The Two Homologous Domains of Human Angiotensin I-converting Enzyme Are Both Catalytically Active*

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Molecular cloning of human endothelial angiotensin I-converting enzyme (kininase II; EC 3.4.15.1) (ACE) has recently shown that the enzyme contains two large homologous domains (called here the N and C domains), each bearing a putative active site, identified by sequence comparisons with the active sites of other zinc metalloproteases. However, the previous experiments with zinc or competitive ACE inhibitors suggested a single active site in ACE. To establish whether both domains of ACE are enzymatically active, a series of ACE mutants, each containing only one intact domain, were constructed by deletion or point mutations of putative critical residues of the other domain, and expressed in heterologous Chinese hamster ovary cells. Both domains are enzymatically active and cleave the C-terminal dipeptide of hippuryl-His-Leu or angiotensin I. Moreover, both domains have an absolute zinc requirement for activity, are activated by chloride and are sensitive to competitive ACE inhibitors, and appear to function independently. However, the two domains display different catalytic constants and different patterns of chloride activation. At high chloride concentrations, the C domain hydrolyzes the two substrates tested faster than does the N domain. His-361,365 and His-959,963 are established as essential residues in the N and C domains, respectively, most likely involved in zinc binding, and Glu-362 in the N domain and Glu-960 in the C domain are essential catalytic residues. These observations provide strong evidence that ACE possesses two independent catalytic domains and suggest that they may have different functions.

Angiotensin I-converting enzyme (kininase II; EC 3.4.15.1) (ACE)* is a zinc metalloprotease which plays an important role in circulatory homeostasis by cleaving the C-terminal dipeptide from angiotensin I (AI), thereby producing the potent vasopressor peptide angiotensin I1 (AII) (1). It also plays a role in circulatory homeostasis by cleaving the C-terminal part of endothelial ACE and thereby producing the dipeptide from angiotensin I (AI), thereby producing the potent vasopressor peptide angiotensin I1 (AII) (1). It also plays a role in circulatory homeostasis by cleaving the C-terminal part of endothelial ACE and thereby producing the dipeptide from angiotensin I (AI), thereby producing the potent vasopressor peptide angiotensin I1 (AII) (1). It also

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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; Hip, hippuryl; HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

pressed in several other cell types including absorptive epithelial cells, neuroepithelial cells, and male germinal cells (3, 4). In somatic tissues, ACE is a glycoprotein composed of a single large polypeptide chain of 140–170 kDa (3, 4). In germinal cells, ACE is synthesized as a lower molecular mass form of 100–110 kDa, which displays catalytic properties for AI similar to the endothelial ACE and is equally inhibited by ACE inhibitors (5, 6).

The primary structure of human endothelial ACE has been recently determined from the cDNA clones obtained from human vascular endothelial cells (7). A single transmembrane segment of 17 amino acids separates a short C-terminal intracellular region of 30 amino acids and a large N-terminal extracellular region of 1230 residues (7, 8). Interestingly, the extracellular part displays a high degree of internal homology between two large homologous domains (called here the N and C domains), suggesting that endothelial ACE results from an ancestral gene duplication. The sequence identity between the two domains is more than 60% over a stretch of 357 amino acids. Each of these two homologous domains contains all residues known to be involved in zinc binding and catalysis of other metalloproteases, identified by sequence comparisons with the active sites of thermolysin and neutral endoproteinase (7). The endothelial ACE therefore contains two putative active sites. A similar organization has also been found for the mouse enzyme (9). Whether only one or both of these putative active sites are catalytically active is however unknown and cannot be determined by sequence analysis (7, 9). Furthermore, two lines of evidence suggest that only one of the two domains is active. First, analysis of the zinc content of endothelial ACE indicated a stoichiometry of one zinc atom/molecule of ACE, suggesting the presence of a single active site in ACE (10, 11). Second, studies using competitive ACE inhibitors similarly only detected a single binding site on each ACE molecule (12–14). Moreover, elucidation of the primary structure of testicular ACE has shown that it corresponds to the C-terminal part of endothelial ACE and therefore contains only the C domain (15–17). This suggests that the C domain is catalytically active but does not indicate the function of the N domain which awaits enzymatic characterization.

In this study, we report the enzymatic characterization of each of the two homologous domains of human endothelial ACE by expressing mutated cDNA in Chinese hamster ovary (CHO) cells. Each domain has been independently expressed after deletion of the other domain. In addition, substitutions of putative critical residues located in the short consensus sequence of zinc metalloproteases His-Glu-X-X-His (Fig. 1) have been introduced by point mutation into the full-length enzyme to inactivate either the N or C domain (Fig. 2). The results demonstrate that both domains are independently active and possess a dipeptidyl carboxypeptidase activity. However, the two sites have significantly different catalytic
were excised from pbACE2 and cloned independently in an M13 vector. A series of mutations were introduced into either the N or C domain. For all full-length mutants, the nucleotide sequence at the junction of this hybrid cDNA was confirmed by DNA sequencing. The cDNA insert was then excised by digestion with EcoRI and inserted into the EcoRI site of pBluescript (Stratagene). The orientation of the plasmid was verified by restriction mapping and sequencing.

Proprietors of human ACE properties, especially in the regulation of their activities by chloride.

MATERIALS AND METHODS

Construction of Expression Plasmids for Truncated Human ACEs

The N-terminal ACE Fragment—The 5' part of the ACE-coding sequence was taken from the plasmid pbACE2 (8) in which the full-length ACE cDNA is constructed from three overlapping cDNA clones xCHDT32, AHEC2111, and XHEC1922 (7) and inserted into the EcoRI site of the plasmid pbACE2 (8) digested with HindIII and XhoI (bp 2578-4024) of the ACE cDNA. The 2.3-kb fragment (bp 9 to 2318) of the ACE cDNA was then purified from a low gelling temperature-agarose gel (FMC BioProducts) and inserted into the expression vector pECE (18) digested with HindIII and SalI that is compatible with XhoI. The resulting expression plasmid encodes a truncated ACE, designated the N fragment, containing the signal peptide, the residues 1-737 of ACE followed by 10 heterologous amino acids provided by the polylinker sequence of pECE (SalI to XbaI). The resulting plasmid was verified by restriction mapping and sequencing.

The C-terminal ACE Fragment—The 5' part of the coding sequence (ie, the signal peptide) used in this construction was an EcolI/SmaI fragment from the clone xCHDT32 (7) corresponding to the first 118 bp of the ACE cDNA. The 3' part of the coding sequence was provided by a Scal/KpnI fragment from the clone xHEC1922 (7) corresponding to bp 1824-4024 of the ACE cDNA. These two fragments were purified from these gels and linked by a 13 nucleotide linker and then inserted into EcolI/KpnI-digested pBluescript, to generate the final plasmid. The resulting plasmid encodes a truncated ACE, designated the C fragment, containing the signal peptide, residues 1-4 of the N terminus of mature ACE followed by two heterologous amino acids (Pro and Trp) encoded by the linker sequence, and the residues 1278-1777 of ACE. The nucleotide sequence at the junction of this hybrid cDNA was confirmed by DNA sequencing. The cDNA insert was then excised by digestion with EcoRI and inserted into the EcoRI site of pECE.

Site-directed Mutagenesis

A 0.5-kb SacI/SalII fragment (bp 1022-1511) of the ACE cDNA and a 1.5-kb SphI/SalI fragment (bp 2578-4024) of the ACE cDNA were excised from pbACE2 and cloned independently in a M13 vector. A series of mutations were introduced into either the N or C putative active site using an oligonucleotide-directed mutagenesis system (Amersham, France) which is based on the method of Taylor et al. (19). A single Gln (GAG) to Asp (GAC) mutation at position of amino acid 362 of a double His (CAT, CAT) to Lys (AAG, AAA) mutation at positions 361 and 365 was introduced into the 0.5-kb cDNA fragment. The same mutations were performed on the 1.5-kb cDNA fragment: Gln-362 (GAG) to Asp (GAT), or His-365 and 366 (CAC, CAC) to Lys (AAG, AAA).

Mutant cDNAs were screened by DNA sequencing and in each case the entire cDNA fragment subcloned in the M13 vector was sequenced to ensure that no other mutations had occurred. The mutated cDNAs were isolated from the replicating form of the M13 recombinant phage, and each was used to replace the corresponding fragment in pEACE, the expression plasmid of wild-type ACE cDNA (8). Six mutants containing mutations on either N or C domain or on both domains were thus constructed (Fig. 2). All expression plasmids were characterized by restriction mapping and sequencing of the mutated regions.

Expression and Characterization of ACE Mutants in CHO Cells

The deleted or mutated ACE cDNAs are under the transcriptional control of the SV40 early promoter in the expression plasmids. The plasmids were introduced by cotransfection with the neomycin resistance plasmid pSVneo into CHO cells as previously described (18). RNA dot blots were used to screen pure cell lines expressing each mutant. Purification of the mutant proteins on lissinopril-coupled Sepharose, immunological characterizations by indirect immunofluorescence on intact cells using the antiseraum Y1 raised against the human kidney ACE, Western blotting, and radioimmunoassays (RIA) were carried out as described for the wild-type recombinant enzyme expressed in the transfected CHO cells (8). Enzymatic studies were performed using hippuryl-His-Leu (Hip-His-Leu) (Biochem, Switzerland) or Al (Sigma) as substrate as previously described (8). The detection and quantification of hippuric acid liberated from the Hip-His-Leu were performed by HPLC. The detection limit of hippuric acid liberated from Hip-His-Leu was 0.006% of substrate hydrolysis. One unit of activity was defined as the amount of enzyme catalyzing the release of 1 μmol of hippuric acid from Hip-His-Leu/min (20). The hydrolysis of Al was followed by HFLC or by RIA of Al. The detection limit of Al was generated from Al was 0.2% of substrate hydrolysis. Kinetic parameters for the hydrolysis of Hip-His-Leu and Al were determined from Lineweaver-Burk plots. Initial velocities were measured during the hydrolysis of the first 5% of substrate. All enzymatic studies were performed under initial rate conditions.

RESULTS

Expression of ACE Mutants in CHO Cells—A series of mutated ACE cDNAs containing only one of the two intact homologous domains were constructed in order to study their enzymatic properties (Fig. 2). They include three mutants having an intact N domain (N fragment, ACE9890, and ACE9893), three mutants having an intact C domain (C fragment, ACE9892, and ACE9893), and two mutants with both N and C domains mutated (ACE9892,9893, and ACE9892,9893,9893). The C domain is absent in the N fragment, and the N domain is absent in the C fragment. All other mutants are full-length and contain point mutations of the two putative zinc-binding histidines or the putative catalytic domain (7). The deleted or mutated ACE cDNAs are under the transcriptional control of the SV40 early promoter in the expression plasmids. The plasmids were introduced by cotransfection with the neomycin resistance plasmid pSVneo into CHO cells as previously described (18). RNA dot blots were used to screen pure cell lines expressing each mutant. Purification of the mutant proteins on lissinopril-coupled Sepharose, immunological characterizations by indirect immunofluorescence on intact cells using the antiseraum Y1 raised against the human kidney ACE, Western blotting, and radioimmunoassays (RIA) were carried out as described for the wild-type recombinant enzyme expressed in the transfected CHO cells (8). Enzymatic studies were performed using hippuryl-His-Leu (Hip-His-Leu) (Biochem, Switzerland) or Al (Sigma) as substrate as previously described (8). The detection and quantification of hippuric acid liberated from the Hip-His-Leu were performed by HPLC. The detection limit of hippuric acid liberated from Hip-His-Leu was 0.006% of substrate hydrolysis. One unit of activity was defined as the amount of enzyme catalyzing the release of 1 μmol of hippuric acid from Hip-His-Leu/min (20). The hydrolysis of Al was followed by HFLC or by RIA of Al. The detection limit of Al was generated from Al was 0.2% of substrate hydrolysis. Kinetic parameters for the hydrolysis of Hip-His-Leu and Al were determined from Lineweaver-Burk plots. Initial velocities were measured during the hydrolysis of the first 5% of substrate. All enzymatic studies were performed under initial rate conditions.

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glutamic acid in either the N or C domain, or in both domains.

The expression vector pCEC (18) was used to express these mutants in CHO cells. This expression system allowed the production of high levels of the wild-type recombinant ACE (8). Stable cell lines expressing each mutant were established. The CHO cells expressed a recombinant mRNA of about 2.5 kb when transfected with truncated ACE cDNA or 4.2 kb when transfected with full-length ACE cDNA containing point mutations. The cellular expression of these mutants was examined by indirect immunofluorescence using the antiserum Y1 raised against the human kidney ACE (21). This antiserum detected each of these ACE mutants at the surface of transfected cells as for the wild-type enzyme (8), except the N fragment which does not contain the C-terminal transmembrane anchor and was secreted into the medium (data not shown). The characterization of all mutants, except the N fragment, was carried out on the protein purified to apparent homogeneity from cell homogenate by affinity chromatography. The full-length mutants had an apparent molecular mass of 170 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis which is identical to that of the wild-type recombinant ACE (8). The C fragment had an apparent molecular mass of 100 kDa. The characterization of the N fragment was performed directly on the dialyzed serum-free medium of transfected cells. It was impossible to obtain sufficient amount of purified mutant by affinity chromatography because this mutant bound very poorly to the affinity gel. Its apparent molecular mass determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting was 135 kDa. The difference between the apparent molecular masses of the N and C fragments and the values predicted from the sequence (85.7 and 81.9 kDa, respectively) is probably due to glycosylation: 15 potential N-glycosylation sites are present in the N fragment and 7 in the C fragment. The full-length mutants and the wild-type recombinant enzyme were immunologically indistinguishable when tested by direct RIA of ACE (data not shown). The concentration of the purified full-length mutants, used in the enzymatic studies, could therefore be measured by RIA as for the wild-type enzyme (8). However the two truncated mutants (the N and C fragments) displayed only partial cross-reactivity with the wild-type recombinant ACE in direct RIA and therefore could not be quantified by this method.

Enzymatic Activity of Mutants Having Mutations in Both N and C Domains—The enzymatic activity of the two full-length mutants containing mutations in both domains ACE<sub>361,365</sub> and ACE<sub>959,963</sub> was assayed using Hip-His-Leu as substrate under the standard assay conditions (5 mM substrate, pH 8.3, 300 mM NaCl, 10 μM ZnSO₄, 37 °C) (20). When tested for 60 min at an enzyme concentration of 0.5 nM, these mutants hydrolyzed 0.6% of the substrate. The enzymatic activity of the two mutants having an intact N domain was therefore at least 100-fold higher than the detection limit of the assay. In addition, the N fragment, in which the C domain is absent, also displayed detectable enzymatic activity when assayed using the concentrated and dialyzed serum-free medium of transfected cells (2.7 ± 0.4 milliunits/10<sup>6</sup> cells/day). The serum-free culture medium of nontransfected CHO cells was used as a control and did not contain any detectable enzymatic activity.

The kinetic parameters of the three mutants containing an intact N domain (the N fragment, ACE<sub>361,365</sub>, and ACE<sub>959,963</sub>) for the hydrolysis of Hip-His-Leu were obtained by assaying their activities over a wide range of substrate concentrations. These three mutants were slightly inhibited at high substrate concentrations (Fig. 3), as previously observed for the wild-type enzyme (8). Kinetic constants were obtained by extrapolation of the linear part of Lineweaver-Burk plots (Table I). The K<sub>m</sub> values were similar for these three mutants and the wild-type enzyme. The K<sub>cat</sub>/K<sub>m</sub> values were determined for the

13-fold lower than the specific activity of the wild-type recombinant ACE, 96 ± 0.3 units/mg (n = 3). When tested for 60 min at an enzyme concentration of 0.5 nM, these mutants hydrolyzed 0.6% of the substrate. The enzymatic activity of the two mutants having an intact N domain was therefore at least 100-fold higher than the detection limit of the assay. In addition, the N fragment, in which the C domain is absent, also displayed detectable enzymatic activity when assayed using the concentrated and dialyzed serum-free medium of transfected cells (2.7 ± 0.4 milliunits/10<sup>6</sup> cells/day). The serum-free culture medium of nontransfected CHO cells was used as a control and did not contain any detectable enzymatic activity.

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**Table I**

| Enzyme  | K<sub>m</sub> (μM) | K<sub>cat</sub> (units/mg) |
|---------|------------------|--------------------------|
| N fragment | 1980 | ND<sup>a</sup>  |
| ACE<sub>361,365</sub> | 2000 | 40 |
| ACE<sub>959,963</sub> | 2000 | 40 |
| C fragment | 2000 | ND<sup>a</sup>  |
| ACE<sub>361,365</sub> | 1590 | 359 |
| ACE<sub>959,963</sub> | 1590 | 364 |

<sup>a</sup> Not determined.

**Fig. 3.** Lineweaver-Burk plots for the hydrolysis of Hip-His-Leu by ACE mutants. The N fragment (○), ACE<sub>361,365</sub> (△), and ACE<sub>959,963</sub> (■) were compared to the C fragment (■), ACE<sub>361,365</sub> (△), and ACE<sub>959,963</sub> (■). Assay mixtures (250 μl) containing 0.15 mM enzyme, 100 mM potassium phosphate, pH 8.3, 300 mM NaCl, and 10 μM ZnSO₄ were incubated for 30 min at 37 °C. The amount of truncated mutants used in the assay was that having the same activity as the corresponding full-length mutants at 5 mM substrate.
two full-length mutants and were about 10-fold lower than for the wild-type enzyme. The small difference observed in the ratio of $K_m$ for mutated and wild-type enzymes (1:10) and in the ratio of the specific activities measured at 5 mM substrate concentration (1:13) was due to the greater substrate inhibition of the mutants than of the wild-type enzyme at 5 mM substrate concentration.

The ability of the N domain to hydrolyze AI was then assayed at pH 7.5, 50 mM NaCl and 1 mM ZnSO$_4$. The three mutants with an intact N domain converted AI to AII as verified by HPLC analysis (data not shown). The kinetic parameters for the hydrolysis of AI by these three mutants were determined (Table II). $K_m$ values were similar for the mutant enzymes and the wild-type enzyme, whereas the $K_{cat}$ values of the two full-length mutants were about 4-fold lower than for the wild-type enzyme.

**Enzymatic Activity of Mutants Possessing an Intact C Domain**—The activities of the three mutants having an intact C domain were tested under standard assay conditions for the hydrolysis of Hip-His-Leu as described above. The two full-length mutants ACE$_{K639/I66}$ and ACE$_{K639/I65}$ were able to hydrolyze the substrate with a specific activity of $89 \pm 10$ units/mg ($n = 3$). This value is close to that of the wild-type enzyme. The truncated mutant, the C fragment, in which the N domain is absent, was also able to hydrolyze Hip-His-Leu. When tested over a wide range of substrate concentrations, these three mutants were also subject to slight inhibition at high substrate concentrations (Fig. 3). The kinetic parameters obtained by extrapolation of the linear part of Lineweaver-Burk plots were close to those of the wild-type enzyme (Table I). Finally, these three mutants converted AI to AII with $K_m$ values similar to that of the wild-type enzyme. The $K_{cat}$ values of the two full-length mutants were about 75% of that of the wild-type enzyme (Table II).

**Factors Influencing the Catalytic Activities of N and C Domains**—To further compare the catalytic properties of the N and C domains, the effects of several factors known to influence the catalytic activity of ACE were analyzed, such as pH, zinc, and chloride concentrations. The effect of competitive ACE inhibitors was also tested. The influence of these factors on the catalytic properties of the ACE mutants was first examined using Hip-His-Leu as substrate. A substrate concentration of 1 mM was chosen in order to avoid any substrate inhibition.

The effect of zinc was examined at pH 8.3 and 300 mM NaCl. No significant change of activity was observed over a wide range of zinc concentrations (1-1000 mM) for all the active mutants (data not shown). The activity of all these mutants was completely inhibited by 10 mM EDTA and restored by 100 mM ZnSO$_4$, indicating that both domains have an absolute zinc requirement for their activity.

The optimum pH was determined over a pH range from 7 to 9 in 0.1 M potassium phosphate buffer containing 300 mM NaCl and 10 mM ZnSO$_4$. The optimum pH for all active mutants was around pH 8.5 (data not shown), which is similar to that of the wild-type recombinant enzyme (8).

The effect of captopril and enalaprilat, two potent inhibitors of ACE (12, 23), was tested at pH 8.3, 300 mM NaCl, 10 mM ZnSO$_4$ in the presence of 1 mM inhibitor. They were all completely inhibited by these two inhibitors.

The influence of chloride on the catalytic activity of each of the two domains was investigated at 1 mM Hip-His-Leu, pH 8.3, and 10 mM ZnSO$_4$. The mutants having an intact N domain and the mutants having an intact C domain behaved differently in response to chloride activation (Fig. 4, Table III). In the absence of added chloride, the three mutants with an intact N domain displayed about 15% of their maximal activity, which was achieved at 10 mM NaCl. They were all slightly inhibited at supraoptimal concentrations of chloride. In contrast, in the absence of added chloride, the three mutants with an intact C domain displayed less than 1% of their activity.

**Table II**

| Enzyme       | $K_m$ (mM) | $K_{cat}$ (s$^{-1}$) |
|--------------|------------|----------------------|
| Wild-type    | 16         | 40                   |
| N fragment   | 16         | ND*                  |
| ACE$_{K639/I65}$ | 18       | 12                   |
| ACE$_{K639/I66}$ | 15       | 11                   |
| C fragment   | 16*        | ND*                  |
| ACE$_{G642}$ | 16*        | 18                   |
| ACE$_{G642/K62}$ | 18*      | 34                   |

* Not determined.

**Table III**

| Enzyme            | No chloride addition | Optimal [Cl$^{-}$] | $K_m$ (mM) | $K_{cat}$ (mM) |
|-------------------|----------------------|--------------------|------------|----------------|
| Wild-type         | 0.8                  | 800                | 180        |                |
| N fragment        | 13.1                 | 10                 | 1.4        |                |
| ACE$_{K639/I65}$  | 14.4                 | 10                 | 1.2        |                |
| ACE$_{K639/I66}$  | 16.8                 | 10                 | 1.1        |                |
| C fragment        | 0.1                  | 800                | 221        |                |
| ACE$_{G642}$      | 0.1                  | 800                | 213        |                |
| ACE$_{G642/K62}$  | 0.2                  | 800                | 217        |                |

* Activity as a percentage of that at optimal chloride concentration.
maximal activity, which was achieved at a high chloride concentration of 800 mM. The level of maximal activity was about 15-fold higher for the full-length mutants having an intact C domain than for those having an intact N domain. Double reciprocal plots of 1/(V - V₀) versus 1/[Cl⁻] (where V₀ is the velocity in the absence of added chloride and V is the velocity at each chloride concentration) yielded an apparent activation constant (Kₐ) of approximately 1.2 mM for the mutants having an intact N domain or 217 mM for those having an intact C domain.

The influence of chloride on the hydrolysis of AI by the N and C domains was assessed at pH 7.5 with 10 μM substrate and 1 μM ZnSO₄. The chloride activation profiles for the hydrolysis of AI confirm the difference in chloride activation of the N and C domains (Fig. 5, Table IV). In the absence of added chloride, the three mutants having an intact N domain displayed approximately 5% of their maximal activity and the three mutants having an intact C domain less than 2%. The concentration of NaCl required for maximal activation was higher for the three mutants having an intact C domain (30 mM) than for the three mutants having an intact N domain (10 mM). No inhibition was observed at supraoptimal chloride concentrations. The level of maximal activity was about 3-fold greater for the full-length mutants having an intact C domain than those having an intact N domain. The enzymatic activity of wild-type enzyme corresponded to the sum of the activities of the full-length mutants having an intact N domain and those having an intact C domain at each chloride concentration. Similar observation was also obtained for Hip-His-Leu hydrolysis at low chloride concentrations (data not shown). However, at high chloride concentrations, the activity of the mutants having an intact C domain was much higher than that of the mutants having an intact N domain (Fig. 4). The exact contribution of the N domain to the overall activity of ACE was difficult to determine, but again the activity of the wild-type enzyme appeared to correspond to the sum of the activities of the two domains (data not shown).

In the studies described above, the three mutants having an intact N domain (the N fragment, ACE₃₆₀, and ACE₃₆₀,₂₆₅) displayed indistinguishable enzymatic properties. Similarly, the three mutants having an intact C domain (the C fragment, ACE₃₆₂, and ACE₃₆₁,₃₆₅) were also enzymatically indistinguishable.

**DISCUSSION**

A peculiar feature of the endothelial ACE molecule, revealed by molecular cloning, is the presence of two homologous domains, each bearing a putative active site (7, 9). This is unexpected because earlier experiments with zinc (10, 11) and also with competitive inhibitors (12-14) suggested the presence of a single active site in ACE. Since it is impossible to predict by sequence analysis whether one or both of these putative active sites are functional (7, 9), enzymatic studies independently performed on each domain are required to resolve the question. This paper reports the enzymatic characterization of six different mutants each possessing only one intact domain, the other domain being either deleted or mutated at putative critical residues.

The results of all previous studies, including the recent elucidation of the primary structure of the testicular enzyme which contains only the C domain (15-17), suggest that only one of the two domains is functional and this is most probably the C domain. However, our results indicate that it is in fact not the case. The N and C domains are both able to cleave the C-terminal dipeptide of Hip-His-Leu or AI and are both sensitive to competitive ACE inhibitors. Moreover, they present similar affinity (Kₐ) for these two substrates, indicating that they probably possess conserved subsites for substrate binding. However, the catalytic constants (Kₐ) are different for the two domains. The Kₐ of the C domain is about 90% for Hip-His-Leu (at pH 8.3 and 300 mM NaCl) or 75% for AI (at pH 7.5 and 50 mM NaCl) of the value of the wild-type enzyme measured under the same conditions. These results are compatible with the observation that the testicular ACE, which contains only the C domain, displays Kₐ, values for hydrolysis of Hip-His-Leu and AI similar to the endothelial ACE (5). The Kₐ value of the N domain, which is about 10% (for Hip-His-Leu at 300 mM NaCl) or 25% (for AI at 50 mM NaCl) of that of the wild-type enzyme, is much lower than the C domain. Two lines of evidence indicate that the N domain is really active and that the activity of the enzymes with a mutated C domain corresponds to the activity of the N domain rather than a residual activity of the mutated C domain. First, the N fragment, in which the C domain is absent, is enzymatically active. Second, mutations in both domains completely inactivate the enzyme. Our results should explain why an 82-kDa N-terminal proteolytic fragment of rabbit lung ACE had enzymatic activity (24).

In addition to the presence of two active domains in ACE, our results establish that a functional zinc-binding site is present in each domain. Each of the two domains indeed has an absolute zinc requirement and is inhibited by EDTA.

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**TABLE IV**

| Enzyme | No chloride addition | Optimal [Cl⁻] | Kₐ |
|--------|---------------------|--------------|----|
| Wild-type | 2.3 | 30 | 2.9 |
| N fragment | 5.5 | 10 | 1.2 |
| ACE₃₆₀ | 6.0 | 10 | 1.5 |
| ACE₃₆₀,₂₆₅ | 7.3 | 10 | 1.2 |
| C fragment | 1.8 | 30 | 5.4 |
| ACE₃₆₂ | 1.5 | 30 | 6.5 |
| ACE₃₆₁,₃₆₅ | 0.7 | 30 | 6.4 |

* Activity as a percentage of that at optimal chloride concentration.
Moreover, the mutations of His-361,365 in the N domain and His-959,963 in the C domain result in complete abolition of the enzyme activity of the corresponding domain, indicating that they are essential amino acids. Considering the similarity between the sequence of ACE containing these residues and the sequence of thermolysin comprising the zinc-binding histidines (Fig. 1), His-361,365 and His-959,963 are most probably two of the zinc-binding residues in each domain of ACE. A third zinc-binding residue, most often glutamic acid, is typically found in zinc metalloendopeptidases (25). Glu-389 in the N domain and Glu-987 in the C domain may be the third zinc-binding residues in the corresponding domain, since their position in the primary structure corresponds to that of Glu-166 of thermolysin (7), which was shown by x-ray structure analysis to coordinate the zinc atom with the 2 histidines (26). However, further site-directed mutagenesis is required to test the involvement of these two glutamic acids of ACE. In addition to the zinc-binding histidines, our mutation experiments indicate that Glu-362 in the N domain and Glu-960 in the C domain are essential for the activity of the corresponding domain. Replacement of each glutamic acid by an aspartic acid, which maintains a negative charge at the same position but retracts the carboxylic group by a distance of approximately 1.4 Å, resulted in a suppression of the catalytic activity. This suggests that these glutamic acids are involved in basic attack of the substrate peptide bond, and correspond to Glu-143 of thermolysin (26).

It is difficult to reconcile the present results, indicating that each domain of ACE contains a functional active site, with previous findings showing the presence of only a single zinc atom (10, 11) and a single binding site for competitive inhibitors in each molecule of ACE (12-15). If we exclude the presence of experimental artifacts in all studies, these discrepancies are consistent with the tertiary structure of ACE being such that only one domain in the whole molecule is able to bind zinc or alternatively, that when one domain binds a zinc atom, the zinc-binding site of the other domain becomes inaccessible. A similar explanation has been recently proposed for rat brain hexokinase, an enzyme with two large homologous domains like ACE, which only appears to have a single hexose-binding site and a single hexose 6-phosphate-binding site. After proteolytic separation of the two homologous halves of the enzyme, a hexose-binding site and a hexose 6-phosphate-binding site have been found on each half of the enzyme (27). However, several arguments strongly suggest that each of the two domains binds a zinc atom in the whole ACE molecule and is enzymatically active. First, truncated proteins harboring only the N or C domain are enzymatically similar to the full-length mutants in which the tertiary structure is probably only minimally affected by the point mutations. This suggests that the N and C domains are both enzymatically active in the whole molecule, not only after their separation. Second, mutation of the catalytic glutamic acid of each domain, which should not affect zinc binding, produces identical results to mutation of the zinc-binding histidines, suggesting that the presence of zinc in one domain does not affect the binding of zinc to the other domain. Finally, the two domains of ACE appear to function independently and additively in the whole molecule, since the activity of the wild-type enzyme is always equal to the sum of the activities of the N and C domains (Fig. 5, Tables I and II).

Taken together, our studies establish that ACE possesses two functional and independent catalytic sites, each being dependent on a zinc cofactor. The two domains both act as a dipeptidyl carboxypeptidase toward Hip-His-Leu and AI but display different catalytic constants for these substrates at least under high chloride concentration conditions. To further compare the catalytic properties of the N and C domains, we investigated the effect of chloride on each domain of ACE. Anion activation is a peculiar property of ACE, known since the discovery of the enzyme (1). It depends on the nature of the anion (chloride being the most efficient), of the substrate, and also on the pH (28-30). The molecular basis of the activating effect of anions remains largely unknown. Chloride may bind to a critical lysine residue located at or near the active site (31), and thereby induce a conformational change that probably stabilizes the enzyme-substrate complex (14, 29-31).

A chloride regulatory site seems to be present in each domain. This is supported by the finding that the hydrolysis of Hip-His-Leu or AI by each domain is greatly stimulated by chloride (Figs. 4 and 5). In addition, the fact that the activation profile of ACE is an additive combination of that of the N and C domains suggests that this stimulation is independent for the N and C domains. However, a segment (residues 572-737 of the ACE) overlapping the two domains is present in all the ACE mutants used in the present study, and the possibility of the presence of a single chloride regulatory site/ACE molecule, located in this region and mediating the effect of chloride for both domains, although unlikely, cannot be completely excluded. Interestingly, the chloride activation profiles are strikingly different for the two domains of ACE. The N domain seems to be much less chloride dependent than the C domain since 1) a significant activity of the N domain is observed in the absence of added chloride at least for Hip-His-Leu whereas the C domain seems to have an absolute chloride requirement; 2) the maximal activation of the N domain is obtained at a lower chloride concentration than that of the C domain (Tables III and IV). On the other hand, chloride appears to be a more potent activator for the C domain than for the N domain since at maximal level of activation the C domain hydrolyzes Hip-His-Leu and AI faster than does the N domain (Figs. 4 and 5). These observations suggest structural and functional differences between the chloride regulatory sites and/or the catalytic sites of the two domains. They also indicate that the chloride concentration is a determining factor for the contribution of each domain to the overall activity of the enzyme and that the C domain is more active than the N domain only at high chloride concentrations. This in vitro observation suggests that under normal physiological conditions, in the vascular and extracellular spaces where the chloride concentration is high, the C domain is probably responsible for the majority of the conversion of AI. However, intracellularly where ACE has been detected (21) and the chloride concentration is low, the N domain may be responsible for a larger part of the hydrolysis of AI and possibly other peptide substrates.

ACE is the only zinc metalloendopeptidase known to date to have two active sites. The enzymes containing two active sites in a single polypeptide usually have two distinct functions based on the major differences in the substrate specificities of the active sites. Apparently, this is not the case for ACE, as the two domains are both able to cleave the C-terminal dipeptide of AI and display kinetic parameters for this substrate within the same order of magnitude. However, the observation that the two domains of ACE display differences in their enzymatic properties, especially in the sensitivity to chloride activation, suggests that there are structural and functional differences between the N and C active sites, and raises the hypothesis that they may have different substrate specificities. This possibility is further supported by the fact that there are large differences among the chloride activation
profiles of the ACE substrates. For example, the hydrolysis of bradykinin is known to be less chloride dependent than that of AI (32). Moreover, synthetic peptide substrates of ACE have been divided into three classes based on the major differences in sensitivity to anion activation (30). Further experiments are therefore required to determine if the differences in catalytic properties between the two domains described here are linked to the differences in the substrate specificities. Furthermore, ACE is also able to act as an endopeptidase cleaving the C-terminal tripeptide or the N-terminal tripeptide of some peptide substrates in vitro (3). The N and C domains may be differently involved in these functions, although this remains to be examined.

Finally, preliminary results indicate that the N and C domains display chloride-related differences in sensitivity to competitive ACE inhibitors. Study of the interaction of competitive ACE inhibitors with each domain will help to elucidate the molecular basis of ACE inhibition. The existence of recombinant ACE and ACE mutants should allow structural studies on each domain and provide valuable information on the mechanisms of peptide hydrolysis and on the evolution of peptidases.

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