.04</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Plasma ACE</td>
<td>0.52</td>
<td>0.20</td>
<td>0.64</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

| Recombinant ACE | 0.52 | 0.20 | 0.64 | 0.00 |
| Plasma ACE | 0.52 | 0.20 | 0.64 | 0.00 |

*Arginine in ultimate position was slowly hydrolyzed at this E/S ratio for recombinant ACE.

The carboxy terminus of this plasma ACE sample was slightly heterogeneous and partially truncated of the dipeptide Gly-Arg, explaining the relative high elution profile of glycine.
acids corresponding to that of the secreted recombinant and the plasma ACE carboxyl terminus, as previously described.

Isolation and Amino Acid Analysis of a Carboxyl-terminal Peptide Issued from the Secreted Form of Recombinant ACE—
The data obtained by carboxypeptidase P and Y hydrolysis were confirmed using a different approach that avoids inherent difficulties due to different reactivities of these enzymes toward some amino acid residues. Quantitative amino acid analysis was performed on the peptide selectively isolated from a S. aureus protease digest of the secreted recombinant ACE form by chromatography on an anhydrotrypsin affinity gel. The peptide purified by HPLC had the following amino acid composition: 105 pmol of arginine, 74 pmol of glutamic acid, 98 pmol of glycine, 68 pmol of alanine, which is consistent with that expected for the sequence AGQR. Again, this result supports a proteolytic cleavage site of the soluble ACE at Arg-1137.

SDS-PAGE and Immunocharacterization of Membrane-bound and Soluble ACE—ACE isoforms purified from human kidney, recombinant CHO cells, human serum, and culture medium of recombinant CHO cells were all analyzed by SDS-PAGE. Fig. 2 shows that the higher Mₖ values correspond to the recombinant membrane-bound protein (lane 2) and the faster migrating species to a soluble form (lane 3). Gel scanning indicated an apparent molecular mass of approximately 170 kDa for membrane-bound ACE and 160 kDa for secreted recombinant ACE. The Mₖ changed considerably following deglycosylation, but the 10-kDa difference in molecular mass between soluble and membrane form persisted (results not shown). Human plasma ACE (lane 4) exhibited an apparent molecular mass higher than that of kidney ACE (lane 1) explained by different glycosylation patterns. Plasma ACE compared with endothelial membrane-bound ACE showed a higher mobility and a difference in molecular mass (results not shown). After deglycosylation (glycopeptidase F treatment), recombinant soluble (lane 5) and plasma ACE (lane 6) had the same Mₖ, suggesting a similar structural backbone.

ACE isoforms were studied by Western blot analysis using specific antibodies (Fig. 3). Antiserum Y₁ recognizes all recombinant and human ACEs. Antiserum 28A, which reacts specifically with the carboxyl terminus of the membrane-bound enzyme, failed to recognize the two soluble forms. All ACE molecules reacted with antiseraum Clq, indicating that all of them contain the N-terminal sequence of human endothelial ACE. Antibodies 5 and 3 were raised against sequences from the N-terminal side of each putative cleavage site. These antibodies are specific to ACE carboxyl-terminal domain and do not recognize the recombinant N-terminal fragment constructed by Wei et al. (7) (results not shown). Results using antisera show that human kidney and membrane-bound recombinant ACE molecules are recognized by each antisera, as expected. Secreted recombinant ACE and human plasma ACE are recognized only by antisera 5 but not by antisera 3, indicating the absence of the corresponding sequence in soluble forms of ACE.

Site-directed Mutagenesis of Arg-1137 and Arg-1227—
ACE₁₁₁₁₁₇, ACE₁₁₁₁₂₇, and ACE₂₈ were designed in order to compare the ability of CHO cells to secrete these mutants with that of the wild-type ACE. The kinetic parameters for Hip-His-Leu hydrolysis of the wild-type and of the ACE mutants, obtained by extrapolation of a Lineweaver-Burk plot, was not significantly different (results not shown). The $K_m$ and the $K_{cat}$ values of the three mutants were similar to that of the wild-type ACE (1.5 μmol and 500 s⁻¹). The effect
of zinc, EDTA, chloride, and captopril on ACE mutant enzyme activity was identical to that of the wild-type ACE.

CHO cell lines from subcloning were first screened according to their cellular ACE content and also by their ability to correctly synthesize ACE. Three mutant clones (ACE_R1137, ACE_R1227, and ACE_2R) expressed various amounts of ACE as follows: 173.4 ± 13.6 (n = 6), 82.4 ± 1.67 (n = 6), and 264.4 ± 13.4 (n = 6) milliunits/min/mg of cellular protein, respectively. Since secretion could be affected by the level of ACE expression, two wild-type ACE cell lines were selected for expression, two wild-type ACE cell lines were selected for their ability to express ACE at a relatively high or low level. These clones, designated thereafter ACE (I) and ACE (II), expressed 437.5 ± 16.4 (n = 14) and 114.1 ± 4.6 (n = 6) milliunits/min/mg of cellular protein, respectively.

The bio synthesis and the secretion process of wild-type and mutant ACE were studied in cell culture lines in the presence of [35S]methionine. In the wild-type ACE (I) and ACE_R1137, 30 min of [35S]methionine labeling followed by 8 h of chase resulted in the appearance of a specific band with an apparent molecular mass of 170 kDa by SDS-PAGE (Fig. 4A, lanes 1-4). Similar results were obtained with ACE (I) and the two other mutants (results not shown). The Triton-solubilized cell lysates were immunoprecipitated with antisera Y1 and 28A to determine whether both ACEs (ACE (I) and ACE (II)) and mutants (R1137, R1227, and 2R) were processed similarly. Y1 reacted with both ACE and mutant ACE were studied in cell culture lines in the presence of [36S]methionine. In the wild-type ACE (I) and ACER, likewise, secreted ACE wild-type recombinant. Similar results were obtained with ACE (I), R1227, and 2R mutants.

The effect of the mutations on the release of ACE was estimated using the ratio of the enzymatic activity in the medium over the enzymatic activity in the cells during 24 h of culture. The results are expressed as a percentage of secreted ACE activity in 24 h (Fig. 5). A variable percentage of secreted ACE was observed among the different cell lines from 4.7 ± 0.4% (n = 6, ACE II) to 12.5 ± 1.2% (n = 14, ACE I), the three mutants having intermediate values (Fig. 5). Then, none of these mutations had grossly altered the release process compared with the ACE (I) and ACE (II). Using only the ACE (I) cell line as control, a misinterpretation would have occurred. In addition, there is a strong correlation (r = 0.96) between the percentage of secreted ACE and its cellular content (data not shown), indicating that the rate of ACE secretion is linearly correlated with the cellular content of the protein, irrespective of the presence or absence of a mutation in Arg-1137, Arg-1227, or indeed both.

The time course of the secretion of wild-type ACE (I) and all mutants was investigated during a 72-h period (Fig. 6). During this period, all enzymes were secreted into the medium in a linear fashion.

**DISCUSSION**

CHO cells transfected with human endothelial ACE cDNA produced membrane-bound ACE and secreted a carboxyl-terminal truncated enzyme by carboxyl-terminal post-translational processing (15). The present study elucidates the proteolytic cleavage site between Arg-1137 and Leu-1138.
that this region contains a sequence motif recognized by a protease site before they can be clarified.

of important for the recognition sequence of the solubilizing enzyme and that further degradation occurs leading to the enzyme. In this case, the absence of a strict requirement for an sequence to Arg-1137 is subject to proteolytic cleavage and a specific protease. A similar mechanism is likely to operate in close proximity to Arg-1137 may be used by the solubilizing enzyme to cleave between Gln-1137 and Leu-1138. In this case, the absence of a single mutation in Arg-1137 or Arg-1227 nor the double mutation (2R) modified ACE secretion. Several hypotheses could account for these results, which contrast somewhat with the cleavage site proposed above.

1) Arg-1227 could be an alternative cleavage site to Arg-1137. However, the secreted mutant ACE_R1227 exhibited the same M, as ACE_R1137. In addition, the mutant ACE_2R, containing the double mutation, is also cleaved and has the same molecular weight as the two individual mutants, suggesting that only one cleavage site is involved in ACE release.

2) The carboxyl terminus of soluble forms of ACE shows that the cleavage site is likely to be located between Arg-1137 and Leu-1138. Since CHO cells are still able to release the mutant ACE_R1137, it is possible that the solubilizing enzyme is able to cleave between Gln-1137 and Leu-1138. In this case, the enzyme would not be strictly dependent on the presence of an arginine residue at the P_i site but could accommodate a glutamine residue at this position. Other amino acids upstream or downstream from the Arg-1137 could also be important for the recognition sequence of the solubilizing enzyme. In this case, the absence of a strict requirement for an arginine residue at P_i would differentiate this enzyme from a trypsin-like protein.

3) Finally, one cannot exclude that a site of cleavage in close proximity to Arg-1137 may be used by the solubilizing enzyme and that further degradation occurs leading to the observed carboxyl terminus in cell cultures and in plasma.

Clearly, these hypotheses require further characterization of the secreted ACE_R1137, R1137, and 2R and another extensive series of site-directed mutagenesis along the proposed cleavage site before they can be clarified.

From these results, it seems likely that the neighboring sequence to Arg-1137 is subject to proteolytic cleavage and that this region contains a sequence motif recognized by a specific protease. A similar mechanism is likely to operate in

CHO cells and in human vascular cells since the same carboxyl-terminal sequence was found in plasma ACE. Maroux’s model of brush border ectoenzymes (32) stipulates that the membrane-spanning hydrophobic sequence is followed by a hydrophilic sequence, forming the fibrillar structure of the “stalk” on which the globular domain bearing the active sites is set. According to this scheme, one can speculate that the cleavage site is localized near the start of the globular domain. The human ACE sequence around the cleavage site Arg-1137-Leu-1138 displays some of the sequence characteristics that govern monoarginyl cleavage (33–35) (Table II), such as a basic residue (lysine) in position P_a and a domain containing several nonpolar amino acids such as alanine in P_b, P'_2, and P'_3. However, a hydrophilic aliphatic residue such as leucine is not suitable in P'_1, for monobasic cleavage, making the proposed ACE cleavage site somewhat unusual.

The human sequence neighboring Arg-1137 can also be compared with homologous sequences of the three other known mammalian ACE species. Rabbit ACE presents a dibasic cleavage site in this position. Some variations have been noted both in mouse and bovine sequences. Interestingly, the leucine residue is found in P'_1, in all species and may therefore play an important role in the cleavage site.

Our data raise the question of the specificity of the protease involved. The production of soluble recombinant ACE was described by Ehlers et al. (36) in CHO cells transfected with human testis ACE cDNA. Sen et al. (37) also observed that ACE synthesized by mouse epithelial cells transfected with rabbit testicular ACE cDNA was partly secreted. Porcine aortic endothelial (38) and bovine aorta cell cultures (39) produced a soluble form of ACE in the medium. An EDTA-sensitive hydrolase has been shown to release soluble ACE in tissues such as lung and kidney (16). However, at the present time, the exact nature of the protease involved in this mechanism and its relation to the present releasing process are unknown.

Various classes of proteins exist as both membrane-bound and soluble isofoms involving two main mechanisms, tissue-specific alternative splicing of a common transcript and post-translational modification. A general post-translational proteolytic mechanism could be envisaged in the case of ACE, as discussed by Ehlers and Riordan (40). The cleavage sequence QRL is similar to the QKL cleavage site of the amyloid precursor protein (APP), which hydrolyses APP within the β amyloid protein (AβP) and prevents deposition of AβP, the principal component of plaques in Alzheimer’s disease (41, 42). Several authors have described an apparent “position specificity” (43–45) of APP cleavage at a fixed distance from the membrane in this proteolytic system. The “APP-secretase” may not only be sequence-specific but also conformation-dependent. Taken together, these results suggest that

![Fig. 6. Time course of ACE medium secretion. Enzymatic activity of the wild-type ACE (I) (n = 3) (●) and mutants R1137 (n = 3) (○) and 2R (n = 3) (■) for 72 h measured in serum-free medium (± S.E.).](image-url)

**TABLE II**

Comparison of human ACE sequence at the Arg-1137, Leu-1138 cleavage site

| Human ACE | QSLAGEQRLATAMKL |
| Rabbit ACE | QSLAGEQRLADAMKL |
| Mouse ACE | QSLAGEQRLADAMKL |
| Bovine ACE | QSLAGEQRLADAMKL |
| AβP | GVEV[QKL]FFAED |
membrane-bound protein release may be a general proteolytic mechanism involving particular signals in each protein rather than a specific mechanism for each protein or each cell type.

Proteolytic release of ACE in CHO cell lines is a useful model for studying the characteristics of solubilizing enzyme and subcellular localization. The fact that plasma ACE exhibits the same carboxyl terminus as the recombinant secreted enzyme suggests that a similar mechanism may operate in endothelial cells. It will remain to be seen whether such a solubilizing enzyme is also present in cultured endothelial cells. It appears to be dependent on the level of the cellular ACE content and not on the rate of the enzymatic secretion process, a finding that has not been previously documented in human vascular endothelial cells.

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