The Two Homologous Domains of Human Angiotensin I-converting Enzyme Interact Differently with Competitive Inhibitors

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The endothelial angiotensin I-converting enzyme (ACE; EC 3.4.15.1) has recently been shown to contain two large homologous domains (called here the N and C domains), each being a zinc-dependent dipeptidyl carboxypeptidase. To further characterize the two active sites of ACE, we have investigated their interaction with four competitive ACE inhibitors, which are all potent antihypertensive drugs. The binding of [3H]trandolaprilat to the two active sites was examined using the wild-type ACE and four ACE mutants each containing only one intact domain, the other domain being either deleted or inactivated by point mutation of the zinc-coordinating histidines. In contrast with all the previous studies, which suggested the presence of a single high affinity inhibitor binding site in ACE, the present study shows that both the N and C domains of ACE contain a high affinity inhibitor binding site (Kd = 3 and 1 x 10^-10 M, respectively, at pH 7.5, 4 °C, and 100 mM NaCl). Chloride stabilizes the enzyme-inhibitor complex for each domain primarily by slowing its dissociation rate, as the Kd values of the N and C domains are markedly decreased (about 30- and 1100-fold, respectively) by 300 mM NaCl. At high chloride concentrations, the chloride effect is much greater for the C domain than for the N domain resulting in a higher affinity of this inhibitor for the C domain. In addition, the inhibitory potency of captopril (C), enalaprilat (E), and lisinopril (L) for each domain was assayed by hydrolysis of Hip-His-Leu. Their Kd values for the two domains are all within the nanomolar range, indicating that they are all highly potent inhibitors for both domains. However, their relative potencies are different for the C domain (L > E > C) and the N domain (C > E > L). The different inhibitor binding properties of the two domains observed in the present study provide strong evidence for the presence of structural differences between the two active sites of ACE.

Angiotensin I-converting enzyme (kininase II; EC 3.4.15.1) (ACE) plays an important role in blood pressure regulation. It is a dipeptidyl carboxypeptidase which converts angiotensin I into the potent vasopressor peptide angiotensin II and inactivates the vasodepressor peptide bradykinin (1, 2). ACE is an unusual zinc-metalloproteinase in that it is activated by chloride and lacks a narrow in vitro substrate specificity (3). ACE is a widely distributed peptidase, predominantly expressed as a membrane-bound ectoenzyme in vascular endothelial cells and also in several other cell types including absorptive epithelial cells, neuroepithelial cells, and male germinal cells (4-6).

Inhibition of ACE is a widely used approach in the treatment of hypertension. The first available competitive inhibitors of ACE were the naturally occurring peptides in snake venom. Clinical studies using the nonapeptide teprotide, the most efficient of these snake venom peptides in vitro, demonstrated the potential of ACE inhibitors as antihypertensive drugs (7). Highly potent inhibitors of ACE which can be taken orally have subsequently been developed. The first of these, captopril, was designed with the help of a theoretical model of the active site of ACE, which was based on its presumed similarity to the known active site of carboxypeptidase A and also with reference to the C-terminal sequences of the venom peptides which compete with substrates (8). In this model, captopril coordinates strongly the zinc atom in the active site of ACE by its sulffhydryl group, which is coupled to the dipeptide Ala-Pro responsible for the specific side chain interactions of this inhibitor with ACE (8). Numerous other inhibitors have subsequently been synthesized using the same model but differing in the nature of their zinc-binding ligands and other interaction groups. Enalaprilat and lisinopril, for example, contain a carboxylate group for coordinating the zinc atom and are analogous to Phe-Ala-Pro and Phe-Lys-Pro, respectively (9). The effectiveness of these inhibitors in the treatment of hypertension and congestive heart failure has been demonstrated in numerous clinical studies (10). The availability of high affinity ACE inhibitors has also provided powerful tools for studying the structure, function, and tissular distribution of this enzyme.

Several studies have been undertaken to examine the molecular mechanisms of ACE-inhibitor interactions by enzymatic kinetic (11) and radiolabeled ligand binding (12-14) approaches. All these studies suggested the presence of a single class of high affinity binding site in ACE, with a stoichiometry of one, a result in agreement with the previous observation of one zinc atom per ACE molecule (15, 16). However, recently, the primary structure of human endothelial ACE (17) and that of mouse kidney ACE (18) have been determined by cDNA cloning, disclosing the presence of two large homologous domains. Each domain contains the consensus sequence of zinc-metallopeptidases His-Glu-X-X-His and therefore a putative catalytic center. By site-directed mutagenesis, we have demonstrated that both domains (called here the N and C domains) 1) bear an independently functional active site, 2) possess a zinc-dependent dipeptidyl carboxypeptidase activity, and 3) are sensitive to competitive ACE inhibitors (19). This study also established that the two
Inhibitor Binding Sites of Angiotensin I-converting Enzyme

Enzymes—Wild type and mutated ACE were produced in stable transfectant Chinese hamster ovary cells, as previously described (19, 20). The wild-type recombinant ACE is enzymatically indistinguishable from human kidney ACE (20). The two truncated mutants (N and C fragments) contain either the N or C domain, the residues 738–1277 or 5–571 of ACE being deleted, respectively (the numbering of ACE residues is according to Sussel et al. (17)) (Fig. 1). The two full-length mutants (ACEK931, ACEK381) contain only one functional domain, the two zinc-binding histidines of either the N or C domain being substituted by lysine residues (Fig. 1). The study was carried out using purified enzymes except in the case of the truncated mutant, N fragment, where the dialyzed serum-free medium of the transfected cells was used (19). The purified wild-type recombinant ACE and the full-length mutants have an apparent molecular mass of 170 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19, 20). The molecular mass of the N and C fragments is 135 and 100 kDa, respectively (19).

Inhibitors—Trandolaprilat or RU44 405 is the diacid form of the orally active ACE inhibitor trandolapril or RU44 570 (21). Trandolaprilat contains a carboxylate group as zinc-binding ligand and was derived from enalaprilat by substituting the proline ring by a saturated isoleucine residue (21). [3H]Trandolaprilat with a specific activity of 66 Ci/mmol was prepared. The purity of the radiolabeled compound was greater than 90% when analyzed by thin silica gel chromatography using chloroform/methanol/acetic acid (90:5:5) as solvent and by high-performance liquid chromatography using an analytical column of Hypersil ODS 5 μm (25 × 0.46 cm, inside diameter) (Biochrom, France) and aceonitrile/10 mM potassium phosphate buffer, pH 3.8, containing 10 μM ZnSO4. Both trandolaprilat and [3H]trandolaprilat were kindly provided by Dr. Hamon (Roussel-Uclaf, France). Captopril was a gift from the Bristol-Myers-Squibb Institute, and enalaprilat and lisinopril were obtained from Merck Sharp and Dohme Research Laboratories.

Enzyme Inhibition Assays—Enzyme activities were measured using His-Glu-His-Leu (Biochem, Switzerland) as substrate as previously described (17). The concentration of the full-length or mutated enzymes was determined by direct radioimmunoassay and also by calculation from enzyme activity measurements using the previously determined kinetic parameters for each enzyme (19, 20). The concentration of the truncated mutants was calculated from enzyme activity measurements by comparison with the full-length mutants, as the catalytic properties of the truncated mutants are indistinguishable from those of the corresponding full-length mutants (19).

The potency of each inhibitor toward each enzyme was determined by establishing dose-dependent inhibition curves at equilibrium. As all the compounds tested are competitive tight binding inhibitors of ACE, enzyme and inhibitor were incubated together for 1 h at 37 °C before the addition of substrate to allow a steady-state equilibrium between the enzyme and inhibitor to be established. The incubations were conducted in duplicate or in triplicate with various concentrations of inhibitor in 235 μl of 100 mM potassium phosphate buffer, pH 8.3, containing 10 μM ZnSO4. At 20 or 300 mM NaCl, 1 mg/ml bovine serum albumin, and 1 × 10−10 or 0.1 × 10−12 M of enzyme. A 1-h incubation was found to be sufficient to reach equilibrium for each enzyme and inhibitor tested, at all inhibitor concentrations. Enzyme activities in the absence of inhibitor did not change during consecutive incubation of at least 5 a indicating that the enzyme was stable during the incubation.

To determine the residual free enzyme concentration, the enzymatic reaction was then started by adding 25 μl of the substrate solution to give a final concentration of 250 μM (corresponding to 0.12–0.15 Kd). Initial velocities were measured during the hydrolysis of the first 5% of substrate. Under these conditions, the enzyme concentration in the assay was too low (<0.2 Kd) to induced significant free inhibitor depletion, and the influence of substrate and dilution can be considered negligible. With the concentration of inhibitor needed to produce 50% inhibition, can be thus be considered to correspond to Kd, the inhibition constant, in the case of competitive inhibition (22).

Trandolaprilat Binding Assay—The dissociation constant(s) Ks and stoichiometry of [3H]trandolaprilat binding for each enzyme (i.e. the binding parameters at equilibrium) were determined by equilibrium dialysis. Dialysis tubing (Polylabo, France) containing enzyme in 0.5 ml of buffer A (100 mM Hepes, pH 7.5, 10 mM ZnSO4, 1 mg/ml bovine serum albumin) with or without NaCl was immersed in tube prefilled with 30 ml of the same buffer containing [3H]trandolaprilat. Sodium chloride concentrations varying from 0 to 300 mM were used to examine the effects of chloride on the binding parameters of each enzyme. All values were measured at one enzyme concentration and at 10 different inhibitor concentrations chosen to produce mole fractions of free enzyme between 10 and 90%. After 20 h at 4 °C under continuous shaking, aliquots of 0.2 ml were taken from both the dialysis bag and the surrounding bath and counted in scintillation vials after addition of PICO-FLUOR 15 scintillation fluid (Packard). All measurements were performed in duplicate. Enzyme activities in the dialysis tubing after 20-h incubation at 4 °C in the absence of inhibitor were measured to ensure the absence of enzyme degradation. The nonspecific binding estimated in the presence of 1 μM unlabeled trandolaprilat remained below 1% of the binding in the absence of unlabeled inhibitor.

The binding parameters were determined by the computer method described by Claire et al. (23), which comprises several iterations to obtain the best-fitted curves for interaction of a ligand with one or two specific and nonspecific binding sites. The standard deviations for the binding parameters were estimated from the experimental data by the computer was less than 15% of the mean values. The results were represented according to Scatchard (24).

Determination of Association and Dissociation Kinetic Constants—Association kinetic experiments were performed in triplicate by incubating 5 × 10−10 M [3H]trandolaprilat (50 mCi/mmol) with or without NaCl, at 37 °C before the addition of substrate to allow a steady-state equilibrium between the enzyme and inhibitor to be established. The enzyme-inhibitor complex was separated from the free inhibitor by addition of 1 ml of cold ethanol (−20 °C), followed by centrifugation. The pellet was resuspended in buffer A and counted. Approximately 80% of the enzyme-inhibitor complex was precipitated, while less than 2% of the free inhibitor was precipitated in the absence of enzyme. The nonspecific binding estimated in the presence of 1 μM of unlabeled

Fig. 1. Diagram of ACE constuctions. The wild-type ACE (ACE) contains the signal peptide (left black box), the two large homologous domains (shaded boxes), and the transmembrane domain (right black box) near the C terminus. The sequence containing the two zinc-binding histidines is shown under each domain. The truncated mutant, N fragment, contains the signal peptide and the N domain. The truncated mutant, C fragment, contains the signal peptide, the C domain, and the C-terminal region including the transmembrane and intracellular domains. For the two full-length mutants, the two zinc-binding histidines have been substituted by lysines. Unchanged amino acids are represented by dashes. The position of the mutated histidines is indicated in the mutant name.

Materials and Methods

Fig. 2. Sensitivity of ACE activity to trandolaprilat. The activity of ACE was measured during the hydrolysis of the first 5% of substrate. Each enzymatic reaction was carried out in triplicate in the presence of 20 and 300 mM NaCl, 1 mg/ml bovine serum albumin, and 1 × 10−10 or 0.1 × 10−12 M of enzyme. A 1-h incubation was found to be sufficient to reach equilibrium for each enzyme and inhibitor tested, at all inhibitor concentrations. Enzyme activities in the absence of inhibitor did not change during consecutive incubation of at least 5 a indicating that the enzyme was stable during the incubation.

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The determination of the inhibitor-binding stoichiometry, performed with three separate preparations of the enzyme, showed that the wild-type recombinant enzyme bound 2.0 ± 0.3 \( (n = 6) \) mol of trandolaprilat per mol of enzyme.

Parallel measurements were conducted on the two truncated mutants (N and C fragments) containing only the N or C domain. Only one class of high affinity binding site was detected in each molecule (Fig. 2). The \( K_d \) values determined in the presence of 100 mM NaCl were 3.1 ± 0.2 \( \times 10^{-10} \) and 1.1 ± 0.1 \( \times 10^{-10} \) M for the N and C fragments, respectively, which are consistent with the \( K_d \) values of the two binding sites of the wild-type enzyme determined under the same conditions (Table I). These results suggest that the affinities of the N and C domains in the truncated mutants for \([3H]\)trandolaprilat are identical to those of the N and C domains in the intact molecule.

Removal of the zinc atom from ACE has been shown to decrease its affinity for enalaprilat by 20,000-fold (12). Alteration of the zinc-binding site of either the N or C domain should result in a dramatic reduction of the affinity of the corresponding domain for \([3H]\)trandolaprilat. Indeed, the two full-length mutants ACEK361.365, ACEK389,393, in which the zinc-binding site of one of the N or C domain was altered by point mutation, contain only one high affinity binding site. The \( K_d \) values in the truncated ACE are identical to those of the corresponding domain in the intact ACE molecule (Table I). Thus ACE possesses two different high affinity binding sites for \([3H]\)trandolaprilat; one is located on each of the two homologous domains.

Effects of Chloride on the Kinetic and Equilibrium Constants of \([3H]\)Trandolaprilat Binding—Previous inhibition studies have shown that chloride influences inhibitor binding to ACE, especially the dissociation rate of the enzyme-inhibitor complex (14, 26). This chloride effect required re-examination because the interpretations of all previous results assumed the presence of one binding site in ACE. Moreover, some results such as the biphasic pattern of the dissociation of the complex at high chloride concentrations (12, 14) may result from the presence of two binding sites. It was thus important to investigate the effects of chloride on inhibitor binding to each domain.

The association of the enzyme-inhibitor complexes follows a monophasic process for each domain. Fig. 3 shows the association curves obtained at 300 mM NaCl. The kinetic constants were determined from the slope of the pseudo-first order plot (Fig. 3). The association rate was only slightly influenced by chloride for both domains (Table II). Identical \( k_{1+} \) values were obtained for the N and C domains in the absence of chloride. The \( K_d \) value of the N domain was not altered and that of the C domain decreased only 2-fold when the chloride concentration was increased from 0 to 300 mM (Table II). The fact that the \( k_{1+} \) values for the two domains are similar explains why the association of trandolaprilat to ACE follows a monophasic kinetic in the previous study (14) and also in the present study (data not shown).

The dissociation of the enzyme-inhibitor complex is also monophasic for each of the two domains. The dissociation curves obtained at 300 mM NaCl are shown in Fig. 4. In contrast with the association rates, chloride has a dramatic effect on the dissociation rate of the enzyme-inhibitor complex for each of the two domains (Table II). The \( k_{1-} \) value of the N domain fell about 30-fold when the chloride concentration was increased from 0 to 20 mM, with no further decrease at higher chloride concentrations. The effect of chloride ap-
The calculated dissociation constants obtained by dividing the dissociation rate constants by the association rate constants are in good agreement with the $K_D$ values determined by equilibrium dialysis (Table III). The affinities of trandolaprilat for the two domains are very different (22-fold) only at high ($\geq$100 mM) or low chloride concentrations. Here again, the $K_D$ values of the N and C domains in the wild-type ACE are similar to those of the corresponding intact domains in the truncated or full-length mutants (data not shown), as observed in the presence of 100 mM NaCl (Table I).

At high chloride concentrations, the dissociation rate of the enzyme-inhibitor complex is greatly enhanced by chloride, which essentially slows the dissociation rate of the enzyme-inhibitor complex.

The calculated dissociation constants obtained by dividing the dissociation rate constants by the association rate constants are in good agreement with the $K_D$ values determined by equilibrium dialysis (Table III). The affinities of trandolaprilat for the two domains are very different (22-fold) only at high ($\geq$100 mM) or low (55 mM) chloride concentrations. Thus, the binding assay is only able to reveal the presence of two different binding sites in the wild-type ACE at these high or low chloride concentrations. Here again, the $K_D$ values of the N domain and 65% on the C domain, with the $k_{-1}$ values of the two domains determined in the same experimental conditions with the two truncated mutants.

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At high chloride concentrations, the dissociation rate of the C domain was lower than that of the N domain, the dissociation of the wild-type enzyme-[$^3$H]trandolaprilat com-

**TABLE I**

<table>
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<tr>
<th>Enzyme</th>
<th>[Enzyme]</th>
<th>$K_{DN}$</th>
<th>$B_N$</th>
<th>$K_{DC}$</th>
<th>$B_C$</th>
<th>[H]Trandolapril bound/mol of enzyme</th>
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<tr>
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<td>$1.5$</td>
<td>$0.86$</td>
<td>$1.04$</td>
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<td>C fragment</td>
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<td>$3.4$</td>
<td>$1.06$</td>
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<td>$0.85$</td>
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<tr>
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<td>$0.88$</td>
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<tr>
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<td>$1.1$</td>
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<td>$0.88$</td>
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**TABLE II**

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<td>$s^{-1}$</td>
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<tr>
<td>5</td>
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<tr>
<td>20</td>
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<tr>
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<tr>
<td>300</td>
<td>8.6</td>
<td>24</td>
</tr>
</tbody>
</table>

**FIG. 3.** Association of [H]trandolaprilat to the N fragment (■) and the C fragment (△). The kinetics of specific binding of [H]trandolaprilat ($5 \times 10^{-5}$ M) to the enzyme ($5 \times 10^{-5}$ M) were determined after incubation in 100 mM Hepes, pH 7.5, at 4 °C, and in the presence of 300 mM NaCl for the indicated times. Inset, a replot of the data according to equation I (see "Materials and Methods"). $B_N$ and $B_C$ are, respectively, the concentration of the enzyme-inhibitor complex at equilibrium (60 min) and that at time t. $k_{+1}$ represents the theoretical curve of the inhibitor dissociation from the wild-type enzyme, assuming that 35% of the bound inhibitor is on the N domain and 65% on the C domain, with the $k_{-1}$ values of the two domains determined in the same experimental conditions with the two truncated mutants.

**FIG. 4.** Dissociation of [H]trandolaprilat from the wild-type enzyme (○), the N fragment (■), and the C fragment (△). Enzyme ($5 \times 10^{-10}$ M) was incubated with [H]trandolaprilat ($1 \times 10^{-10}$ M) for 4 h in 100 mM Hepes, pH 7.5, at 4 °C, and in the presence of 300 mM NaCl. The dissociation kinetic experiment was started by adding $10^{-6}$ M of unlabeled trandolaprilat. The ratio of the bound [H]trandolaprilat at time t ($E-C$) to the bound inhibitor is on the N domain and 65% on the C domain, with the $k_{-1}$ values of the two domains determined in the same experimental conditions with the two truncated mutants.
The dissociation curve obtained in the presence of 300 mM NaCl is shown in Fig. 4. The first rapid dissociation step, involving about 35% of the bound inhibitor, corresponds mainly to the dissociation of the N domain. The second slow phase corresponds to the dissociation of the C domain. The experimental data are in agreement with the calculated values of the bound inhibitor on the N and C domains (35 and 65%, respectively) according to the $K_d$ values of the N and C domains under the conditions of this experiment. Furthermore, the experimental data are also in accordance with the theoretical dissociation curve (Fig. 4) assuming that 35 and 65% of the bound inhibitor were on the N and C domains, respectively, with $k_1$ values of $2.4 \times 10^{-4}$ and $1.4 \times 10^{-5}$ s$^{-1}$, as determined using the truncated mutants.

**Table III**

<table>
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<tr>
<th>[Cl$]^{-}$</th>
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<th>$K_d = k_1/k_2$</th>
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<td>C fragment</td>
<td>N fragment</td>
</tr>
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<tr>
<td>300</td>
<td>2.9</td>
<td>0.32</td>
<td>2.8</td>
</tr>
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</table>

* Values were determined by equilibrium dialysis in 100 mM Heps, pH 7.5, at 4 °C as described under “Materials and Methods” and are the average of two or three independent determinations which varied by less than 15%.

Values were derived from the rate constants presented in Table II.

The inhibitory potency of these inhibitors was determined for the wild-type recombinant ACE and the truncated or full-length mutants by enzymatic assay using Hip-His-Leu as substrate. The $K_i$ values of trandolaprilat for the N and C domains ($3.1 \times 10^{-10}$ and $0.29 \times 10^{-10}$ M) (Table IV), measured by enzyme inhibition assay at 300 mM NaCl in potassium phosphate buffer, pH 8.3, at 37 °C, were consistent with the $K_d$ values determined by $[^3]$Htrandolaprilat binding assay with Heps buffer, pH 7.5, at 4 °C ($2.9 \times 10^{-10}$ and $0.32 \times 10^{-10}$ M) (Table III). Similar result was obtained at 20 mM NaCl (Tables III and IV). For all the inhibitors tested, the $K_i$ values of the truncated mutants were identical to those of the corresponding full-length mutants (data not shown).

The results, shown in Table IV, indicate that captopril, enalaprilat, and lisinopril are all potent inhibitors for both the N and C domains with $K_i$ values within the nanomolar range. Lisinopril, trandolaprilat, enalaprilat, but not captopril inhibit preferentially the C domain more than the N domain, at least at high chloride concentration, as the $K_i$ values of these inhibitors were, respectively, about 18-10, 4-fold lower for the C domain than for the N domain at 300 mM NaCl. However, this difference in affinities to the two domains was also observed for captopril and enalaprilat at low chloride concentration.

**Table IV**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>[Cl$]^{-}$</th>
<th>$K_i$</th>
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<tbody>
<tr>
<td></td>
<td>mM</td>
<td>$\times 10^5$, M</td>
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<tr>
<td>Wild-type</td>
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<td>13</td>
</tr>
<tr>
<td>N fragment</td>
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<td>8.9</td>
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<td>C fragment</td>
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DISCUSSION

We have recently demonstrated by site-directed mutagenesis that ACE possesses two independent active sites and that their activities are inhibited by captopril and enalaprilat (19). This finding was unexpected since the results of all the previous studies of the molecular interaction of ACE with competitive inhibitors suggested the presence of a single high affinity binding site in the ACE molecule (11-14). The present study establishes that both domains of ACE contain a high affinity binding site for these inhibitors and that these two domains display different properties and different chloride sensitivities for inhibitor binding.

The presence of two different high affinity binding sites in ACE is supported by several lines of evidence: 1) $[^3]$Htrandolaprilat binding to the wild-type ACE reveals the presence of two classes of high affinity binding site and a stoichiometry of two molecules of inhibitor bound per enzyme molecule; 2) the two truncated mutants, lacking either the N or C domain, each contain only one class of binding site and present a stoichiometry of one; 3) the binding affinities of the N and C domains for $[^3]$Htrandolaprilat in the truncated mutants are consistent with those of the N and C domains in the wild-type enzyme, suggesting that each domain independently binds an inhibitor molecule; 4) alteration of the zinc-binding site in either the N or C domain destroys the corresponding inhibitor binding site. In addition, we demonstrate that the catalytic activities of both the N and C domains are inhibited by captopril, enalaprilat, lisinopril, and trandolaprilat with $K_i$ values within the nanomolar range.
Therefore, the present observations differ from previous findings and, in particular, from a previous study of our group on \(^{3}H\)trandolaprilat binding to human kidney ACE. This report suggested a single binding site in ACE with a \(K_D\) value of \(4.4 \times 10^{-10}\) M, when determined at 300 mM NaCl and at room temperature after a 24-h incubation time (14). In the present study, two binding sites with \(K_D\) values of \(2.9 \times 10^{-10}\) and \(0.32 \times 10^{-10}\) M were observed. The dissociation constants of these two studies may be due to degradation of the enzyme in the conditions of the previous experiment. Indeed, we observed a 30 to 70% decrease in enzyme activities of human kidney ACE and recombinant ACE when incubated for 24 h at room temperature (data not shown). No degradation was observed at 4 °C. In addition, the detection of two different binding sites in ACE requires an appropriate range of inhibitor concentrations to produce mole fractions of free enzyme between 10 and 90%. If the inhibitor concentration range is too narrow, only one binding site will be identified.

Given the low \(K_D\) values of the N and C active sites for captopril, enalaprilat, and lisinopril, previous studies using the corresponding radiolabeled inhibitors should have been able to detect the presence of two high affinity binding sites in ACE, whereas they detected only one (12, 13). Several explanations for this discrepancy can be proposed. First, the two sites present similar affinities for the inhibitors under some experimental conditions, leading to the detection of a single class of binding site in the wild-type enzyme, as was the case for \(^{3}H\)trandolaprilat at 20 mM NaCl (Table III). Another possibility is that even if the affinities of the two binding sites are very different, as in the case of trandolaprilat at high (\(\geq 100\) mM) or low (\(\leq 5\) mM) chloride concentrations (Table III), a single class of binding site will be detected if the various concentrations of inhibitor are not appropriately chosen, as discussed above. Our results indicate that the difference in affinities between the two domains is greater at high chloride concentration (300 mM) for trandolaprilat, enalaprilat, and lisinopril, whereas for captopril, the difference is larger at low chloride concentration (20 mM) (Table IV).

Finally, the stoichiometry of 1 mol of inhibitor bound per mol of enzyme found in the previous studies (11–14) may be due to inaccurate determination of the enzyme concentration, partial occupation of the two binding sites because of a too narrow range of inhibitor concentrations, or to partial degradation of enzyme during incubation, as discussed above. A recent study on the binding of lisinopril (27) detected two binding sites in human kidney and rabbit lung ACE, and a single binding site in human and rabbit testis ACE which contain only the C domain (28–30). However, only one of these two sites in somatic ACE appeared to be a high affinity binding site, and the affinity of the second site was not determined.

The choice of the appropriate experimental conditions is therefore crucial in determining the stoichiometry and affinity of inhibitor binding for ACE by the equilibrium dialysis method. In the present study, this was greatly facilitated by separate determination of the affinities of the two domains using ACE mutants, each containing only one intact domain. The availability of these mutants is also essential to determine the inhibitory potency of competitive inhibitors for each of the two domains of ACE by steady-state kinetic method. The results presented in Table IV show that the \(K_I\) values of four inhibitors tested for the wild-type ACE are very similar to those for the C domain at 300 mM NaCl using Hip-His-Leu as substrate, because under these conditions the catalytic activity of the C domain is greater than that of the N domain and accounts for the major part of ACE activity, as previously observed (19). Since the previous steady-state inhibition studies investigating the potency of these inhibitors for ACE (11–14, 27) were generally performed under conditions where the activity of the C domain is greater than that of the N domain, they reported mainly the potency of these inhibitors for the C domain, whereas their potency for the N domain could not be assessed using the intact ACE molecule. However, the potencies of ACE inhibitors for the N domain, which were determined using ACE mutants containing only the intact N domain, can be assumed to be equivalent to those of the N domain in the wild-type ACE only if the two domains of ACE function independently. This is most likely the case, since the binding of trandolaprilat to either domain does not seem to affect the binding to the other domain as shown by the fact that the binding affinities of the two domains in the truncated or full-length mutants are essentially the same as those of the two domains in the wild-type enzyme (Table I). These findings are in good agreement with our previous results showing that the wild-type enzyme activity was always equal to the sum of the activities of the two domains assessed separately (19), and further support the concept that the two active sites of ACE function independently.

The present study also shows that the two high affinity binding sites in ACE display different binding properties, especially different chloride sensitivities. The binding of \(^{3}H\)trandolaprilat to both domains was greatly enhanced by chloride, as the \(K_D\) values were decreased by about 30- and 500-fold for the N and C domains, respectively, by the presence of 300 mM NaCl. The major effect of chloride is to decrease the dissociation rate of the enzyme-inhibitor complex for both domains, since the association rates of the two domains are only slightly influenced by chloride (Table II). The main difference between the two domains is that the stabilizing effect of chloride is much greater for the C domain (about 1100-fold decrease in its \(k_{-1}\) value when the chloride concentration increases from 0 to 300 mM) than for the N domain (about 30-fold). Moreover, this effect of chloride reaches a plateau at a much lower concentration for the N domain (20

| TABLE V Comparison of the inhibitor binding properties of the N and C Domains |
|-----------------------------------------------|-----------------|-----------------|------------------|
| | \(K_D\) | \(k_{+1}\) | \(k_{-1}\) | \(K_I\) | 20 mM | 300 mM |
| N domain | \(2.9 \times 10^{-10}\) M | \(8.6 \times 10^{-10}\) M | \(24 \times 10^{-10}\) M | 3.1 | 28 | 28 |
| C domain | 0.32 | 4.3 | 1.4 | 0.29 | 60 | 522 |

\* Parameters were determined in the presence of 300 mM NaCl.
\* Values were measured using Hip-His-Leu as substrate.
\* The \(K_D\) value of trandolaprilat determined without chloride addition divided by that at 20 or 300 mM NaCl.
\* T, trandolaprilat; L, lisinopril; E, enalaprilat; C, captopril. Relative inhibitory potency was determined at 20 and 300 mM NaCl. The same order was obtained.
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mm) than for the C domain (≥300 mM). Chloride activation of substrate hydrolysis by the N and C domains showed a similar phenomenon (19). A consequence of these differences between the two domains is that the relative binding affinity of trandolapril toward the two domains depends on the chloride concentration: its binding affinity for the C domain becomes higher than that for the N domain at chloride concentrations higher than 20 mM, because of the greater chloride effect on the C domain (Table III). Moreover, the difference in the binding affinities of the two domains is mainly due to the differences in their dissociation rates, since the association rates of the two domains are very similar (Table II). Another consequence of the different effects of chloride on the dissociation rates of the two domains is that the dissociation kinetic of the wild-type enzyme appears to be biphasic at high chloride concentrations (Fig. 4).

The different chloride sensitivities of the two domains for trandolapril binding can probably be extended to other inhibitors such as captopril, enalaprilat, and lisinopril, since the $K_d$ values of these inhibitors for the N and C domains are also differently influenced by chloride (Table IV). Moreover, the association kinetics of these inhibitors to the native ACE were found to be monophasic (12, 13), suggesting that the two domains of ACE display similar association kinetics. In the previous dissociation kinetic studies, where it was assumed that ACE contained a single active site, it was observed that enalaprilat, lisinopril, and trandolaprilat had biphasic dissociation kinetics (12, 14). These observations can now be interpreted as reflecting the presence of two binding sites with different dissociation kinetics. The previous studies also showed that chloride enhanced inhibitor binding to ACE (12–14, 26) by decreasing the dissociation rate of the ACE-inhibitor complex (14, 26). The present results indicate that this property is conserved for the two active sites of ACE, suggesting that the mechanism of enzyme-chloride interaction for the two active sites is probably similar.

The observed difference in chloride sensitivities for inhibitor binding between the two domains raises the question whether the two active sites of ACE are equally inhibited under physiological conditions (about 100 mM chloride in plasma) after drug intake. The present in vitro study shows that the $K_d$ values of trandolapril for the two domains are within the $10^{-16}$ to $10^{-10}$ range at 100 mM NaCl, and suggests that trandolapril binds both active sites after drug intake. Similarly, captopril, enalaprilat, and lisinopril are all high affinity binding inhibitors for the two domains at high chloride concentrations (≥200 mM) (Table IV), indicating that both active sites should be readily inhibited in vivo by these inhibitors for hydrolysis of angiotensin I.

Interestingly, several structural differences between the two active sites appear to be independent of chloride binding. The relative potency of the four inhibitors tested is different for the two domains (trandolapril > lisinopril > enalaprilat > captopril for the C domain and trandolaprilat > captopril > enalaprilat > lisinopril for the N domain), at 300 mM as well as 20 mM NaCl. The fact that the replacement of Ala in enalaprilat by Lys in lisinopril in the P$^1$ position results in enhanced binding for the C domain, but decreased binding for the N domain suggests structural difference of the two domains at their S$^1$ substrate binding subsite. In addition, captopril is more potent than enalaprilat for inhibiting the N domain, but less potent than enalaprilat for the C domain. Captopril and enalaprilat differ in that enalaprilat contains an additional residue (Phe) in the P$^1$ position and a weaker zinc-coordinating ligand (a carboxylate group, captopril contains a sulfhydryl group). The present results suggest that the phenylalanine residue in the P$^1$ position of enalaprilat may interact better with the C domain than with the N domain or that the N domain may prefer a strong zinc-coordinating inhibitor than the C domain. In our previous study, we suggested that the two active sites of ACE may have different functions since they display different catalytic properties (19). The presence of structural differences between the two active sites revealed by interaction with different inhibitors provides additional evidence to support this hypothesis. It has been recently reported that endothelial ACE but not testicular ACE efficiently hydrolyzes the N-terminal tripeptide of LH-RH, an in vitro ACE substrate, suggesting that the N domain may be specifically involved in this endoproteolytic cleavage (27). Using ACE mutants, we found that LH-RH was indeed better hydrolyzed by the N domain than by the C domain. The N domain may therefore have a specific in vitro function. Further experiments with a large number of ACE inhibitors may help to elucidate the structural and functional differences between the two active sites and thereby help to design specific inhibitors for each active site which will be useful to investigate the physiological functions of each of the two active sites of ACE.

In conclusion, the present study demonstrates that ACE contains two different high affinity binding sites for competitive inhibitors. The difference in the potencies of an inhibitor for the two domains depends both on the chloride concentration and on the structure of the inhibitor. The difference in the characteristics of trandolapril binding between the two domains at 300 mM NaCl and the other marked differences in the inhibitor binding properties summarized in Table V provide strong evidence for the existence of structural differences between the two active sites of ACE. The availability of ACE mutants, each containing only one intact domain, provides powerful tools for studying the structural and functional differences of the two active sites and thereby to understand the catalytic mechanism of ACE at molecular level.

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