Expression and Characterization of Recombinant Human Angiotensin I-converting Enzyme

EVIDENCE FOR A C-TERMINAL TRANSMEMBRANE ANCHOR AND FOR A PROTEOLYTIC PROCESSING OF THE SECRETED RECOMBINANT AND PLASMA ENZYMES

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Lei Wei, François Alhenc-Gelas, Florent Soubrier, Annie Michaud, Pierre Corvol, and Eric Clauer
From the U. 36 Institut National de la Santé et de la Recherche Médicale, Collège de France, 3 rue d’Ulm, 75005 Paris, France

Chinese hamster ovary (CHO) cells have been transfected with either a full-length cDNA encoding human angiotensin I-converting enzyme (kininase II; EC 3.4.15.1) (ACE) or a mutated cDNA, in which the last C-terminal 47 amino acids, including the putative transmembrane domain, are not translated. Cell lines expressing high levels of the wild-type ACE or the mutant were established. The cells transfected with the wild-type cDNA (CHO-ACE) express a membrane-bound ectoenzyme with an intracellular C terminus, as shown by indirect immunofluorescence using an anti-serum (28A7) raised against a synthetic peptide corresponding to the deduced C terminus of ACE. This enzyme is structurally, immunologically, and enzymatically identical to human kidney ACE. In addition, CHO-ACE cells also produce a secreted form of the enzyme. Neither this secreted form nor the enzyme purified from human plasma was recognized by the antiserum 28A7, indicating that they undergo a truncation in the C-terminal region. On the other hand, the transfected cells expressing the C-terminally truncated mutant (CHO-ACEΔc), do not retain ACE in the plasma membrane, but secrete it into the medium. These results indicate that ACE is anchored to the plasma membrane by the predicted C-terminal transmembrane domain, and the secreted form is derived from the membrane bound form by a post-translational proteolytic cleavage of the C-terminal region.

Angiotensin I-converting enzyme (kininase II; EC 3.4.15.1) (ACE) is a zinc-metallopeptidase which plays an important role in blood pressure regulation by converting the inactive decapeptide angiotensin I (AI) into the potent vasopressor octapeptide angiotensin II (AII). This peptidase is also able to hydrolyze bradykinin, a vasodilator peptide, and various neuropeptides. ACE is a widely distributed enzyme, predominantly identified as a membrane-bound ectoenzyme in vascular endothelial cells, various absorptive epithelial cells, and neuroepithelial cells and also as a so-called “soluble” enzyme in plasma and other body fluids.

ACE has been purified and characterized from many different tissues and species. Plasma and membrane-bound ACEs have been shown to display similar physicochemical and enzymatic properties and to be indistinguishable immunologically. However, little is known about the nature of the membrane anchorage of cellular ACE and the mechanism of synthesis and secretion of plasma ACE. The recent determination of the complete amino acid sequence of human ACE (10) sheds some light on the possible nature of the anchorage. The protein contains two hydrophobic segments located at the N terminus and near the C terminus, respectively (Fig. 1). The N-terminal hydrophobic segment is most likely the signal peptide, since N-terminal sequencing of the mature protein did not identify this sequence (10). Thus, the 17 amino acid C-terminal hydrophobic segment, which is located at position 1230–1247, probably anchors the enzyme into the plasma membrane. This hypothesis is compatible with the demonstration that a soluble form of epithelial ACE, cleaved from the plasma membrane by trypsin has a similar size to the membrane-bound enzyme, and conserves an identical N-terminal amino acid sequence, but appears to be more hydrophilic. Nevertheless, no direct evidence of such a type of anchorage exists. Other evidence indicates that plasma ACE originates from the endothelial cells, but the exact relationship between plasma ACE and membrane-bound endothelial ACE is not known, and several hypotheses can be proposed. The plasma ACE could be translated from an mRNA species lacking the sequence encoding the C-terminal region of the endothelial ACE, resulting from an alternative splicing at the 3′ end of the gene, but the presence of a single mRNA species of 4.3 kb in endothelial cells (10) is not consistent with this possibility. The other possible mechanisms of production of the secreted form include leakage of intact ACE from the plasma membrane, or post-translational enzymatic cleavage of the hydrophobic anchor either before or after reaching the surface of the cell.

In this paper, recombinant human ACEs have been expressed in Chinese hamster ovary (CHO) cells by transfection with cDNA encoding either the wild-type ACE or a mutated ACE deleted for the C terminus. The cell line expressing the intact sequence not only produces a membrane-bound form of ACE, which was characterized and compared to the human renal native ACE, but also secretes ACE into the medium.

The cell line expressing the truncated ACE produces only a secreted form of ACE. These secreted forms of ACE purified from human plasma were characterized and compared to membrane-bound enzymes. The results show that ACE is

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† The abbreviations used are: ACE, angiotensin I-converting enzyme; AI, angiotensin I; AII, angiotensin II; CHO, Chinese hamster ovary; kb, kilobase; bp, base pairs; RIA, radioimmunoassay; Fa, 2-furanacryloyl; Hip, hippuryl; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
anchored to the plasma membrane by the C-terminal hydrophobic segment, and the secreted form is derived from the membrane-bound form by a post-translational proteolytic cleavage in the C-terminal region.

MATERIALS AND METHODS

Construction of the Expression Plasmid for Wild-type ACE—An ACE cDNA containing the entire coding sequence for human endothelial ACE was constructed from three overlapping cDNA clones: xCHDT32, xHEC2111, and xHEC1922 (2). The plasmids were digested with BamHI and the resulting fragments were subcloned into the EcoRI site of pBluescript (Stratagene) to generate pbCHDT32, pbHEC2111, and pbHEC1922, respectively. The different DNA fragments used for this construction were purified from LGT agarose gels (Seaplaque, FMC Corporation). A 0.2-kb fragment (bp 9–225 of the ACE cDNA) was excised from the plasmid pbCHDT32 by digestion with XbaI (in the polylinker of pBluescript, 5' to the cDNA insert) and Smal (bp 225 of the ACE cDNA). This fragment was ligated with a 37-nucleotide synthetic blunt end linker (bp 226–262 of the ACE cDNA) containing a BsmI site at its 3' end (bp 256 of the ACE cDNA). This intermediate construction was then digested with BamHI (5' to the cDNA fragment) and BamHI and BsmI, and this 0.25-kb fragment was ligated into pbHEC2111 and pbHEC1922. The resulting plasmid, designated pbACE1, extends from bp 9 to bp 3018 of the ACE cDNA. The 3' part of the ACE cDNA was provided by the plasmid pbHEC1922. A 1.5-kb fragment (bp 2578–4024 of the ACE cDNA) was isolated after digestion with Spel (bp 2578) and HindIII (in the polylinker of pBluescript, 3' to the cDNA insert), and was subcloned into an M13 vector. A stop codon (TAA) was introduced into the cDNA at the position corresponding to amino acid 1231 (tryptophan) by site-directed mutagenesis, using the Ampligen kit based on the method of Taylor et al. (15). Mutant cDNAs were sequenced by screening, and the entire 1.5-kb insert was sequenced to ensure that no other mutation had occurred. The mutated Spel (Spel I) fragment was isolated from M13 and used to replace the corresponding part of ACE cDNA in peACE to generate peACE1 (16). This expression plasmid was characterized by restriction mapping and sequencing of the mutated region to ensure that the mutation had been introduced correctly.

Expression of Recombinant ACEs in CHO Cells—The expression plasmids peACE and peACE1 were introduced by cotransfection with the neomycin resistance plasmid pSV2neo into CHO cells as described previously (14). Single colonies of primary G418-resistant transformants were assayed for the expression of ACE by RNA dot blot (16), radioimmunoblot (RIA) of ACE, and enzymatic assays (see below). Cell lines expressing ACE were selected and purified by subcloning and dilution.

ACE Purification—Human kidney ACE was purified from renal microsomes by chromatography on phenyl-Sepharose followed by inhibitor (lisinopril) affinity chromatography, as described previously (10). Human plasma ACE was purified from fresh frozen human plasma essentially by the same procedure but with two modifications: 1) as most of plasma ACE did not bind to the phenyl-Sepharose matrix, the samples passed through the column were subjected to further purification; 2) after affinity chromatography, the purification was completed by gel filtration on Ultrogel AcA-44 to apparent homogeneity.

Membrane-bound recombinant ACE was purified from cell extracts. Transfected CHO cells were collected after extensive washing with cold phosphate-buffered saline. Cells were resuspended in 1 ml of 5 mM potassium phosphate, pH 8, and treated for 24 h with 8 mM Chaps detergent (Serva, Germany). The samples were centrifuged at 105,000 × g for 5 min, the supernatant was collected, and KCl and ZnSO4 were added to a final concentration of 350 mM and 10 μM, respectively. The enzyme activity was measured by the free inhibitor, the dissociation step was reduced to 48 h by the use of chlороdeoxy-filler buffer (18).

Secreted recombinant ACE was purified from the culture medium. The medium was concentrated from flasks of transfected CHO cells grown for 2 days in serum-free medium and concentrated by ultrafiltration. ZnSO4 and KCl were added to the concentrated medium, as described above; and the purification was then performed, as described above.

Immunological Characterization of Recombinant ACEs—The direct RIA of ACE was performed as described previously (9).

Two different rabbit antisera were used to characterize ACE. Antiserum Y1 was obtained from rabbit immunized against pure human ACE (19), and antiserum 28A7 was raised against a synthetic peptide corresponding to the last 20 amino acids of the ACE C-terminal sequence (Fig. 1). These antisera were used at dilution of 1:20 as primary antibody for indirect immunofluorescence studies, performed on the intact cells or on the permeabilized cells as described (20). The secondary antibody was a fluorescinated goat anti-rabbit antibody ( Biosys, France). For permeabilization, the transfected cells were washed in 4% paraformaldehyde in 0.05% sodium cacodylate, and then permeabilized by treatment with the nonionic detergent Triton X-100 (0.5%, 3 min).

Recombinant and native enzymes were analyzed by SDS-PAGE followed by Western blotting. SDS-PAGE was carried out on a 7.5% polyacrylamide gel (21). After electrophoresis, proteins were stained with silver nitrate or transferred to a polyvinylidene difluoride membrane (Millipore) which was then incubated in 10 mM Tris buffer, pH 8, containing 150 mM NaCl, 0.05% Tween 20, and 20% fetal calf serum for 20 min prior to overnight incubation at 4°C with antiserum Y1 (1:5000 dilution) or antiserum 28A7 (1:1000). The secondary antibodies were a fluorescinated goat anti-rabbit antibody.

Enzymatic Characterization—The enzymatic activity of ACE was determined by using 2-fluoracetyl-Phe-Gly-Gly (Fa-Phe-Gly-Gly) as described by Hofmioost et al. (22). The standard assay conditions were 50 mM Tris, pH 7.5, 300 mM NaCl, and 25°C. ACE catalytic activity was also determined by the hydrolysis of the substrate hippuryl-His-Leu (Hip-His-Leu). The standard assay conditions were 5 mM substrate, 100 mM potassium phosphate, pH 8.3, 300 mM NaCl, and 37°C, as described by Cushman and Cheung (23). The velocities at other pH values (6-9) were measured in 100 mM potassium phosphate, 1 mM substrate, and 300 mM NaCl. The velocities at other concentrations of chloride (0–800 mM) were measured at pH 8.3 with 1 mM substrate. ZnSO4 (10 μM) and bovine serum albumin (1 mg/ml) were added to the assay solution for purified enzyme samples. After incubation for 30–60 min, the amount of hippuric acid liberated from the substrate was determined by HPLC using a procedure modified from Horichi et al. (24). A 25 × 0.46 cm (inside diameter) column packed with 10 μM Nucleosil C18 (Société Française Chromo Colonne, Neuilly-Puisances, France) was used. The mobile phase consisted of a mixture of acetonitrile and 10 mM potassium phosphate buffer, pH 3 (24:76). The absorption was measured at 210 nm and calibrated with a standard solution of hippuric acid. One unit of activity was defined as the amount of enzyme catalyzing the release of 1 μmol of hippuric acid from Hip-His-Leu per minute (23).

When ACE was assayed for Al hydrolysis, the standard assay conditions were 100 μM Al, 50 mM Hapes, pH 7.5, 60 mM NaCl, 1 μM ZnSO4, and 1 mg/ml bovine serum albumin, 37°C. After 10–30 min incubation, conversion of Al to AI was measured qualitatively by HPLC or quantitatively by RIA of Al as described below. HPLC analysis was carried out on an analytical column of Hypersep ODS 5 μm (25 × 0.46 cm, inner diameter), with phosphate buffer containing 20% methanol as the mobile phase (25). The samples were eluted isocratically at 45°C using a mixture of acetonitrile and 100 mM triethylammonium phosphate buffer, pH 3 (21:79), and the absorbance was monitored at 210 nm. The amount of Al liberated by incubation of AI with ACE was measured directly by RIA using the monoclonal antibody 28A7 which was a gift from Dr. B. Pau (Immundiagnostic Laboratory, Sanofi, France). The RIA was performed in 100 mM Tris buffer, pH 7.5, containing I
nm EDTA, 0.02% Na$_2$CO$_3$, and 2 mg/ml bovine serum albumin (RIA buffer). 10$^2$-labeled AII (10,000 cpm, 2000 Ci/mmol) in 100 ml of buffer and 100 ml of antibody (1:1,000,000 dilution of a 3 mg/ml stock solution) were added to 100 ml of the samples to be tested. Free angiotensins were precipitated using a charcoal-dextran suspension and counted as described (25). Fifty percent displacement was achieved with 20 pg of AII, and the sensitivity of the assay was 2 pg of AII in 100 ml of sample solution. All measurements were made in triplicate. Because the antibody slightly cross-reacted with AI (0.4%), incubations containing only AI without enzyme were also performed in parallel, and the amount of immunoreactive AI was subtracted from the AI measurement.

Kinetic parameters for the hydrolysis of Fa-Phe-Gly-Gly, Hip-His-Leu, and AI under the standard assay conditions were determined from Lineweaver-Burk plots. Initial velocities were measured during the first 5% of substrate hydrolysis. Assays were performed in triplicate. The detection limit of hippuric acid liberated from Hip-His-Leu corresponded to 0.006% of substrate hydrolysis. The detection limit of AI generated from AI corresponded to 0.2% of substrate hydrolysis.

Inhibition studies for Hip-His-Leu and AI hydrolyses were performed under the standard conditions above using 10 $\mu$M captopril (a gift from Bristol-Myers-Squibb Institute), 10 $\mu$M enalaprilat (a gift from Merck Sharp and Dohme), or 10 $\mu$M EDTA as inhibitors. The dose-response curves for captopril and enalaprilat inhibition of the hydrolysis of Hip-His-Leu were established by incubating 0.05 nM enzyme with 1 mM substrate, 300 mM NaCl, at pH 8.3 in the presence of 0.01–100 nM inhibitor.

RESULTS

Expression of Wild-type and C-terminally Truncated ACEs in CHO Cells—The expression plasmid peACE, which directs the synthesis of the wild-type ACE, contains the sequence of the SV40 early promoter, then the full-length ACE cDNA, followed by the SV40 sequence for polyadenylation. The entire coding sequence of the cDNA is identical to the published cDNA sequence. The first translation initiation codon ATG is preceded by a 14-nucleotide 5' untranslated region. The termination codon TGA is followed by 80 nucleotides corresponding to a part of the 3'-untranslated region without polyadenylation signal.

The expression plasmid peACE$^{\text{COOH}}$ is identical to peACE except for a two-base mutation replacing the codon TGG (Trp-1231) by the stop codon TAA. The 17-amino-acid C-terminal hydrophobic segment and the 30-amino-acid C terminus of ACE therefore are not translated (Fig. 1). Thus, this construction can be used to analyze the role of these C-terminal sequences in the anchorage and secretion of ACE.

Stable cell lines expressing large amounts of each of these recombinant ACEs were established and designated CHO-ACE and CHO-ACE$^{\text{COOH}}$. Both cell lines contain a recombinant ACE mRNA with an estimated size of 4.2 kb as assessed by Northern blot analysis (data not shown). This result is consistent with the size of the cDNA inserted into the expression vector (4 kb) and the use of the vector polyadenylation signals.

To assess the cellular localization of the recombinant enzyme in both cell lines, indirect immunofluorescence was performed using the antiserum Y1 directed against the human kidney ACE. On intact CHO-ACE cells, the recombinant ACE appears to be localized at the cell surface (Fig. 2a). No staining was detected with intact CHO-ACE$^{\text{COOH}}$ cells, suggesting that the C-terminally truncated recombinant enzyme is not present stably at the surface of the cells. No staining was observed in control nontransfected CHO cells.

To determine the orientation of recombinant ACE in the plasma membrane of CHO-ACE cells, we used the antiserum 28A7 directed against the peptide of the C terminus (Fig. 1). This antiserum reacted specifically with the purified membrane-bound form of the kidney enzyme (see below). There was no staining with this antiserum on intact CHO-ACE cells. However, when the cells were permeabilized with Triton X-100 before incubation with the antiserum, a positive signal was observed (Fig. 2b). This suggests that the C terminus of the molecule is localized intracellularly and that the recombinant enzyme is incorporated into the cell membrane as an ectoenzyme.

The synthesis rate of recombinant ACE by CHO-ACE cells was monitored by direct RIA. CHO-ACE cell extracts contain 1.94 ± 0.29 pg of recombinant ACE per 10$^6$ cells (n = 5).

![Fig. 1. Diagram of human ACEs encoded by the cDNA constructions. Human ACE, encoded by the full-length cDNA, comprises the signal peptide of 29 amino acids and the mature protein of 1277 residues containing a hydrophobic segment of 17 amino acids near the C terminus. The sequences HEMGH correspond to the location of the two active sites. An antisera, designated 28A7, was raised against a synthetic peptide (C-terminal peptide) corresponding to the C-terminal 20 amino acids. The C-terminally truncated ACE (ACE$^{\text{COOH}}$) comprises the signal peptide and 1230 residues. A stop codon was introduced into the cDNA at the first amino acid of the C-terminal hydrophobic domain by site-directed mutagenesis.](image1)

![Fig. 2. Indirect immunofluorescence of CHO-ACE cells. a, immunofluorescence in intact cells with the antiserum Y1 raised against human kidney ACE; b, immunofluorescence in Triton X-100 permeabilized cells with the antiserum 28A7 raised against the C-terminal peptide.](image2)
Interestingly, CHO-ACE cells also secrete recombinant enzyme into the culture medium at a rate of 0.39 ± 0.07 μg/10^6 cells per day.

The synthesis and release of recombinant enzyme by CHO-ACE<sub>ACO</sub> cells was compared with those of CHO-ACE cells (Table I). Around 7-fold less ACE is present in CHO-ACE<sub>ACO</sub> than in CHO-ACE cell homogenate, and the amount of ACE secreted into the culture medium by CHO-ACE<sub>ACO</sub> cells is about 8-fold greater than by CHO-ACE cells after 24 h collection. Thus, the presence of the hydrophobic sequence limits ACE secretion into the culture medium.

ACE was not detected in the cell extract or in the culture medium of wild-type CHO cells.

Characterization of the Membrane-bound Recombinant ACE—Membrane-bound and secreted forms of wild-type recombinant ACE were purified and characterized separately. The membrane-bound form of wild-type recombinant ACE was purified from homogenates of CHO-ACE cells by affinity chromatography. The purified ACE migrated as a single molecular species with an apparent molecular mass of 170 kDa on SDS-PAGE (Fig. 3), similar to the native human kidney ACE (10).

The membrane-bound form of the recombinant enzyme and human kidney enzyme were found to be indistinguishable in direct RIA of ACE. The two enzymes produced parallel displacement curves and, when the amounts of enzymes were normalized in activity units, a similar displacement was observed for both enzymes (Fig. 4). The amount of recombinant ACE therefore was estimated by RIA for enzymatic studies, using the pure kidney ACE as the standard.

To analyze the enzymatic activity of membrane-bound recombinant ACE, the best known physiological substrate, AI, and two most commonly used synthetic substrates (Fa-Phe-Gly-Gly and Hip-His-Leu) were used. Recombinant ACE was found to be able to convert AI into AI1 and to hydrolyze Fa-Phe-Gly-Gly and Hip-His-Leu. The conversion of AI into AI1 was demonstrated by HPLC analysis (Fig. 5). This conversion could be inhibited completely by 10 μM captopril (Fig. 5) or 10 μM EDTA. The kinetic parameters (K<sub>m</sub> and K<sub>cat</sub>) of the

![Fig. 4. Direct RIA of ACE. Displacement curves of pure membrane-bound recombinant ACE (0.11-7.2 milliunits) (solid symbols) and pure human kidney ACE (0.14-9 milliunits or 1.56-100 ng) (open symbols). B/Bo is the ratio of bound [125I]-labeled ACE in the presence and absence of unlabeled enzyme and is plotted on a logit-log scale. The amount of enzyme is plotted on a logarithmic scale.](image)

![Fig. 5. HPLC analysis of AI to AI1 conversion by recombinant ACE. a, elution profile of standard AI and AI1; b, elution profile of the hydrolysis of AI (100 μM) by pure membrane-bound recombinant ACE (0.5 nM) for 20 min in the same conditions as described in the legend of Table II and in the absence (solid curve) or the presence (dashed curve) of captopril (10 μM).](image)

### Table I

<table>
<thead>
<tr>
<th>Cell</th>
<th>ACE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Culture medium&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>CHO-ACE (n = 5)</td>
<td>1.94 ± 0.29</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>CHO-ACE&lt;sub&gt;ACO&lt;/sub&gt; (n = 5)</td>
<td>0.28 ± 0.05</td>
<td>3.27 ± 0.56</td>
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<sup>a</sup>Determined by direct RIA.

<sup>b</sup>24-h collection of serum-free culture medium.

![Fig. 3. SDS-PAGE of human ACE expressed in CHO-ACE cells. Lane 1, 0.2 μg of recombinant ACE purified from cell homogenate by affinity chromatography; lane 2, cell homogenate containing 0.2 μg of recombinant ACE. On the left, the arrows indicate the positions of molecular mass markers (in kDa), and on the right, the position of recombinant ACE (in kDa).](image)

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>K&lt;sub&gt;cat&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td>Recombinant</td>
<td>Kidney</td>
<td>Recombinant</td>
</tr>
<tr>
<td>Fa-Phe-Gly-Gly</td>
<td>174 μM</td>
<td>320 s&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hip-His-Leu</td>
<td>1540 s&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>408 s&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>16 s&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>408 s&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup>Assay conditions: Fa-Phe-Gly-Gly, 50 mM Tris, pH 7.5, 300 mM NaCl, 10 mM ZnSO<sub>4</sub>, 25 °C; Hip-His-Leu, 100 mM potassium phosphate, pH 8.5, 300 mM NaCl, 10 μM ZnSO<sub>4</sub>, 37 °C; angiotensin I, 50 mM Hepes, pH 7.5, 50 mM NaCl, 1 μM ZnSO<sub>4</sub>, 37 °C.

<sup>b</sup>Previously obtained in the same assay conditions (10).

### Recombinant Angiotensin I-converting Enzyme

![5543](image)
lung and kidney converting enzymes (26, 27).

We next studied the zinc requirement of the recombinant enzyme. Under standard assay conditions for the hydrolysis of Hip-His-Leu, 0.5 nM recombinant enzyme was inhibited completely by 10 μM EDTA, and the activity was restored by adding 100 μM ZnSO₄. No alteration in enzymatic activity was observed over a zinc concentration range of 1–1000 pM. For subsequent experiments, 10 μM ZnSO₄ was added routinely to the assay solution.

The optimum pH for the hydrolysis of Hip-His-Leu was determined in a pH range from 6 to 9 in 0.1 M potassium phosphate buffer containing 300 mM NaCl and 1 mM substrate. The pH profile of ACE activity is similar for both recombinant and kidney enzymes with an optimum pH around 8.5 (Fig. 6a).

The effects of chloride on the hydrolysis of Hip-His-Leu were investigated at pH 8.3 and 1 mM substrate (Fig. 6b). The chloride activation profile of the recombinant enzyme is identical to that of the kidney enzyme. In the absence of added chloride, both enzymes possess less than 1% of their maximal activity, which is obtained at 800 mM NaCl. A double-reciprocal plot of activity versus chloride concentration yields an apparent constant (Kₘ) for chloride activation of 180 mM for both enzymes.

Finally, the effects of captopril and enalaprilat, two potent competitive inhibitors of ACE (28, 29) on the hydrolysis of Hip-His-Leu were examined at pH 8.3, 300 mM NaCl, 1 mM substrate, and 0.05 nM enzyme. The dose-dependent inhibition curves for recombinant and kidney enzymes are similar (Fig. 6c). IC₅₀ values for the two enzymes (1.3 nM for captopril and 0.7 nM for enalaprilat) are identical.

**Characterization of Secreted Recombinant and Plasma ACEs**—The secreted form of the wild-type recombinant ACE and the C-terminally truncated recombinant enzyme (ACE_cTrunc), were each purified to homogeneity from culture media. The C termini of these secreted recombinant ACEs were examined by Western blot analysis using the antiserum 28A7 directed against the C-terminal peptide (Fig. 7a). As expected, the purified membrane-bound recombinant ACE and human kidney ACE react with the antiserum 28A7, and the C-terminally truncated recombinant enzyme does not, indicating that this antiserum reacts specifically with the C terminus of the membrane-bound enzyme. But most interestingly, the secreted form of the wild-type recombinant enzyme is not recognized by the antiserum 28A7, indicating that the enzyme secreted by CHO cells transfected with the wild-type ACE cDNA is processed at its C terminus. Similarly, the C terminus of the human plasma enzyme was examined using the antiserum 28A7. As for the secreted form of the wild-type recombinant ACE, the plasma enzyme does not react with this antiserum (Fig. 7b), indicating that it also differs from the membrane-bound enzyme by a truncation in the C-terminal region. As shown in Fig. 7, the antiserum Y1 recognizes all recombinant and native ACEs. The two secreted recombinant enzymes are indistinguishable from the membrane-bound recombinant enzyme in direct RIA of ACE (using a polyclonal antibody raised against human kidney ACE) (data not shown). This allows us to measure by this method the concentration of these recombinant enzymes used in the enzymatic studies.
The catalytic properties of the two secreted recombinant ACEs were compared to those of the membrane-bound recombinant enzyme using Hip-His-Leu as substrate (Table III). The kinetic parameters of the two secreted recombinant ACEs are similar to those of membrane-bound recombinant ACE. Chloride activation profiles are identical for the two secreted recombinant ACEs and the membrane-bound recombinant ACE, with maximal activation at 800 mM NaCl (pH 8.3 and 1 mM Hip-His-Leu) and an apparent activation constant \((K'_a)\) around 180 mM. Both secreted recombinant ACEs are inhibited completely by 1 \(\mu\)M captopril or enalaprilat, or by 10 \(\mu\)M EDTA.

**DISCUSSION**

A stable cell line (CHO-ACE) producing a substantial amount of recombinant ACE has been established by transfection of human ACE cDNA into CHO cells. The purified recombinant ACE displays structural, immunological, and enzymatic properties indistinguishable from the native human kidney enzyme. Both enzymes have an apparent molecular mass of 170 kDa, compared with the 146.6 kDa predicted from the amino acid sequence (10), suggesting that they both contain approximately 14% carbohydrate. Both enzymes are immunologically indistinguishable when tested with polyclonal antibodies raised against the human kidney ACE. Finally, the recombinant ACE is fully active and displays enzymatic properties identical to those of the kidney enzyme, including kinetic parameters for hydrolysis of synthetic substrates and AI, pH profile of activity, chloride activation, absolute zinc requirement, and inhibition by competitive ACE inhibitors. The expression system described here is therefore convenient for structure-function studies of ACE.

This system was used to examine the function of the 17-amino-acid C-terminal hydrophobic segment. Previous work has suggested that the membrane-bound enzyme is attached to the membrane at or near its C-terminal end, since the size of the enzyme did not change detectably and the N-terminal amino acid sequence of the enzyme remained intact when it was solubilized from the membrane by trypsin treatment (11). Hydropathy analysis of the sequence of human endothelial ACE revealed two highly hydrophobic segments in the molecule, one is located at the N terminus and the other near the C terminus. The N-terminal hydrophobic segment has the characteristics of a signal peptide, and N-terminal sequencing of human kidney enzyme has revealed that this signal peptide is cleaved off during maturation and therefore is not involved in membrane anchoring (10). The present study clearly shows that the C-terminal hydrophobic segment located 30–47 residues upstream of C terminus is the transmembrane anchor for the following reasons: 1) The deletion of the C terminus of ACE by introducing a stop codon at the first residue of the hydrophobic segment leads to the production of an unbound form of ACE. 2) The wild-type recombinant enzyme is expressed as an ectoenzyme inserted in the plasma membrane with its C-terminal end located on the cytoplasmic side of the plasma membrane, as demonstrated by immunofluorescence using the antisera 28A7 directed specifically against the C terminus of ACE. The large N-terminal region upstream of the transmembrane domain is therefore located outside the cell. This region of 1230 residues comprises two large highly homologous domains (10, Fig. 1) which are both enzymatically active.

Other known mechanisms for membrane attachment are not consistent with the results of present and previous studies. A number of plasma membrane proteins are known to be attached to the membrane by a phosphorylinsitol anchor at the C terminus (30). The previous observations that the membrane-bound ACE is resistant to hydrolysis by phosphorylindolinositol-specific phospholipases but is readily solubilized by the detergent Triton X-100 are not in favor of a phosphorylindolinositol anchorage mechanism (11, 31). Indeed, this mechanism cannot be involved in the membrane anchoring of ACE because the addition of the phosphorylindolinositol anchor generally is preceded by deletion of approximately 20 residues of the C terminus of the molecule (30), and experiments with the antisera 28A7 indicate that no C-terminal deletion has occurred in the membrane-bound ACE. Another known mechanism for membrane attachment is binding by an amphiphilic helix, as described for carboxypeptidase E (32). However, the hydrophathy profile of ACE is more compatible with the occurrence of a transmembrane domain than with this last mechanism.

ACE is also a brush-border peptidase. Most of these ector- peptidases characterized to date are attached to the membrane by one of the two common mechanisms: either by a hydrophobic sequence near their N terminus, such as for the neutral endopeptidase (33, 34), dipetidylpeptidase IV (35), and aminopeptidase N (36, 37), or by a C-terminal phosphorylindolino- stol anchor, such as the renal dipetidase (38) and aminopeptidase P (39). ACE differs from these peptidases by being anchored via its C-terminal hydrophobic sequence, like lactose-phlorizin hydrolase, another brush-border enzyme (40).

Interestingly, the CHO cells transfected with the wild-type ACE cDNA secrete ACE into the culture medium, although they mainly produce a membrane-bound form of the enzyme. The secreted form of the recombinant enzyme is similar enzymatically to the membrane-bound form, but its C terminus is structurally different. This secreted enzyme is not recognized by the antisera 28A7, which recognizes specifically the C terminus of ACE, indicating that the C terminus of the secreted enzyme is deleted. The observation that the secreted and membrane-bound forms of the enzyme are both synthesized by CHO cells transfected with the wild-type cDNA suggests that these two forms are translated from a common mRNA and that the secreted form is derived from the membrane-bound form by a post-translational event. It is unlikely that the secreted ACE is released by leakage of intact ACE through the plasma membrane, since the C terminus of secreted ACE is deleted. The secreted form presumably is derived from the membrane-bound form by enzymatic cleavage occurring intracellularly or at the plasma membrane. The nature of this putative proteolytic enzyme is unknown. It has been reported that an EDTA-sensitive endogenous hydrolase was able to release ACE from pig kidney membrane, but so

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**TABLE III**

<table>
<thead>
<tr>
<th>Recombinant ACEs</th>
<th>Kinetic parameters*</th>
<th>Effects of chloride*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted form of wild-type ACE</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>(K_e)</td>
<td>1540</td>
<td>360</td>
</tr>
<tr>
<td>(K_{cat})</td>
<td>800</td>
<td>%</td>
</tr>
<tr>
<td>Activity without added chloride</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>(K'_a)</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>ACE(_{COOH})</td>
<td>1590</td>
<td>358</td>
</tr>
<tr>
<td>(K_e)</td>
<td>860</td>
<td>&lt;1</td>
</tr>
<tr>
<td>(K_{cat})</td>
<td>182</td>
<td></td>
</tr>
</tbody>
</table>

* Determined at 300 mM NaCl as described in Footnote a of Table II.

* Assay conditions were as in Fig. 6.

2 L. Wei, F. Alenc-Gelas, P. Corvol, and E. Clauser, unpublished results.
fat this enzyme has not been identified (11). Although ACE can be solubilized readily from the membrane by trypsin, there is no dibasic cleavage site between the most C-terminal cysteine and the hydrophobic anchor. Further studies are needed to detect the exact cleavage site(s) and the cellular localization and nature of the putative processing enzyme.

The circulating plasma enzyme is believed to be secreted by vascular endothelial cells (8, 12). The mechanisms responsible for ACE secretion by vascular endothelial cells are probably similar to those in CHO cells, as the C terminus of proteases are responsible for the production of plasma ACE in serum-free medium. Cells transfected with the wild-type ACE cDNA release a C-terminally truncated form of ACE in serum-free medium.