Molecular Basis of Exopeptidase Activity in the C-terminal Domain of Human Angiotensin I-converting Enzyme

INSIGHTS INTO THE ORIGINS OF ITS EXOPEPTIDASE ACTIVITY*

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Proteolytic processing is a primary means of biological control. Exopeptidases use terminal anchoring interactions to restrict cleavage at peptide substrate N or C termini. In contrast, internal peptide bond targeting by endopeptidases is through context-driven recognition. Angiotensin I-converting enzyme (ACE), a zinc metalloproteinase, has tandem duplicate catalytic domains, N- and C-terminal, each of which is a dual specificity enzyme with exo- and endocarboxypeptidase activities. The mechanisms by which ACE evolved from its endopeptidase ancestors as a dual specificity enzyme have not been defined. Based on kinetic studies of wild-type and mutant forms of the C-terminal catalytic domain of human ACE and of the ACE substrates angiotensin I, substance P, and bradykinin, as well as considerations of the ACE x-ray structure, we provide evidence that the acquisition of its exopeptidase activity is due to novel evolutionary specializations. These involve not only interactions between the S2’ subsite cognate for the C-terminal substrate P2’ side chain, acting in concert with carboxylate-docking interactions with Lys1087 and Tyr1096, but also electrostatic selection against a cationic C-terminal substrate carboxylate. With a blocked C terminus, substrate side chain interactions are dominant in cleavage site selection. In the evolution of obligate exopeptidases from endopeptidase ancestors, mutations that destroy context-driven peptide bond targeting are likely to have followed the acquisition of terminal docking interactions. Evolutionary intermediates between endopeptidases and obligate exopeptidases could therefore have been dual specificity proteinases like ACE.

Angiotensin (Ang)I-converting enzyme (ACE; EC 3.4.15.1) is a zinc metalloproteinase of the gluzincin family. ACE has a unique chloride activation mechanism that has been identified in life forms from invertebrates to mammals (1). An internal duplication in the ancestral ACE gene resulted in a molecule with two catalytic domains, N- and C-terminal. Most mammalian tissues contain ACE with two catalytic domains; however, the use of an internal promoter has additionally led to the expression of an ACE with a single catalytic domain (C-domain) in the testis (1). Vertebrate ACE is a type I membrane protein; its catalytic domains are extracellular (1).

ACE primarily cleaves a C-terminal dipeptide from substrates and is also known as peptidyl dipeptidase A. Important physiological substrates of ACE are Ang I and bradykinin. Ang I is hydrolyzed by ACE to form the potent vasopressor octapeptide Ang II. Bradykinin is inactivated by ACE, thus abrogating its vasodilatory function (1). Thus, by regulating Ang II and bradykinin levels, ACE plays a critical role in cardiovascular homeostasis, and its inhibitors are effective and widely used drugs for the treatment of hypertension and heart failure (2). ACE also hydrolyzes a wide range of polypeptide substrates, including substance P (Sub P), luteinizing hormone-releasing hormone, acetyl-Ser-Asp-Lys-Pro, and neurotensin (1).

In contrast to Ang I and bradykinin, the C termini of Sub P and luteinizing hormone-releasing hormone are amidated. Studies by Skidgel et al. (3), Cascieri et al. (4) and Yokosawa et al. (5) were the first to show that, for substrates with amidated C termini, ACE not only displays exopeptidase (i.e. dipeptidyl carboxypeptidase) activity, but also acts as an endopeptidase. Given that many metallopeptidases closely related to ACE, such as nephrilysin and endothelin-converting enzyme, are endopeptidases (6, 7), it is likely that ACE evolved from an endopeptidase ancestor. Moreover, given that ACE retains endopeptidase activity, evolution of its carboxypeptidase activity is not due to acquisition of a confined substrate-binding cleft that can accommodate substrates with only a dipeptide- leaving group C-terminal to the scissile bond. Rather, it is generally believed that evolutionary acquisition of C-terminal carboxylate-docking residues in the ACE substrate-binding site stabilize the ground state so that registration of substrates with a C-terminal carboxylate is restricted to only a solitary mode that limits substrate processing to cleavage of the C-terminal dipeptide. The recent high resolution structure determined for the human ACE (h-ACE) C-domain co-crystallized with lisinopril (its transition-state inhibitor) bound revealed close molecular contacts, both direct and indirect, between Lys1087 and Tyr1096 and the carboxylate group (equivalent to the C-terminal carboxylate group of peptide substrates) of the inhibitor (8). Using site-specific mutagenesis and a variety of ACE substrates, we examined the role of the carboxylate-docking interactions in effecting substrate registration and in stabilizing the ground
and transition states of the h-ACE C-domain. We show novel evolutionary specializations that, in concert with carboxylate-docking interactions, reinforce the dipeptidyl carboxypeptidase activity of the h-ACE C-domain.

EXPERIMENTAL PROCEDURES

Construction of the h-ACE C-domain Gene—An h-ACE C-domain gene with unique restriction sites was chemically synthesized and cloned into the expression vector pDNA3 (Invitrogen) as described by us previously (9). The synthetic gene encodes (in the following order) amino acid residues 1–29 (signal peptide), a 31-residue c-Myc epitope sequence, a 611–612 residue sequence recognized by a commercially available anti-c-Myc antibody (9E10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and residues 611–1201 of human somatic ACE. This synthetic gene does not include the transmembrane sequence found in somatic ACE. Mutations were constructed in the synthetic gene by site-directed mutagenesis and confirmed by DNA sequence analysis.

Expression and Purification of the Recombinant h-ACE C-domain Gene and Its Mutants—COS-7 cells (American Type Culture Collection) were cultured and transfected as described previously (9). Briefly, cells were cultured under an atmosphere of 5% CO₂ at 37 °C and transfected with plasmid DNA using the Gene Pulser system (Bio-Rad). Twelve hours after transfection, cells were washed and further cultured in serum-free Dulbecco's modified Eagle's medium (Invitrogen) for 72 h. Conditioned medium obtained from cells expressing and releasing the h-ACE C-domain or its mutants was collected and used as the starting point of the purification. The following ACE C-domain mutations were made: Y1096F, K1087A, R1098Q, and K1087A/Y1096F.

h-ACE and its mutants were purified by ion-exchange HPLC using a Bio-Scale Q2 column (Bio-Rad) as described previously (9). Briefly, conditioned medium containing the h-ACE C-domain or its mutants was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 20 mM NaCl. The dialyzed medium (5–10 ml) was applied to a Bio-Scale Q2 column, which was developed using a 45-min linear NaCl gradient (20–500 mM) at a flow rate of 1 ml/min. Two 1-ml fractions with the highest activity were pooled. The purity of recombinant ACE in the pooled peak fractions was ~50%, with bovine serum albumin as the only detectable contaminant. ACE was not further purified to remove the contaminating bovine serum albumin since the stability of the pure ACE C-domain at 4 °C was markedly lower than that of partially purified ACE containing bovine serum albumin.

Determination of Kinetic Constants for the h-ACE-Ang I Reaction—To determine the kcat (Michaelis constant) and Vmax (maximal velocity) values for the h-ACE C-domain and its mutants, initial velocities (v) were determined as described by us previously (9). Twelve concentrations of substrate (Ang I or its analogs; ranging between 7.5 and 150 μM) were mixed with the wild-type or mutant h-ACE C-domain or its mutants (K1087A, Y1096F, R1098Q, and K1087A/Y1096F) at 37 °C in 50 mM HEPES (pH 7.5) containing 50 mM NaCl and 10 mM ZnSO₄ (final volume of 50 μl) for 30–45 min. For each peptide substrate, the enzyme concentration was adjusted to ensure that <15% of the substrate was utilized at the lowest substrate concentration. Under these conditions, product formation was linear with respect to time over the duration of the incubation. As described previously (9), the formation of the Phe8–Xaa9 bond in the substrate, but the absence of cleavage at other sites. ΔGbind, Vmax, and Kcat values were calculated (using nonlinear regression with KaleidaGraph (Synergy Software, Reading, PA)) using v = Vmax × |S|/(Kcat + |S|), where |S| is the concentration of the peptide substrate. The overall rate constant kcat was calculated using kcat = Vmax × [E]₀, where [E]₀ is the total enzyme concentration and was determined by Western and analyses using the h-ACE C-domain as a standard. Values represent means ± S.E. of triplicate determinations. ΔΔGbind, ΔΔGcat, and ΔΔGv were calculated as described by Wells (10) and represent the difference between the wild-type and mutant enzymes in free energy required to form the enzyme-substrate complex (ES) from E + S, to convert the E-S complex to the transition-state complex (ES'), and to stabilize the transition-state complex (E.I.S') (either energy required to form ES', E-S, or E + S, respectively).

Results and Discussion

Role of C-terminal Carboxylate-Docking Interactions in the Hydrolysis of Sub P—The undecapeptide Sub P is amidated at its C terminus and, as a result, lacks negative charge at this position. This has been proposed to weaken or prevent docking of the substrate with ACE, via its carboxylate, and thus to allow alternative registration of the substrate with the extended substrate-binding site of the enzyme (11). Consistent with this proposal, we determined here that ACE not only degraded Sub P to release the C-terminal dipeptide (evident from the formation of Sub P–1–7) and Sub P–1–9) (Fig. 1 and Table I). Tripeptidyl carboxypeptidase and endopeptidase activities were estimated by determining the rate of Sub P–1–8) and Sub P–3–11 formation, respectively (see Fig. 1). Bradycinidegrada- tion by the h-ACE C-domain and its mutants K1087A, Y1096F, and K1087A/Y1096F was estimated by determining the rate of substrate degradation (initial concentration of ~125 μM, instead of product formation, using methods similar to those described for Sub P degradation; in these experiments, the fragmentation pattern was not quantified.

RESULTS AND DISCUSSION

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We next studied the role of the carboxylate-docking interactions by mutating the h-ACE C-domain residues Tyr1096 and Lys1098 to Phe and Ala, respectively. Based on x-ray crystal structures of human testicular ACE-lisinopril and ACE-captopril complexes (8, 12), the Y1096F mutation, which deletes the side chain hydroxyl group of Tyr, is expected to eliminate its ability to interact with the substrate C-terminal carboxylate or amide groups as well as with the captopril and lisinopril C-terminal carboxylate groups. The K1087A mutation, which deletes the charged amino group (and shortens the side chain length) from the Lys side chain, is also expected to eliminate its ability to interact, directly and indirectly, with the substrate C-terminal carboxylate or amide group as well as with the
captopril and lisinopril C-terminal carboxyamide groups. Indeed, Fig. 2 shows that, as expected, the Y1096F mutation produced a decrease (~20-fold) in captopril binding affinity (1/K), indicating, by experiment, its importance for captopril docking. The low activity of the K1087A C-domain made it difficult to accurately determine the binding affinity of captopril for this mutant; however, at a concentration of 40 mM, captopril inhibited K1087A C-domain activity by only ~80% (data not shown).

Table I shows that single or double site-specific mutations of the h-ACE C-domain carboxylate-docking residues Tyr1096 and Lys1087 to Phe and Ala, respectively, produced a decrease in dipeptidyl carboxypeptidase activity (e.g. 83 ± 0.57% in the wild-type h-ACE C-domain versus 36 ± 0.78% in Y1096F/K1087A (n = 3); p < 0.0001) and an increase in substrate hydrolysis at other sites. With Y1096F/K1087A, endopeptidase (at the Pro2–Lys3 bond) and tripeptidyl carboxypeptidase activities were higher by 3- and 5.8-fold, respectively, than with the wild-type h-ACE C-domain. These findings suggest that hydrogen-bonding interactions between the C-terminal amide group of Sub P and Tyr1096 and Lys1087 of the h-ACE C-domain are a significant part of the substrate registration strategy.

Sub P free acid hydrolysis was reduced by 1.8-, 34-, and 72-fold with mutants Y1096F, K1087A, and Y1096F/K1087A, respectively, compared with the wild-type h-ACE C-domain (Table I), indicating the functional importance of the C-terminal carboxylate-docking interaction. Remarkably, however, with respect to Sub P free acid hydrolysis, the dipeptidyl carboxypeptidase activity of the h-ACE C-domain remained the sole mode of substrate hydrolysis, even in the Y1096F/K1087A h-ACE C-domain (Table I). These findings indicate that interactions of a substrate C-terminal carboxylate other than the carboxylate-docking interaction with Tyr1096 and Lys1087 are important for substrate registration. The h-ACE C-domain-

lisinopril co-crystal x-ray structure shows that the surface of the substrate-binding cleft, which contains the S' subsites, is negatively charged (8). It is tempting to speculate that the negatively charged C-terminal carboxylate group of the substrate generates unfavorable electrostatic interactions with most registration strategies other than that which, by virtue of interactions with Tyr1096 and Lys1087, stabilizes the substrate for the hydrolysis of a C-terminal dipeptide. Steric hindrance due to restricted S’ subsites appears to play little, if any, role in positioning the C-terminal substrate residue in a P2’ registration because endopeptidase activity is readily noted in a number of ACE substrates with a C-terminal amide group, as observed, for example, with Sub P (Table I).

Given these considerations, we questioned whether, in addition to its well appreciated role in determining peptide bond cleavage efficiency, the interaction of the side chain of the C-terminal residue of the substrate with ACE could also be an important facet of substrate registration. We (9) and others (13) have previously shown that a P2’ Arg side chain in a substrate helps to stabilize the Michaelis h-ACE C-domain-substrate complex (i.e. the ground state). Here, we have shown that the h-ACE C-domain hydrolyzed [Arg11]Sub P exclusively via initial cleavage of a C-terminal dipeptide (Table I). In comparison, Sub P (which contains a C-terminal Met at position 11) underwent initial cleavage via the excision of C-terminal di-, tri-, or nonapeptides. These findings highlight the importance of both carboxylate-docking and C-terminal residue side chain interactions in determining substrate registration. Interestingly, positional effects have also been demonstrated in the nephrilisin reaction (14).

The finding that [Arg11]Sub P was hydrolyzed by the carboxylate docking-incompetent h-ACE C-domain mutant Y1096F/K1087A exclusively via initial cleavage of a C-terminal tripeptide (Table I) additionally suggests that ACE has a previously unappreciated S3’ subsite that has a preference for an Arg side chain over a Met side chain. For reasons discussed above, this site cannot be utilized if the substrate is able to dock productively via the C-terminal carboxylate interaction. Nevertheless, it is evident from our findings that it can be readily targeted with compromise of the carboxylate interaction. This and other potential interactions between substrate and S’ subsites are expected to be crucial in determining the site at which the endopeptidase activity of ACE becomes manifest when it interacts with peptide substrates containing an amidated C terminus. In addition, differences in preferences, not only in P1’, P2’, but also in P3’–Pn’, between the ACE C- and N-terminal domains may explain why the ACE N-terminal domain displays higher endopeptidase activity with the naturally amidated substrate luteinizing hormone-releasing hormone than does its C-domain.

**C-terminal Carboxylate-docking Interactions Are Critical in the Hydrolysis of Ang I but Not Bradykinin.—**The hydrolysis of Ang I by ACE differs from that of Sub P in some important respects. With Ang I, ACE is an obligate exopeptidase (dipeptidyl carboxypeptidase), as evidenced by the fact that cleavage is restricted to its Phe3–His8 bond to yield Ang II and the dipeptide His-Leu. Indeed, alternative substrate registration modes are not observed even when the C-terminal carboxylate group in Ang I is amidated. These findings indicate that interactions between the ACE extended substrate-binding site and Ang I are not cognate in any registration mode other than that
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Hydrolysis of Sub P and its analogs by the h-ACE C-domain and its mutants was studied by incubating each enzyme (between 2 and 10 ng) with 125 μM substrate for 20 min at 37 °C. The reaction buffer was 50 mM HEPES (pH 7.5) containing 50 mM NaCl and 10 mM ZnSO₄ (final volume of 60 μl). Values represent means ± S.E. of triplicate determinations. ND, not detected.

| Substrate | Enzyme | Sub P-(3–11) | Sub P-(1–8) | Sub P-(1–7) + Sub P-(1–9) |
|-----------|--------|--------------|-------------|---------------------------|
| Sub P     | Wild-type | 32 ± 0.17 (11%) | 16 ± 0.20 (5.6%) | 230 ± 1.6 (83%) |
| Sub P     | Y1096F  | 210 ± 0.58 (44%) | 57 ± 0.30 (12%) | 210 ± 5.3 (44%) |
| Sub P     | K1087A  | 64 ± 0.34 (35%) | 56 ± 0.20 (30%) | 66 ± 1.3 (35%) |
| Sub P     | K1087A/Y1096F | 48 ± 0.54 (33%) | 47 ± 0.31 (32%) | 53 ± 1.1 (36%) |
| Sub P-COO⁻ | Wild-type | ND (0%) | ND (0%) | 650 ± 5.4 (100%) |
| Sub P-COO⁻ | Y1096F  | ND (0%) | ND (0%) | 350 ± 4.1 (100%) |
| Sub P-COO⁻ | K1087A  | ND (0%) | ND (0%) | 19 ± 0.4 (100%) |
| Sub P-COO⁻ | K1087A/Y1096F | ND (0%) | ND (0%) | 9 ± 0.5 (100%) |
| [Arg¹¹]Sub P | Wild-type | ND (0%) | ND (0%) | 138 ± 1.4 (100%) |
| [Arg¹¹]Sub P | Y1096F  | ND (0%) | ND (0%) | 116 ± 0.78 (100%) |
| [Arg¹¹]Sub P | K1087A  | ND (0%) | ND (0%) | 62 ± 0.28 (11%) |
| [Arg¹¹]Sub P | K1087A/Y1096F | ND (0%) | ND (0%) | 350 ± 2.4 (100%) |

| Substrate | Enzyme | Sub P-(3–11) | Sub P-(1–8) | Sub P-(1–7) + Sub P-(1–9) |
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| [Arg¹¹]Sub P | K1087A/Y1096F | ND (0%) | ND (0%) | 350 ± 2.4 (100%) |

* Formation of Sub P-(3–11), Sub P-(1–8), and Sub P-(1–7) + Sub P-(1–9) represents endopeptidase, tripeptidyl carboxypeptidase, and dipeptidyl carboxypeptidase activities, respectively (Fig. 1). The percentage of total activity (endopeptidase, tripeptidyl carboxypeptidase, and dipeptidyl carboxypeptidase) that is due to a particular mode of substrate cleavage is shown in parentheses.

[Table I](#)

![Graph](#)

**FIG. 2.** Inhibition of wild-type and Y1096F (○) ACE C-domains by the ACE inhibitor captopril. Assays with captopril were performed in 20 mM HEPES (pH 7.5) containing 50 mM NaCl and 10 mM ZnSO₄ at 37 °C with 30 μM [Phe₉]Ang I as substrate in a total volume of 50 μl. The incubation period was 30 min. Prior to the addition of substrate (5 μl), the enzyme was preincubated with inhibitors for 30 min. Values are means S.E. of three independent determinations for each inhibitor. K₅₀ values for the wild-type (●) and Y1096F (○) ACE C-domains (C-ACE) used in the abscissa calculations were from Table II.

which releases a C-terminal dipeptide. Moreover, the occurrence of a P₈-P₉ Pro in the product Ang II prevents its degradation by further C-terminal dipeptide cleavage. Here, we took advantage of these properties of the ACE–Ang I interaction, which are useful in determining the kinetics of the ACE carboxylate-docking interaction with respect to its dipeptidyl carboxypeptidase activity.

As shown in Table II, with respect to Ang I hydrolysis, the Y1096F/K1087A mutation, which is expected to eliminate carboxylate-docking interactions, caused a >10,000-fold decreased k₅₀/K₅₀ (Table II), i.e. the carboxylate-docking interactions appear to stabilize the h-ACE C-domain-Ang I transition state by >5.7 kcal/mol (ΔGₕ). From studies of the inhibitory properties of dipeptides, Cushman and co-workers (15) reported that H₃N⁺-His-Leu-COO⁻ is >1000-fold less avidly bound to ACE than is H₃N⁺-Phe-Arg-COO⁻. Given that Phe-Arg is the C-terminal dipeptide structure of the ACE substrate bradykinin, it seems reasonable to question whether these suboptimal P₈ His and P₉ Leu side chain interactions of Ang I with ACE increase the relative importance of the C-terminal carboxylate-docking interactions with respect to transition-state stabilization. To address this question, we studied the kinetics of Ang I, [Phe₉]Ang I, [Arg¹⁰]Ang I, and [Phe₉,Arg¹⁰]Ang I hydrolysis by the h-ACE C-domain and its carboxylate docking-compromised mutants. As shown in Table II, the specificity constants (k₅₀/K₅₀) for ACE-mediated hydrolysis of the [Phe₉]Ang I, [Arg¹⁰]Ang I, and [Phe₉,Arg¹⁰]Ang I analogs were markedly increased over that for the native Ang I peptide, changes likely due to enhanced transition-state stabilization given that the K₅₀ values were largely unaltered. Indeed, compared with Ang I, we found that the Gibbs free energies (ΔGₕ) required to stabilize the transition state were decreased with [Phe₉]Ang I (by 1.7 kcal/mol), [Arg¹⁰]Ang I (by 1.5 kcal/mol), and [Phe₉,Arg¹⁰]Ang I (by 3.0 kcal/mol). These results indicate that, within the context of Ang I, the P₈ His and P₉ Leu are much less effective at stabilizing the transition state than the P₈ Phe and P₉ Arg, respectively. The additive effects of these residue changes on ΔGₕ (i.e. observed ΔGₕ ~ calculated ΔGₕ) observed ΔGₕ = 3.0 kcal/mol; calculated ΔGₕ = ΔGₕ⁰ + ΔGₕ₂ + ΔGₕ₃ + ΔGₕ₄ (HBD) + ΔGₕ₅ (1,10D) further indicate that the P₈ Phe and P₉ Arg side chains stabilize the transition state independently of each other.

Consistent with an important role for transition-state stabilization by the P₈ Phe and P₉ Arg side chains, we also observed that increases in ΔGₕ produced by the ACE mutation Y1096F (which is expected to decrease the strength of the carboxylate-docking interaction) were greater for Ang I (ΔGₕ = 2.5 kcal/mol) than for [Phe₉]Ang I (ΔGₕ = 1.4 kcal/mol), [Arg¹⁰]Ang I (ΔGₕ = 1.3 kcal/mol), and [Phe₉,Arg¹⁰]Ang I (ΔGₕ = 1.2 kcal/mol) (Table III). These findings illustrate that, even with large, otherwise cognate (K₅₀ for Ang I of ~50 μM) polypeptide substrates, the reinforcing effect of carboxylate-docking interactions on transition-state stability is inversely linked to how optimal the substrate C-terminal dipeptide structures are. (Although not specifically studied here, it is possible that P₈ and P₉ specificities are also important in this regard.) Thus, when precise positioning of the scissors bond is not possible because of less than ideal C-terminal side chains, the carboxylate-docking interaction compen-
in conjunction with an amidated C terminus, the P3 domain-dependent Hydrolysis of Ang I Analogs—

amidated substrate, potential S\textsubscript{3}'-P\textsubscript{3}' Arg interactions are important for activity with carboxylate docking-compromised h-ACE C-domain mutants, but that the context provided by the rest of the substrate is no less important. Stabilization of the Transition State by the P\textsubscript{2}' Arg in an Ang I Analog Requires the Cl\textsuperscript{−} -binding Side Chain of Arg\textsuperscript{1098}\textsuperscript{\textsubscript{K}} in the h-ACE C-domain—More than 2 decades ago, Riordan and co-workers (13) reported that the affinity of Cl\textsuperscript{−} binding to ACE is increased with substrates containing a basic P\textsubscript{2}' side chain, particularly that of arginine. Recently, we identified Arg\textsuperscript{1098} as its mutants K1087A, Y1096F, and K1087A/Y1096F. Values represent means ± S.E. of triplicate measurements. Experimental details are given under “Experimental Procedures.” WT, wild-type h-ACE C-domain.

The S\textsubscript{1}'-P\textsubscript{3}' Arg Interaction in Y1096F/K1087A h-ACE C-domain-dependent Hydrolysis of Ang I Analogs—In the studies with Sub P analogs described above, we concluded that the h-ACE C-domain has a previously unappreciated S\textsubscript{3}' subsite that has a preference for an Arg side chain that can be readily targeted with compromise of the C-terminal carboxylate-docking interaction. Examination of other ACE substrates indicated that Pro-Xaa bonds are readily cleaved by ACE (e.g. ACE cleaves the Pro–Phe\textsuperscript{8} bond in the degradation of bradykinin); thus, the C-terminal structure of Ang I (i.e. Pro–Phe–His–Leu–COO\textsuperscript{−}) provides an opportunity to critically examine whether, in conjunction with an amidated C terminus, the P\textsubscript{3}' Arg is both necessary and sufficient to cause scissile bond retargeting when carboxylate-docking interactions are compromised (as in the h-ACE C-domain mutant Y1096F/K1087A). Specifically, we tested whether Y1096F/K1087A could hydrolyze [Arg\textsuperscript{10}]Ang I amide, yet not Ang I amide (which contains Leu at position 10), at the Pro–Phe\textsuperscript{8} bond to yield the N-terminal fragment Ang I-(1–7) and to release a C-terminal tripeptide. However, no Ang I-(1–7)-forming activity was detected using either substrate, even after prolonged incubations with high levels of Y1096F/K1087A. In this setting, the absence of detectable tripeptidyl carboxypeptidase activity suggests that, with a C-terminal amidated substrate, potential S\textsubscript{3}'-P\textsubscript{3}' Arg interactions are not sufficient in themselves to stabilize the ground and/or transition states of Y1096F/K1087A. Collectively, these findings and the contrasting data with Y1096F/K1087A-dependent cleavage of [Arg\textsuperscript{11}]Sub P (described above) suggest that S\textsubscript{3}'-P\textsubscript{3}' Arg interactions are important for activity with carboxylate docking.
compromised by a 0.7–1.7 kcal/mol increase in $\Delta G_p^\dagger$ following the R1098Q substitution in the h-ACE C-domain. In distinct contrast, the decreases in $\Delta G_p^\dagger$ produced by the P$_1$' His-to-Phe substitution in both Ang I and [Arg$^{10}$]Ang I were accentuated by a further 0.8–1.7 kcal/mol decrease in $\Delta G_p^\dagger$ by the same R1098Q mutation. These findings support the notion that the Arg$_{1098}$-bound Cl$^-$ forms an integral part of the S$_2$ subsite of the h-ACE C-domain that helps to stabilize both the ground and transition states by binding the P$_2$' Arg side chain of ACE substrates.

Conclusion—Here, we have shown that C-terminal carboxylate- and amide-docking interactions between the h-ACE C-domain and its polypeptide substrate greatly influence substrate registration and catalytic efficiency for the cleavage of C-terminal dipeptides. The functional importance of these interactions is, however, exquisitely dependent on the nature of the substrate, particularly in terms of the primary structure of its C terminus.

Three distinct features of the substrate-binding site serve to specialize the h-ACE C-domain as a dipeptidyl carboxypeptidase. First is the occurrence of C-terminal carboxylate-docking residues Lys$_{1087}$ and Tyr$_{1096}$. Second is the S$_2$ subsite cognate for the substrate C-terminal P$_2$' Arg side chain. Third is a collective surface formed by the S$_1$–S$_n$ subsites (8) that selects against the negatively charged C-terminal carboxylate group of the substrate. We propose that these features determine substrate registration and regulate catalytic efficiency in the following ways.

(i) For substrates with suboptimal P$_1$' and P$_2$' side chains, as in Ang I, the negatively charged C terminus selects against alternative registration modes, and the carboxylate-docking interactions greatly stabilize the transition state with the P$_{12}$–His$_8$ bond in the P$_1$–P$_2$' orientation. Thus, in this case, the loss of carboxylate-docking interactions with the enzyme causes a >10,000-fold loss of hydrolytic activity.

(ii) For substrates with an optimal P$_1$' side chain, as in bradykinin, the negatively charged C terminus prevents alternative registration modes, but here the carboxylate-docking interactions are not as crucial for transition-state stabilization because the P$_2$' Arg side chain appropriately positions the C terminus of the substrate. Moreover, our data suggest that an important feature of the S$_n$ subsite is an Arg$_{1098}$-bound Cl$^-$, which serves to stabilize the substrate P$_2$' Arg side chain. Natesh et al. (8) argued against this possibility, based on the x-ray crystal structure of an h-ACE C-domain-lisinopril complex. However, our current mutagenesis data and previous kinetic findings (9, 13) suggest otherwise. We believe that, because the P$_2$' Pro side chain of lisinopril is markedly different in length and charge from the arginine side chain, this static co-crystal structure may not allow a definitive extrapolation of the ground- and transition-state orientations of the P$_2$' Arg side chain within the ACE substrate-binding site. In this regard, it is interesting to note that ACE2, an enzyme closely related to ACE, displays considerable conformational changes between the ground and transition states (16).

(iii) For substrates with suboptimal P$_1$' and P$_2$' side chains and with amidated C termini, as in Sub P, weaker carboxylate-docking and P$_1$' and P$_2$' side chain interactions with the enzyme lead to reduced dipeptidyl carboxypeptidase activity. Here, because the substrate C terminus is neutral, alternative registration modes are permissive, and endopeptidase activities are enhanced. The extent to which, as well as the site at which, endopeptidase activity occurs is likely to depend on the ability to form productive S-P and S$^\prime$-$P^\dagger$ interactions within the substrate, at sites other than those that produce a registration mode leading to the release of a dipeptide.

Our studies indicate that the h-ACE C-domain does not absolutely require a carboxylate-docking interaction to efficiently stabilize the transition state. For example, [Arg$^{11}$]Sub P hydrolysis rates were minimally influenced by the K1087A/Y1096F mutation (Table I); however, the registration mode was altered. Thus, the K1087A/Y1096F h-ACE C-domain appears to function efficiently as an endopeptidase despite defective carboxylate-docking interactions. The endopeptidase activity of ACE is becoming more appreciated as detailed investigations explore ACE-dependent hydrolysis of polypeptides at internal cleavage sites. For example, recently, Hu et al. (17) reported that ACE degrades amyloid $\beta$-peptide (1–40) by cleaving the Asp$^\gamma$–Ser$^\gamma$ bond.

The endopeptidase activity of the h-ACE C-domain is likely to be an ancestral trait since, as indicated above, many metalloproteinases closely related to ACE (for example, nephrisin and endothelin-converting enzyme) are endopeptidases (6, 7). Its presence in the modern day enzyme may thus be vestigial, as there is no compelling evidence that endopeptidic processing of substrates such as Sub P has any primary biological role. More importantly, our studies point to two novel evolutionary specializations that, independently of carboxylate-docking interactions,
reinforce the dipeptidyl carboxypeptidase activity of the h-ACE C-domain. First, there is an S_2/H subsite with a bound Cl^− that binds the substrate P_{2}^′ Arg side chain such that the scissile bond is optimally positioned for efficient substrate hydrolysis. Whereas we have demonstrated this effect with peptide substrates with arginine as the terminal residue, it remains to be determined whether a P_{2}^′ Arg side chain can also stabilize the ground and transition states when substrate registration on the h-ACE C-domain results in endopeptidase activity. Second, an S′ subsite network (at least from S_3' to S_9') exists that selects against the positioning of a charged C-terminal carboxylate group of a peptide substrate in any registration mode other than that which liberates a C-terminal dipeptide.

Collectively, these findings suggest that the targeting of internal cleavage sites within large polypeptide substrates by the h-ACE C-domain (as an endopeptidase) is dictated entirely by cognate S-P and S′-P′ enzyme-substrate interactions. In this situation, because the S′ subsites are not presented with a negatively charged C terminus, mutually reinforcing effects (carboxylate-docking interactions and negatively charged distal S′ subsites) do not force an exopeptidase registration mode. Hence, in the evolution of the h-ACE C-domain, endopeptidase activity with protein substrates may not have been greatly compromised since new specializations developed independently to allow acquisition of highly efficient dipeptidyl carboxypeptidase activity for peptide substrates containing a negatively charged carboxyl group at their C termini.

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