Angiotensin (Ang) I-converting enzyme (ACE, EC 3.4.15.1) is a Zn\(^{2+}\) metalloprotease with two homologous catalytic domains. Both the N- and C-terminal domains are peptidyl dipeptidases. Hydrolysis by ACE of its decapptide substrate Ang I is increased by Cl\(^{-}\), but the molecular mechanism of this regulation is unclear. A search for single substitutions to Glu among all conserved basic residues (Lys/Arg) in human ACE C-domain identified R1098Q as the sole mutant that lacked Cl\(^{-}\) dependence. Cl\(^{-}\) dependence is also lost when the equivalent Arg in the N-domain, Arg\(^{1098}\), is substituted with Gln. The Arg\(^{1098}\) to Lys substitution reduced Cl\(^{-}\) binding affinity by \(-100\)-fold. In the absence of Cl\(^{-}\), substrate binding affinity (Kd) of and catalytic efficiency (kcat/Km) for Ang I hydrolysis are increased 6.9- and 32-fold, respectively, by the Arg\(^{1098}\) to Gln substitution, and are similar (<2-fold difference) to the respective wild-type C-domain catalytic constants in the presence of optimal [Cl\(^{-}\)]. The Arg\(^{1098}\) to Gln substitution also eliminates Cl\(^{-}\) dependence for hydrolysis of tetrapeptide substrates, but activity toward these substrates is similar to that of the wild-type C-domain in the absence of Cl\(^{-}\). These findings indicate that: 1) Arg\(^{1098}\) is a critical residue of the C-domain Cl\(^{-}\)-binding site and 2) a basic side chain is necessary for Cl\(^{-}\) dependence. For tetrapeptide substrates, the inability of R1098Q to recreate the high affinity state generated by the C\(^{-}\)-C-domain interaction suggests that substrate interactions with the enzyme-bound Cl\(^{-}\) are much more important for the hydrolysis of short substrates than for Ang I. Since Cl\(^{-}\) concentrations are saturating under physiological conditions and Arg\(^{1098}\) is not critical for Ang I hydrolysis, we speculate that the evolutionary pressure for the maintenance of the Cl\(^{-}\)-binding site is its ability to allow cleavage of short cognate peptide substrates at high catalytic efficiencies.

Angiotensin I (Ang I)\(^{1}\)-converting enzyme (ACE, EC 3.4.15.1) belongs to the gluzincin family (clan MA) of metalloproteases and is a peptidyl dipeptidase with broad substrate specificity (1). Peptide hydrolysis is activated by monovalent anions such as Cl\(^{-}\); this feature is unique among metalloproteases (2). The somatic form of human ACE has two homologous catalytic domains. These N- and C-domains most likely are the result of an ancient gene duplication event that occurred during vertebrate evolution (3, 4). Invertebrate ACE has a single, Cl\(^{-}\)-sensitive catalytic domain (5). The physiological substrates of ACE include Ang I, bradykinin, substance P, and AcSDKP (2); however, there are significant differences between the catalytic domains in catalytic efficiencies for the hydrolysis of these substrates (6, 7).

Cl\(^{-}\) dependence of hydrolysis is substrate-specific. For example, the degree of enzyme activation by Cl\(^{-}\) and its apparent dissociation constant (Kd,app) associated with this activation is high for Ang I and low for bradykinin (8, 9). To define the molecular determinants in the substrate structure responsible for these differences, Riordan and colleagues (8, 9) studied the hydrolysis of a collection of tripeptide substrates with varying C-terminal dipeptide structures. They observed that a P1\(_{-}\)- or P2\(_{-}\)-(Arg/Lys)\(^{2}\) in the tripeptide substrate was necessary and sufficient for high affinity Cl\(^{-}\) binding. Bradykinin, like these substrates, but unlike Ang I, contains a basic residue in its C-terminal dipeptide. Also, hydrolytic activity of ACE for tripeptide substrates with a P1\(_{-}\)- or P2\(_{-}\)-(Arg/Lys)\(^{2}\) is less affected by Cl\(^{-}\) than for those that lack a basic P1\(_{-}\) or P2\(_{-}\) residue. Because chemical modification of ACE that leads to methylation of Lys residues has selective effects on hydrolytic activity for furanacryloyl-FGG-COO\(^{-}\) (inactivated by >99%) and furanacryloyl-FFR-COO\(^{-}\) (activity reduced by \(-50\%\)) it was proposed that a critical Lys is part of the Cl\(^{-}\)-binding site of ACE (10).

The study by Shapiro and Riordan (10) had provided only circumstantial evidence that a critical Lys was involved in Cl\(^{-}\)-binding, and in principle it is best to consider both types of basic residues as possibilities. To identify the Cl\(^{-}\)-binding residue(s) in ACE we made single substitutions with Glu of all conserved arginines or lysines in the human ACE C-domain. Here we show that the primary site of Cl\(^{-}\) binding in the C-domain is Arg\(^{1098}\) irrespective of whether the substrate is the decapptide Ang I or a short peptide with or without a basic residue at its C terminus. We show for the first time that anion-substrate interactions are critical for the hydrolysis of short substrates suggesting that the substrate itself forms an important part of the Cl\(^{-}\)-binding site. Finally, kinetic studies with position 1098 C-domain mutants provide insights into the molecular mechanism of ACE activation by Cl\(^{-}\).
EXPERIMENTAL PROCEDURES

Construction of Human ACE C- and N-domain Gene Constructs—Homology between exons 4–11 and exons 17–24 in the human ACE gene was established by Hubert et al. (4). Nucleotide sequence similarities between exons 1 and 14, 2 and 15, 3 and 16, and 12 and 25 are 53, 41, 59, and 55%, respectively. Shaded areas represent sequences that are strictly conserved between human N- and C-domains. A filled circle indicates Arg and Lys residues conserved in all known ACE primary structures.

Human ACE C- and N-domain gene construct design was based on the region of gene duplication shown in Fig. 1. The gene constructs were made by strategies reported previously (11, 12), chemically synthesized and cloned into the shuttle expression vector pcDNA3 (Invitrogen). The C-domain gene construct encodes amino acids 29 to 1 (signal peptide), 1 to 4, and 611 to 1201 of human somatic ACE (14) with an 8-residue FLAG epitope recognized by a commercially available antibody (M2, Sigma) at the C terminus. The N-domain gene construct encodes amino acids 29 to 1 (signal peptide) and 1–609 of human somatic ACE with an 8-residue FLAG epitope at the C terminus. Mutations were constructed in the synthetic gene by the technique of restriction fragment replacement or site-directed mutagenesis (QuikChange Site Directed Mutagenesis Kit, Stratagene), and mutants were fully sequenced (13) to check for the presence of intended mutations and the absence of unintended mutations.

Transfection of COS-7 Cells—COS-7 cells (ATCC) were cultured under an atmosphere of 5% CO₂ at 37 °C and transfected with plasmid DNA using the Gene Pulser system (Bio-Rad). 12 h after transfection, cells were washed and further cultured with serum-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) for 48–72 h. C- and N-domains and their mutants released into conditioned media were collected and used as the starting point of the purification. The following mutants were made: K919Q-, R924Q-, R942Q-, R944Q-, K1030Q-, K1044Q-, K1065Q-, K1077Q-, K1087Q-, R1098Q-, and R1098K-C-domain and R500Q-N-domain.

Purification of Recombinant Human ACE C and N-domains and Their Mutants—An anti-FLAG M2 affinity gel was used to purify re-
The kinetic constants were determined in 50 mM HEPES buffer, pH 7.5, containing varying concentrations of NaCl and 10 μM ZnSO₄ at 37 °C. Kₘ and Vₘₐₓ values were determined by nonlinear regression. Values are mean ± S.E. of three independent determinations.

<table>
<thead>
<tr>
<th>[NaCl] (mM)</th>
<th>C-domain ACE</th>
<th>R1098Q</th>
<th>ΔΔG°</th>
<th>N-domain ACE</th>
<th>R500Q</th>
<th>ΔΔG°</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>420 ± 11</td>
<td>61 ± 6.4</td>
<td>-1.2</td>
<td>180 ± 21</td>
<td>100 ± 3.2</td>
<td>-0.36</td>
</tr>
<tr>
<td>Kₘ --------</td>
<td>8.4 ± 0.0037</td>
<td>39 ± 1.9</td>
<td>0.95</td>
<td>52 ± 4.4</td>
<td>130 ± 2.2</td>
<td>-0.56</td>
</tr>
<tr>
<td>Kₘ/Kₘ</td>
<td>0.02</td>
<td>0.64</td>
<td>2.1</td>
<td>0.29</td>
<td>1.3</td>
<td>-0.92</td>
</tr>
<tr>
<td>20</td>
<td>47 ± 1.5</td>
<td>100 ± 4.8</td>
<td>0.47</td>
<td>23 ± 2</td>
<td>47 ± 1.4</td>
<td>0.44</td>
</tr>
<tr>
<td>Kₘ</td>
<td>52 ± 1.3</td>
<td>50 ± 4.5</td>
<td>0.47</td>
<td>23 ± 2</td>
<td>56 ± 3.3</td>
<td>0.663</td>
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<tr>
<td>Kₘ/Kₘ</td>
<td>1.1</td>
<td>0.5</td>
<td>0.49</td>
<td>2.7</td>
<td>1.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* For Kₘ, Kₘ cat, and Kₘ/Kₘ comparisons, ΔΔG is ΔΔG binding, ΔΔG cat, and ΔΔG cat and ΔΔG cat, respectively. Units for Kₘ, Kₘ cat, and Kₘ/Kₘ are μM, s⁻¹, and s⁻¹ μM⁻¹, respectively.

Chloride-binding Site of ACE C-domain

**Table I**

**Fig. 2.** Effects of NaCl on the Ang II forming activity of C- and N-domains and their R1098Q and R500Q mutants, respectively. Activity assays were carried out in 50 mM HEPES buffer, pH 7.5, containing 0–100 mM NaCl, 10 μM ZnSO₄, and 20 μM Ang I as substrate at 37 °C. Generated Ang II was quantified by HPLC using pure standards. Enzyme activity is expressed as picomoles of Ang II formed per mg of enzyme protein. For these studies, purified enzyme preparations (see “Experimental Procedures”) were first extensively dialyzed against 50 mM HEPES buffer, pH 7.5, containing 10 μM ZnSO₄ at 4 °C to remove Cl⁻. NaCl was then added to some samples to achieve NaCl concentrations of 1 to 100 mM. Effects of NaCl on the activity of wild-type C-domain (●) and its R1098Q mutant (○) are shown in panel A and of wild-type N-domain (●) and its R500Q mutant (○) are shown in panel B. Values are means of duplicate measurements. This figure shows theoretical curves for data generated with C- and N-domains, but not for R1098Q and R500Q.

is the total enzyme concentration. Apparent dissociation constants (K_d,app) for Cl⁻ and other anions were calculated from activation curves generated by NaCl titration (or corresponding sodium salt). The saturation curves were computer fitted by a nonlinear regression analysis of
anions. Activity assays were carried out in 50 mM HEPES buffer, pH 7.5, containing 10 mM ZnSO₄ and 20 mM NaCl (Fig. 2). The N-domain had an ~2-fold higher catalytic efficiency for Ang I hydrolysis than did the C-domain. This difference was mainly due to a 2-fold lower $K_{\text{cat}}$ for Ang I hydrolysis by the N-domain.

**Activation of C-domain Ang II Forming Activity by Halide Ions**—Removal of Cl⁻ by extensive dialysis results in a reversible inactivation of C- and N-domains. Addition of Cl⁻ to reconstituted these catalytic domains and maximal activity was attained at ~20 mM NaCl (Fig. 2). $K_{\text{d,app}}$ for Cl⁻ activation of Ang  

![Image](http://www.jbc.org/)

**RESULTS**

Enzymatic Activity of C- and N-domains—Kinetic constants for Ang I conversion to Ang II by C- and N-domains are summarized in Table I. At saturating Cl⁻ concentration levels (20 mM NaCl), the N-domain had an ~2.5-fold higher catalytic efficiency for Ang I hydrolysis than did the C-domain. This difference was mainly due to a 2-fold lower $K_{\text{cat}}$ for Ang I hydrolysis by the N-domain.

![Image](http://www.jbc.org/)
I hydrolysis by C- and N-domains was 0.5 and 1.1 mM, respectively (Figs. 2 and 3). Addition of 20 mM NaCl increased catalytic efficiencies of C- and N-domains for Ang I hydrolysis 55- and 9.3-fold, respectively (Table I). At a concentration of 20 mM, Cl\(^-\) increased the affinity (1/\(K_m\)) of Ang I binding to the N- and C-domains to an equivalent level (8-fold). The difference between these domains in the Cl\(^-\) effect on catalytic efficiency was mainly due to differences in catalytic rate enhancement (Cl\(^-\) increased \(k_{cat}\) for the C- and N-domains 6.2- and 1.2-fold, respectively).

The C-domain can be fully reactivated not only by Cl\(^-\) but also by F\(^-\), Br\(^-\), and I\(^-\) (Fig. 3). Activation of this ACE domain by CH\(_3\)COO\(^-\) (sodium salt) was <5% of that seen with Cl\(^-\) (data not shown). The apparent dissociation constants, \(K_{d,app}\), between halides for the C-domain differed widely (Fig. 3). An inverted bell-shaped relation was seen between ionic size and \(K_{d,app}\) with Cl\(^-\) and Br\(^-\) being the most tightly bound, and I\(^-\) and F\(^-\) binding with 10–100-fold lower affinity.

Identification of Arg\(^{1098}\) as a Cl\(^-\) Binding Residue in the C-domain—Cl\(^-\)-dependent activation of ACE is conserved during ACE evolution. Hence we reasoned that the critical residue responsible for coordinating this Cl\(^-\) would also be conserved in each ACE domain and between species. In Cl\(^-\)-activated \(\alpha\)-amylases, the negatively charged Cl\(^-\) is primarily coordinated by the positively charged side chain of either Arg or Lys (16, 17). To identify the Arg or Lys residue responsible for Cl\(^-\) dependence, all Arg and Lys residues in the C-domain that are conserved in all known ACE primary structures were individually changed to Gln by mutagenesis. The positions of these conserved residues in human ACE are shown in Fig. 1. Conservative substitutions (i.e. Lys in place of Arg, and vice versa) were allowed but none were observed.

Six lysines and four arginines are conserved in human ACE C-domain (Fig. 1). These are: Lys\(^{919}\), Arg\(^{924}\), Arg\(^{942}\), Arg\(^{944}\), Lys\(^{1030}\), Lys\(^{1044}\), Lys\(^{1065}\), Lys\(^{1077}\), Lys\(^{1087}\), and Arg\(^{1098}\). Mutants containing a single Gln substitution of these residues were expressed in COS-7 cells. The protein expression level of each mutant, except K1077Q, was >60% of the wild-type enzyme. The expression level of K1077Q was ~15% of the wild-type enzyme. In these mutations, the glycosylation pattern was unchanged from that in the wild-type enzyme, indicating that their post-translational modification was intact. Enzyme activity of each mutant is shown in Fig. 4A.

Following extensive dialysis in Cl\(^-\)-free buffer, Ang II forming activity of each mutant was examined in 0 or 20 mM NaCl (Fig. 4B). Like the wild-type C- and N-domains, all C-domain mutants, except R1098Q, exhibited a >10-fold increase in activity associated with the change in Cl\(^-\) concentration from 0 to 20 mM. In 0 mM NaCl, R1098Q had ~60% of the activity seen in 20 mM NaCl. The selective loss of Cl\(^-\) dependence in the Ang I hydrolytic activity of R1098Q suggested that Arg\(^{1098}\) was the Cl\(^-\) binding residue in the C-domain.

Effect of Cl\(^-\) on Ang I Hydrolysis by R1098Q-C-domain and R500Q-N-domain ACE—The residue in the N-domain, equivalent to Arg\(^{1098}\) of the C-domain, is Arg\(^{500}\). The effect of Cl\(^-\) on the R1098Q-C-domain and R500Q-N-domain Ang II forming activity is summarized in Table I and Fig. 2. In Ang I hydrolysis assays carried out in the absence of Cl\(^-\), the Arg\(^{1098}\) to Gln substitution produced a 6.9-fold decrease in \(K_m\), a 4.7-fold increase in \(k_{cat}\), and a 32-fold increase in \(k_{cat}/K_m\) (\(\Delta G^{‡} = -2.1\) kcal mol\(^{-1}\)). Under identical conditions the Arg\(^{500}\) to Gln substitution produced a 1.8-fold decrease in \(K_m\), a 2.5-fold increase in \(k_{cat}\), and a 4.5-fold increase in \(k_{cat}/K_m\) (\(\Delta G^{‡} = -0.76\) kcal mol\(^{-1}\)). Increasing Cl\(^-\) concentration levels to 20 mM produced minimal changes in the \(k_{cat}/K_m\) of Ang I hydrolysis by R1098Q (22% decrease) or R500Q (7.7% decrease).

Therefore, the Arg\(^{1098}\) to Gln and Arg\(^{500}\) to Gln substitutions lead to a loss of Cl\(^-\) dependence (Fig. 2). In the presence of 20 mM NaCl, R1098Q and R500Q had ~45% of the catalytic efficiency of their respective wild-type controls.

**Fig. 5. Effects of Cl\(^-\) on tetrapeptide substrate hydrolysis by the C-domain (filled symbols) and its R1098Q mutant (open symbols).** In A, activity was measured with Ac-AFGG-COO\(^-\) (squares), Ac-AFAG-COO\(^-\) (circles), and Ac-AFHL-COO\(^-\) (triangles). In B, activity was measured with Ac-AFFK-COO\(^-\) (squares), Ac-AFAK-COO\(^-\) (circles), and Ac-AFAK-COO\(^-\) (triangles). In C, activity was measured with Ac-AFFR-COO\(^-\) (squares), Ac-AFHR-COO\(^-\) (circles), and Ac-AFHR-COO\(^-\) (triangles). Activity assays with purified enzymes were carried out in 50 mM HEPES buffer, pH 7.5, containing 0 to 100 mM NaCl, 10 \(\mu\)M ZnSO\(_4\), and 30 \(\mu\)M substrate at 37°C. Generated Ac-AF-COO\(^-\) was quantified by HPLC using pure standards. Values are means of duplicate measurements. This figure shows theoretical curves for data generated with ACE C-domain, but not for R1098Q.
The kinetic constants were determined in 50 mM HEPES buffer, pH 7.5, containing 10 μM ZnSO4, NaCl (0 to 100 mM) and 30 μM substrate at 37 °C. Kd(app) and Vmax for Cl−-dependent activation of the C-domain (from data in Fig. 4) and Kd values for substrate hydrolysis at saturating Cl− concentration levels (>5-fold higher than Kd(app)) were determined by nonlinear regression. Vmax was the enzyme activity in the absence of Cl−. Values are the means of two independent determinations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kd(app)</th>
<th>Kd(m)</th>
<th>Vmax</th>
<th>Vmax [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcAFFR</td>
<td>1.6</td>
<td>45</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>AcAFFR</td>
<td>3.4</td>
<td>45</td>
<td>21</td>
<td>9.4</td>
</tr>
<tr>
<td>AcAFHR</td>
<td>5.0</td>
<td>145</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>AcAFAK</td>
<td>8.7</td>
<td>104</td>
<td>17</td>
<td>4.3</td>
</tr>
<tr>
<td>AcAFHK</td>
<td>20</td>
<td>500</td>
<td>12</td>
<td>2.0</td>
</tr>
<tr>
<td>AcAFHL</td>
<td>30</td>
<td>200</td>
<td>19</td>
<td>3.7</td>
</tr>
<tr>
<td>AcAFFK</td>
<td>37</td>
<td>140</td>
<td>17</td>
<td>2.2</td>
</tr>
<tr>
<td>AcAFAG</td>
<td>40</td>
<td>166</td>
<td>28</td>
<td>5.8</td>
</tr>
<tr>
<td>AcAFGG</td>
<td>73</td>
<td>1,000</td>
<td>20</td>
<td>1.3</td>
</tr>
</tbody>
</table>

a Substrate concentration used for these experiments was <Km and thus, for each substrate, variations in enzyme activities associated with changes in NaCl concentration reflect changes in kcat/Km.

**Effect of the Arg1098 to Lys Substitution in the C-domain on Chloride-binding Site of ACE C-domain**

As a percentage of Cl−-binding site during hydrolysis of the C-domain, Arg1098 is critical for the Cl−-enzyme interaction that leads to enzyme activation whereas the critical residue was previously thought to be a Lys; 2) that Cl− activates the C-domain by disrupting an intramolecular interaction involving Arg1098 that keeps the enzyme in a low affinity state with respect to Ang I binding; and 3) that Cl− binding to the enzyme is influenced by multiple determinants in a substrate, on both sides of the scissile bond. These findings suggest an interaction between the anion and the substrate in addition to the anion-enzyme interaction. Surprisingly, however, anion-substrate interactions appear to be important only for the hydrolysis of short tetrapeptide substrates and not for the decapeptide Ang I. These observations provide new insights into the molecular mechanism of ACE activation as well as the selective retention of the Cl−-binding site during ACE evolution.

**Discussion**

Novel aspects of this study include the findings: 1) that a single conserved Arg in the ACE C-domain, Arg1098, is critical for the Cl−-enzyme interaction that leads to enzyme activation whereas the critical residue was previously thought to be a Lys; 2) that Cl− activates the C-domain by disrupting an intramolecular interaction involving Arg1098 that keeps the enzyme in a low affinity state with respect to Ang I binding; and 3) that Cl− binding to the enzyme is influenced by multiple determinants in a substrate, on both sides of the scissile bond. These findings suggest an interaction between the anion and the substrate in addition to the anion-enzyme interaction. Surprisingly, however, anion-substrate interactions appear to be important only for the hydrolysis of short tetrapeptide substrates and not for the decapeptide Ang I. These observations provide new insights into the molecular mechanism of ACE activation as well as the selective retention of the Cl−-binding site during ACE evolution.

**ACE Constructs**—At the beginning of these studies we considered the possibility that a Cl− binding interaction with one domain could influence the activity of the other domain and thus contribute to the identification of the critical Cl−-binding residue. Hence, a study of separated domains was undertaken.

In the study by Wei et al. (6), the N- and C-domain fragments used contained considerable overlapping sequences and one could not exclude the possibility that a single Cl−-binding site in the region that was present between the two active sites was important for both active sites. We constructed N- and C-domains based on the gene region that was duplicated (Fig. 1).

A direct comparison of catalytic efficiencies for Ang I hydrolysis by N- and C-domains shows that at 20 mM NaCl the N-domain is ~2.5-fold more active than the C-domain (2.7 and 1.1 s−1 μM for the N- and C-domains, respectively). Catalytic efficiencies for Ang I hydrolysis by separated N- and C-domains were not reported by Wei et al. (6). However, studies with the two full-length mutants in which one or the other active site was inactivated, where interdomain interactions were possible, showed that at 50 mM NaCl the C-domain has a 2.6-fold higher catalytic efficiency for the hydrolysis of Ang I than does the N-domain (0.73 and 1.9 μM s−1 for the N- and C-domains, respectively) (6). Our data for Ang I hydrolysis shows that maximal activation by Cl− of both the N- and C-domains occurs at Cl− concentration levels between 10 and 20 mM, with Kd(app) values for Cl− of 1.1 and 0.5 mM for the N- and C-domains, respectively. Wei et al. (6) reported a similar (1.2 mM) Kd(app) for Cl−-dependent activation of Ang I hydrolysis for the N-domain fragment and the full-length mutant with an inactivated C-domain. However, they observed a ~4.5-fold higher Kd(app) (5.4 mM) for the C-domain fragment as well as the full-length mutant with an inactivated N-domain. Since assay conditions were similar it is possible that the presence of additional sequences on the C-domain fragment studied by Wei et al. (6) mimicked the influence of the entire N-terminal domain on the Cl− effect. The recombinant N- and C-domain proteins described in this study are catalytically efficient at hydrolyzing Ang I and display a remarkable degree of Cl− activation that is observed with native somatic ACE. Differences between these separated domains in relative reaction rates for Ang I hydrolysis and in Cl− activation constants for this reaction are not identical to those observed between the domains in full-length somatic ACE and could reflect the contribution of interdomain...
interactions to the catalytic reaction.\textsuperscript{3} 

Identification and Properties of the C-domain Cl\textsuperscript{−} binding Site—The chloride ion produces a 2.5 kcal mol\textsuperscript{−1} decrease in the free energy required for stabilization of the C-domain-Ang I transition-state complex. This decrease in free energy is due to Cl\textsuperscript{−}-dependent changes in both $K_m$ and $k_{cat}$. Arg\textsuperscript{1098} is likely to be the key Cl\textsuperscript{−}-binding residue in the C-domain because Cl\textsuperscript{−}-dependent enzyme activation: 1) is selectively lost in R1098Q but not in other conserved Arg or Lys C-domain mutants; 2) is also lost when the equivalent Arg in the N-domain, Arg\textsuperscript{500}, is substituted with Gln; and 3) persists in the R1098K mutant. The Arg to Gln substitution represents a 17% decrease in side chain accessible area retaining much of the hydrophobic portion of the side chain while replacing the positive charge with an uncharged polar end (18). The lack of Cl\textsuperscript{−}-dependent activation in R1098Q and R500Q suggests an ionic interaction between Cl\textsuperscript{−} and the side chains of Arg\textsuperscript{1098} in the C-domain and Arg\textsuperscript{500} in the N-domain. In porcine pancreatic $\alpha$-amylase, the side chain amine of Arg\textsuperscript{357} coordinates the Cl\textsuperscript{−} in a bidentate mode. This interaction is of higher affinity (>10-fold) than in \textit{Aleromonas haloplanctis} and \textit{Thermomonomospora curvata} $\alpha$-amylases, in which the equivalent Cl\textsuperscript{−} coordinating side chain is Lys, a residue that can only provide an unidentate coordination (16, 17). The finding that the R1098K mutant is Cl\textsuperscript{−}-dependent and coordinates the Cl\textsuperscript{−} with a ~100-fold lower affinity than its wild-type control further indicates that Arg\textsuperscript{1098} is the primary Cl\textsuperscript{−} binding residue in the C-domain. The difference in Cl\textsuperscript{−} binding affinities between Arg\textsuperscript{1098}, and Lys\textsuperscript{1098}, C-domains is, however, much greater than that seen between wild-type porcine pancreatic and \textit{A. haloplanctis} $\alpha$-amylases. The inability of Cl\textsuperscript{−} to increase R1098K-dependent Ang I hydrolysis to the same level as that seen with the wild-type C-domain (V\textsubscript{max} for R1098K is ~8% of that of wild-type C-domain) as well as the marked decrease in Cl\textsuperscript{−} binding affinity associated with the Arg\textsuperscript{1098} to Lys substitution could suggest that the environment of the guanido group at position 1098 in the C-domain is optimized for the Arg side chain and not for other basic side chains. In this regard, it is interesting to note that in \textit{A. haloplanctis} $\alpha$-amylase, substituting the native Lys\textsuperscript{357} with Arg causes a 10-fold decrease in Cl\textsuperscript{−} binding affinity, rather than an increase (16).

Kinetic analyses indicate that Cl\textsuperscript{−} binding to the C-domain causes this domain to adopt a structure that can bind Ang I with an ~9-fold higher affinity (here, the comparison was made between 0 and 20 mM NaCl). Because the kinetic constants $K_m$ and $k_{cat}/K_m$ for Ang I hydrolysis by the C-domain at an optimal Cl\textsuperscript{−} concentration are similar to those observed with R1098Q in the absence of Cl\textsuperscript{−}, we propose that Cl\textsuperscript{−}-bound Arg\textsuperscript{1098} is not important in Ang I binding \textit{per se} but that Cl\textsuperscript{−} causes the enzyme to adopt structures that allow higher affinity substrate binding during the initial and rate-limiting steps of catalysis. Mechanistically, these conclusions are consistent with the view that in the absence of Cl\textsuperscript{−}, intramolecular interactions within the C-domain keep critical regions of the substrate-binding site poorly accessible to the substrate. Cl\textsuperscript{−} binding to Arg\textsuperscript{1098} or replacement of the Arg basic side chain with a neutral one disrupts this interaction allowing the enzyme to reach a new conformation in which substrate binding interactions between Ang I and ACE are greatly improved. The inability of Cl\textsuperscript{−} to modify Ang I binding to R1098Q or its subsequent hydrolysis suggests that Cl\textsuperscript{−} binding to sites on the C-domain other than Arg\textsuperscript{1098} either does not occur or is not relevant for the catalytic reaction that leads to Ang I hydrolysis.

Activation experiments with halides show differential effects of these monovalent anions on binding and hydrolysis of Ang I. All halide ions can be accommodated in the anion-binding site of the C-domain, but with differing affinities. Binding affinity was the highest for Cl\textsuperscript{−} and Br\textsuperscript{−} but was ~10-fold lower for I\textsuperscript{−} and ~100-fold lower for $\Gamma^−$ suggesting that optimal electrostatic and van der Waals distances are achieved with Cl\textsuperscript{−} and Br\textsuperscript{−}. Loss of this binding selectivity between halides associated with the Arg\textsuperscript{1098} to Lys substitution further suggests that the geometry of the anion-ligating residues within the anion-binding site is a crucial design feature of the C-domain. It is known that ACE (8, 9) and its C-domain (present data) are anion- and not cation-activated. It is tempting to speculate that the Arg\textsuperscript{1098}–X intramolecular interaction that keeps the enzyme in a low affinity state involves amino-aromatic or amino-polar bridges. Cl\textsuperscript{−}, but not Na\textsuperscript{+}, can disrupt these bridges by interacting with the positively charged guanido group of Arg\textsuperscript{1098}.

Based on chemical modification studies, Shapiro and Riordan (10) proposed that a critical Lys is present in the Cl\textsuperscript{−}-binding site of rabbit ACE. The Lys-modified ACE was relatively inactive at hydrolyzing a tripeptide with a P$_2$-Gly but had substantial hydrolytic activity for a tripeptide with a P$_2$-Arg. In the course of analyzing our C-domain mutants we observed a conserved Lys, Lys\textsuperscript{1087}, which when substituted with Gln produces a 500-fold greater decrease in catalytic efficiency for the hydrolysis of a peptide substrate with a P$_2$-Leu or -Gly than for one with a P$_2$-Arg (data not shown). The phenotypic similarity between the Lys-modified rabbit ACE and K1087Q indicates that Lys\textsuperscript{1087} is the critical residue proposed by Shapiro and Riordan (10). However, Lys\textsuperscript{1087} is unlikely to be important for Cl\textsuperscript{−} binding because hydrolysis by K1087Q of a variety of P$_2$-modified substrates is Cl\textsuperscript{−}-dependent (data not shown). We believe that this unusual structural influence of Lys\textsuperscript{1087} on ACE catalytic activity explains the apparent misidentification of this residue as the Cl\textsuperscript{−}-binding site, as will be detailed in a separate publication.

Nature of Substrate and the Cl\textsuperscript{−} Effect—Early studies with full-length rabbit ACE showed that differences in Cl\textsuperscript{−}-dependent enzyme activation are substrate dependent (8, 9); in particular, it was proposed that a P$_1$- or P$_2$-(Arg/Lys) in a substrate is necessary and sufficient for high affinity Cl\textsuperscript{−} binding. Using tetrapeptide substrates with identical P-positioned residues (acyetyl-AF) but with variable P\textsuperscript{′} dipeptide structures, we re-examined Cl\textsuperscript{−}-dependent activation of the C-domain because the earlier studies had been carried out with two-domain somatic ACE. Affinity for Cl\textsuperscript{−} binding to the C-domain was 25- and 46-fold higher using Ac-AFAR-COO$^-\textsuperscript{−}$ as substrate compared with Ac-AFAG-COO$^-\textsuperscript{−}$ and Ac-AFGG-COO$^-\textsuperscript{−}$, respectively. These results confirm earlier findings that Cl\textsuperscript{−} binding to the C-domain is greatly influenced by the nature of the substrate P\textsuperscript{′} dipeptide structure with the highest affinity Cl\textsuperscript{−}-C-domain interaction observed with P$_2$,-Ala,-Phe, or -His indicated that the influence of the P$_2$ basic residue on Cl\textsuperscript{−} binding can be modified (by ~2–4-fold) by the nature of the P$_1$ residue.

In the course of these comparisons we were struck by the substantial effect of overall substrate structure on Cl\textsuperscript{−} binding.
For example, Cl⁻ binding affinity for the C-domain when DRVYI-HPFHL-COO⁻ (Ang I) is used as substrate is ~60-fold higher than with AcAFHL-COO⁻, and it is ~3-fold higher than with AcAFFAR-COO⁻. If the C-terminal dipeptide is the site on the short substrate that interacts with the anion, as discussed above, it is quite likely that a similar interaction occurs when the substrate is an N-terminal extended form of a short substrate as is the case between DRVYIHPFHL-COO⁻ (Ang I) and AcAFHL-COO⁻. Why then should Cl⁻ binding to the C-domain be of much higher affinity with Ang I as substrate than with the substrate AcAFHL-COO⁻? We hypothesize that the affinity of Cl⁻ binding to the enzyme is directly related to the strength of two bridging interactions: enzyme-anion and anion-substrate. The latter interaction is in turn dependent on the strength of the enzyme-substrate interaction. Consistent with this hypothesis is the finding that at saturating levels of Cl⁻ (50 and 150 mM NaCl for Ang I (from Ref. 11) and AcAFHL-COO⁻, respectively) the affinity of substrate binding to the C-domain is ~10-fold higher with Ang I than with AcAFHL-COO⁻. Implicit in these views is the idea that the substrate forms an important part of the Cl⁻ binding site and that the affinity of Cl⁻ binding is not solely dependent on the substrate C-terminal dipeptide structure.

With tetrapeptide substrates such as Ac-AFAR/R/K-COO⁻ and Ac-AFFR/R/K-COO⁻ potential interactions with Cl⁻ include hydrogen-bonding interactions with main chain N-H groups in the substrate and with the P₂'-Arg or -Lys side chains. Similar hydrogen-bonding interactions between Cl⁻ and main chain N-H groups of Ang I and AcAFHL-COO⁻ are also possible. Tetra- and penta-coordination of Cl⁻ has been described (17) and X-H—Cl⁻ hydrogen bonds are likely to be strong (19). These enzyme-anion-substrate interactions may be particularly important for the binding of short substrates to the C-domain where direct enzyme-substrate contacts are likely to be fewer than are possible with larger cognate substrates such as Ang I. In the gluzincin nephrilysin, for example, docking interactions between the enzyme and the substrate C-terminal carboxylate group are critical for binding short tripeptide substrates but are not evident for larger substrates such as Ang I (20).

With respect to AcAFHL-COO⁻ hydrolysis, the R1098Q mutant is Cl⁻ independent. However, in marked contrast to the findings with Ang I, the hydrolitic activity of R1098Q for AcAFHL-COO⁻ is ~3% of that of the wild-type C-domain under optimal Cl⁻ conditions. For AcAFHL-COO⁻, an interaction between Cl⁻ and substrate is therefore likely to be important for hydrolysis and hence the Arg₁⁰⁹⁸-coordinated Cl⁻ must form an important part of the substrate-binding site. Similar arguments may be made for other tetrapeptide substrates used in this study. With these short substrates, the functional importance of the anion-substrate interaction contrasts markedly with that noted for Ang I, for which substantial anion-substrate interactions can be demonstrated, but are functionally unimportant. Finally, the findings presented here, in our opinion, give no clues as to why substrate hydrolysis is maximally activated to different extents by Cl⁻, depending on substrate sequence. This important issue remains to be resolved.

Conclusions—Modifier binding to an allosteric site induces conformational changes that inhibit or activate the enzyme but this site is distinct from the active site. Is Cl⁻ an allosteric modulator of the C-domain? The most direct evidence for allosteric kinetics is that the enzyme may be made insensitive (by mutagenesis or chemical modification) to the modifier without loss in catalytic activity, thereby providing evidence that the modifier-binding site is different from the substrate-binding site or catalytic site (21). In the case of the C-domain the answer is complex. With Ang I as substrate, in the absence of Cl⁻, substituting the Cl⁻ binding cationic side chain of Arg₁⁰⁹⁸ with a neutral side chain causes an increase in substrate binding and hydrolysis to a level that is similar to that seen with the wild-type enzyme in the presence of optimal Cl⁻. Here, the simplest point of view is that Cl⁻ activates the C-domain via an allosteric mechanism, which most likely changes enzyme conformation. However, given that Cl⁻ binds to Ang I (for reasons discussed above) we must conclude that Cl⁻-dependent activation of Ang I hydrolysis does not occur by a simple allosteric mechanism.

Given that ACE is an ectoenzyme located in the extracellular environment, it is likely that physiologically it exists as an anion-enzyme complex. Thus, the anion switch that keeps ACE in a high affinity state is likely to be perpetually in the “on” position. The loss of Cl⁻ dependence without a decrease in catalytic efficiency for Ang I associated with the Arg₁⁰⁹⁸ to Gln substitution does not provide a reason for maintaining the anion-binding site during ACE evolution. As a regulatory mechanism the importance of the enzyme-bound Cl⁻ could reside in its ability to bind the substrate and increase the affinity of the enzyme-substrate complex. This effect is likely to influence the hydrolysis of short substrates, which have fewer interactions with ACE, more markedly than large cognate substrates. It is tempting to speculate that evolutionary conservation of the Cl⁻-binding site must have been linked to the need to preserve high binding affinities and catalytic efficiencies for short cognate peptide substrates of the C-domain necessary for maintaining an evolutionary advantage. In this regard, it is known that testicular ACE, which only contains the C-domain, plays a critical role in male fertility (22). Are enzyme-bound Cl⁻ interactions with substrates of testicular ACE important? The answer to this question must await the identification of physiological substrates for this ACE isoform.

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REFERENCES
Arg1098 Is Critical for the Chloride Dependence of Human Angiotensin I-converting Enzyme C-domain Catalytic Activity

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