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# N-domain Selectivity of Angiotensin I-Converting Enzyme as Assessed by Structure–Function Studies of its Highly Selective Substrate, *N*-Acetyl-Seryl-Aspartyl-Lysyl-Proline

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**ABSTRACT.** The physiological functions of angiotensin I-converting enzyme (ACE) are not limited to its cardiovascular role. ACE constantly degrades *N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), a natural circulating regulator of the hematopoietic stem cell proliferation, and thereby may be involved in hematopoietic stem cell regulation. AcSDKP is hydrolyzed 50-fold faster by the N-domain active site compared to the C-domain active site. The aim of the present study was to investigate which aminoacid residues from AcSDKP are required to ensure N-domain specificity. Several peptides were designed by progressively increasing the length of the peptidic chain from a tripeptide to a pentapeptide. Kinetic studies of the wild-type ACE and of the two ACE mutants containing a single active domain (N- or C-domain) were performed using Bz (benzoyl) Asp-Lys-Pro, benzoyl-glycyl (Bz-Gly)-Asp-Lys-Pro, and Bz-Gly-Ser-Asp-Lys-Pro (with its intermediate product Bz-Gly-Ser-Asp) as substrates. The unexpected importance of an aspartic acid in the P1 position was discovered, as well as the interaction of the P2 and P3 positions in the substrate to increase or decrease N-domain specificity. Substrates longer than five residues may involve interdependence between subsites. Finally, the discovery of highly specific and novel N-domain substrates cannot be predicted from single subsite mapping, but may require other approaches such as combinatorial peptide libraries. BIOCHEM PHARMACOL **57**;6:611–618, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** ACE N-domain; ACE C-domain; peptidyl dipeptidase; AcSDKP; stem cells

ACE† (peptidyl-dipeptidase A, EC 3.4.15.1), a type I ectoprotein, is a zinc metalloenzyme, member of the gluzincins, defined by an HExxH motif and a glutamic acid residue as the third zinc ligand [1, 2]. Two ACE isoenzymes exist in mammalian tissues: a widely distributed somatic isoform consisting of two homologous domains named the N-domain of the C-domain according to their location in the single-chain protein, each of them containing an active site, a germinal form expressed exclusively in mature spermatids. Germinal ACE corresponds to the C-domain of somatic ACE, with the exception of a short specific N-terminal sequence [3, 4].

The primary specificity of ACE is to remove carboxyl-terminal dipeptides from the carboxyl terminus of an oligopeptide substrate, although other atypical cleavages have been described [5]. ACE plays a central role in blood pressure regulation by acting on two main physiological substrates, angiotensin I and bradykinin [6, 7]. However, the physiological functions of ACE are not limited to its cardiovascular role; it may be involved in hematopoietic stem cell regulation by constantly degrading AcSDKP, a natural circulating regulator of hematopoietic stem cell proliferation [8, 9]. In addition, testicular ACE plays a primordial role in male reproduction, as shown by the male hypofertility which results from its gene inactivation [10–12], but the substrate involved in this function is unknown.

The two domains of somatic ACE share a high degree of homology, particularly at the active centers, but differ in some properties. A series of ACE mutants containing a single active domain was used to demonstrate these differences. First, the two active sites are activated differently by chloride ions: the C-domain active site is far more sensitive to chloride concentration for the hydrolysis of some substrates such as the synthetic tripeptide Hip-His (histidyl)-Leu (leucine) [13]. Second, while both domains cleave bradykinin, Ang I, and substance P, the rate of hydrolysis differs: for example, Ang I is converted about three times faster by the C-domain [14]. Interestingly, AcSDKP is

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† *Abbreviations:* ACE, angiotensin I-converting enzyme; N-domain, full-length ACE with C-domain catalytic site inactivated by substitution of the two zinc-binding histidyls ($H_{959}$ and $H_{963}$) by lysyl residues; C-domain, full-length ACE with N-domain catalytic site inactivated by substitution of the two zinc-binding histidyls ($H_{361}$ and $H_{365}$) by lysyl residues; AcSDKP, *N*-acetyl-seryl-aspartyl-lysyl-proline; and Hip, hippuryl (benzoyl-glycyl).

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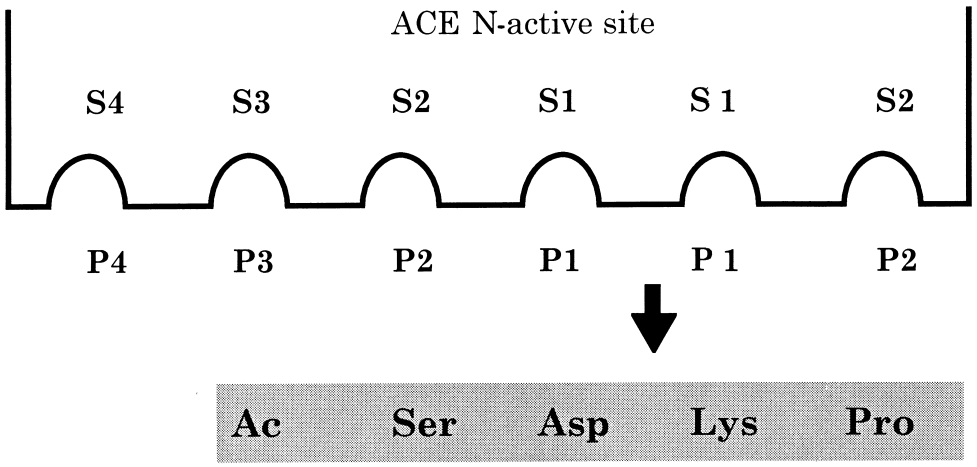
**TABLE 1. ACE substrate peptides derived from N-Ac-Ser-Asp-Lys-Pro**




| | | | | | |
|---|---|---|---|---|---|
| | | Bz | Gly | **Lys** | **Pro** |
| | | Bz | **Asp** | Lys | Pro |
| | Bz | **Gly** | **Asp** | Lys | Pro |
| Bz | **Gly** | **Ser** | **Asp** | Lys | Pro |
| | | Bz | Gly | Ser | Asp |

The aminoacid residues of a peptide substrate situated towards the NH2 end from the cleavage site are numbered as P, while those towards the COOH end are numbered as P′ according to the respective S1-S′ subsites of the enzyme they occupy [20].

hydrolyzed 50-fold faster by the N-domain active site compared to the C-domain and constitutes a natural and specific substrate of the ACE N-domain [8]. Recently, Erdös *et al.* determined that angiotensin 1–7 (Asp-Arg-Val-Tyr-Ile-His-Pro) appears to be a specific substrate of the N-domain with a catalytic efficiency, $k_{cat}/K_m$, 100 times greater than that for the C-domain. However this peptide is very slowly hydrolyzed by the N-domain (catalytic constant: 0.45 $sec^{-1}$) and inhibits the C-domain of ACE [15]. Finally, it was previously shown that the potency of an ACE inhibitor appears to depend, at least in part, on the substrate, and that captopril displays a partial selectivity for inhibiting AcSDKP hydrolysis compared with that of Ang I [16]. On the contrary, Keto-ACE inhibits bradykinin and Ang I hydrolysis by the C-domain at concentrations approximately 47 and 38 times lower, respectively, than by the N-domain [15].

These functional differences indicate that despite the high level of primary sequence homology between the N- and C-domains, structural and functional differences do exist between the two active sites of somatic ACE. It is even possible that unknown N- or C-domain-specific substrates exist and are important during development or angiogenesis. The search for new putative specific substrates requires a better understanding of the subsite differences between the two active sites of somatic ACE.

The aim of the present study was to investigate which aminoacid residues in the sequence of AcSDKP are required to ensure N-domain specificity. This peptide introduces, in the N-domain active site, a basic charge in S′1, an acidic charge in S1, and an alcohol function in S2 subsites. It has been shown that carboxyl-terminal tripeptide residues play a predominant role in ACE hydrolysis with a relative importance of P′2 > P′1 > P1 for peptide binding while the order is reversed for substrate specificity (P1 > P′1 > P′2) [17–19]. Thus, the aminoacid requirement for substrate seems to be confined to the aminoacid residues composing the C-terminal tripeptide. In a previous work, we examined the hydrolysis of hippuryl dipeptides mimicking the carboxydipeptide Lys-Pro of AcSDKP (Hip-Lys-Pro) or of captopril (Hip-Ala-Pro). Both peptides exhibit a strong affinity for the N- and C-domains of ACE and are hydrolyzed efficiently by both wild-type ACE and ACE containing either an active N- or C-domain, with $k_{cat}$ values comparable to that for Ang I hydrolysis [16]. We hypothesize that this loss of selectivity may result from the lack of an aspartic acid residue in the P1 position (antepenultimate carboxyl-terminal position), one strictly required for N-domain specificity. In the present study, several peptides were designed to further examine N-domain selectivity by progressively increasing the length of the peptidic chain (Table 1).

The results of this study delineate the subsite preferences of each ACE active site domain and the selectivity of the N-domain active site. An unexpected importance of the S2

subsite and the involvement of S3 subsites for N-domain efficacy were discovered.

## MATERIALS AND METHODS

### *Materials*

**ENZYMES.** Wild-type somatic ACE and the two ACE mutants containing only one functional active site were obtained through stable expression from Chinese hamster ovary cells transfected with the appropriate ACE cDNA, as reported previously [13]. The two ACE mutants were full-length enzymes with either the N- or C-domain catalytic site inactivated by substitution of the two zinc-binding histidyl residues ($ACE_{K361,\ 365}$ and $ACE_{K959,\ 963}$). These mutants are hereafter referred to as N-domain or C-domain according to their location in the single-chain protein, indicating the sole catalytically active domain. The construction of the ACE cDNAs, their expression in Chinese hamster ovary cells, and the purification of the corresponding membrane-bound proteins have been previously described [21]. ACE concentration was quantified by direct radioimmunoassay [22] or deduced from its enzymatic activity determined using Hip-His-Leu as substrate according to Cushman and Cheung [23]. The released Bz Gly was quantified by HPLC as described previously [24]. A relative molecular mass of 170,000 for ACE was used for the calculation of $k_{cat}$ values.

**PEPTIDES.** BzGly was from Sigma Chemical Co. BzAsp-Lys-Pro, BzAsp, BzGly-Asp-Lys-Pro, BzGly-Ser-Asp-Lys-Pro, BzGly-Asp, BzGly-Ser, and BzGly-Ser-Asp were synthesized by Neosystem. The purity of these peptides, determined by HPLC, was greater than 90%.

### *Methods*

First, the HPLC conditions for quantification on a Waters apparatus directed by a millennium chromatography manager were determined for each new substrate. Second, optimal conditions for hydrolysis of each substrate (pH dependence and chloride activation) were adjusted as described previously [16]. For all forms of ACE, optimal cleavage was observed around pH 6–7, and the optimal chloride concentration was determined for each substrate. Finally, kinetic studies of wild-type ACE and of the ACE mutants were performed on each substrate; kinetics parameters were calculated from Michaelis-Menten plots using ENZFITTER software, values being mean ± standard error from three independent determinations.

#### *BzAsp-Lys-Pro and BzGly-Asp-Lys-Pro Hydrolysis*

Reactions were performed in 10 μM $ZnSo_4$, 1 mg/mL BSA, 100 mM HEPES pH 6 with 50 mM NaCl for BzDKP or pH 6.5 with 20 mM NaCl for BzGDKP using 1-2 $\times$ $10^{-9}$ M or 2-10 $\times$ $10^{-9}$ M of wild-type ACE or the N- or C-domain, respectively. Reactions were initiated by the addition of BzDKP or BzGDKP in a total volume of 250 μL and incubated at 37°C to produce 5–10% substrate hydrolysis. Reactions were stopped by the addition of 12% $H_3PO_4$ (50 μL). BzD and BzDKP, or BzGD and BzGDKP, were resolved and quantified by reverse-phase HPLC on a 5 μm Puresil C18 column (Waters) in 25% (v/v) acetonitrile and 10 mM potassium phosphate, pH 3, at a flow rate of 1 mL/min. Retention time was 3.5, 2.3, 4.0, and 2.6 min for BzD, BzDKP, BzGD, and BzGDKP, respectively, with a detection at 228 nm. Initial velocities were measured over a substrate concentration range of 5–200 μM for these two substrates.

#### *BzGly-Ser-Asp-Lys-Pro and BzGly-Ser-Asp Hydrolysis*

BzGSDKP was incubated with wild-type ACE or the N- or C-domain active forms of ACE, and the rate of hydrolysis was calculated from the products identified as BzGSD and BzG (Hip). Optimal conditions of pH and NaCl concentrations were determined for each step of hydrolysis by the three ACE forms. For the hydrolysis of BzGSDKP with production of BzGSD by all forms of ACE, optimal cleavage was observed at pH 6.5 with an NaCl concentration of 20 mM. For the hydrolysis of BzGSD with production of BzG at optimal pH 6, a concentration of 50 mM NaCl was chosen. Reactions were performed using 0.2–0.5 $\times$ $10^{-9}$ M for BzGSDKP or 2–10 $\times$ $10^{-9}$ M for BzGSD of the three forms of ACE under the same conditions of hydrolysis and HPLC described above, with the only change being in acetonitrile concentration: 11% in place of 25%. Retention time was 17.0. 5.4, 12.7, and 10.1 min for BzGSD, BzGSDKP, BzG, and BzGS, respectively. Initial velocities were measured over a substrate concentration range of 10–1500 μM for these two substrates.

## RESULTS

The binding affinity ($K_m$) and turnover ($k_{cat}$) of substrates by the three forms of ACE varied according to the AcSDKP amino acid substitutions. The specificity constant ($k_{cat}$)/$K_m$) reflects enzyme preference. Peptides composed of 3 to 5 aminoacid residues were designed to clarify the substrate specificity. Kinetic studies of the wild-type ACE and of the two ACE mutants were performed using BzAsp-Lys-Pro, BzGly-Asp-Lys-Pro, and BzGly-Ser-Asp-Lys-Pro (with its intermediate product BzGly-Ser-Asp) as substrates. This allowed us to assess the role played by aminoacid residues at each sequence position in conferring N-domain specificity. The inhibitory potency of the dipeptide Lys-Pro generated from the hydrolysis of these substrates was previously reported using Ang I as substrate [16]. Apparent $K_i$ values were high (1.37, 0.52, and 3.3 mM for wild-type ACE, the N-domain, and the C-domain, respectively) and, therefore, had negligible effect on the determination of the kinetic constants reported below.

**TABLE 2. Kinetic parameters ($k_{cat}/K_m$) of different ACE recombinant forms acting on synthetic and natural substrates**

| ACE | AcSDKP* | BzDKP† | BzGDKP† ($\mu M/sec$) | BzGSDKP† | BzGSD† |
|---|---|---|---|---|---|
| Wild-type | 0.30 | 0.34 ± 0.03 | 0.11 ± 0.02 | 0.85 ± 0.13 | 0.016 ± 0.002 |
| N-domain | 0.50 | 0.39 ± 0.03 | 0.08 ± 0.01 | 0.99 ± 0.14 | 0.004 ± 0.001 |
| C-domain | 0.01 | 0.35 ± 0.07 | 0.01 ± 0.00 | 0.53 ± 0.14 | 0.060 ± 0.016 |

* Values are from Rousseau *et al.* [8].
† Present study: values are means ± standard error from three independent experiments.

### *BzAsp-Lys-Pro (BzDKP)*

To identify a hippuryl-dipeptide potentially more specific for the N-domain active site than Hip-Lys-Pro (BzGKP), Benzoyl-**Asp**-Lys-Pro (Bz**D**KP) was synthesized. This made it possible to test the importance of an aspartic acid residue as the antepenultimate COOH-terminal residue (P1) (Table 1).

The $K_m$ values calculated from Michaelis-Menten plots of Bz**D**KP hydrolysis by wild-type ACE, the N- and the C-domain were 120 ± 14, 177 ± 41, and 58 ± 26 μM, respectively, with $k_{cat}$ values of 40 ± 1, 65 ± 9, and 14 ± 2 $sec^{-1}$. The $K_{cat}/K_m$ value for Bz**D**KP hydrolysis by the N-domain was 1.1-fold higher than for the C-domain (Table 2). For BzGKP the results were reversed, with a $k_{cat}/K_m$ ratio 2.6-fold higher for the C-domain than for the N-domain [16]. These results show a gradual increase in the N-domain efficacy (ratio of $k_{cat}/K_m$, N-domain vs $k_{cat}/K_m$, C-domain) when efficacy values for the hippuryl dipeptides BzGAP and BzGKP (0.25 and 0.38) are compared with that of Bz**D**KP (1.46).

### *BzGly-Asp-Lys-Pro (BzGDKP)*

To determine the importance of the NAcSer motif located in the P2/P3 position in the AcSDKP structure (Table 1), this moiety was substituted by BzGly, and the corresponding peptide (Bz**GD**KP) studied. The $K_m$ values for Bz**GD**KP hydrolysis by wild-type ACE and the N- and C-domains were 206 ± 30, 132 ± 22, and 355 ± 59 μM, respectively, with $k_{cat}$ values of 24 ± 6, 10 ± 1.7, and 2.8 ± 0.8 $sec^{-1}$. In comparison with the peptide Bz**D**KP, the $K_m$ values were conserved but the $k_{cat}$ decreased, especially for the C-domain. The $k_{cat}/K_m$ value for Bz**GD**KP hydrolysis by the N-domain active site was 10-fold higher than that for the C-domain (Table 2); under the same conditions, the $k_{cat}/K_m$ value for AcSDKP was 50-fold higher for the N-domain. This difference is attributable to a better affinity of AcSDKP for the enzyme, as shown by the lower $K_m$ value (40 μM).

### *Bz-Gly-Ser-Asp-Lys-Pro (BzGSDKP)*

A pentapeptide extending the AcSDKP structure was designed to investigate whether the N-active site of ACE acts on a substrate such as an endopeptidase, as in the case of the N-terminal endopeptidase cleavage of LH-RH [5], or via its classical peptidyl dipeptide hydrolase activity.

Bz**GSD**KP was incubated with wild-type ACE and the N- and C-domains. The only products identified were BzGSD and BzG. BzGS was never detected, showing that the three forms of ACE hydrolyze BzGSDKP in two consecutive steps via its classical peptidyl dipeptide hydrolase activity.

The kinetic parameters of the hydrolysis of Bz**GSD**KP into BzGSD were determined. The $K_m$ value of hydrolysis by wild-type ACE, N- and C-domains were 344 ± 37, 400 ± 85, and 773 ± 155 μM, respectively, with corresponding $k_{cat}$ values of 277 ± 25, 408 ± 123, and 327 ± 27 $sec^{-1}$. The $k_{cat}/K_m$ value for Bz**GSD**KP hydrolysis by the N-domain active site was 1.8-fold higher than for the C-domain (Table 2), a value lower than that found for Bz**GD**KP hydrolysis. This shows that N-domain efficacy decreases for a pentapeptide. A surprising increase in the $K_{cat}$ value for the three forms of ACE was observed.

The hydrolysis of BzG from BzGSD by N- and C-domain hydrolysis, the second hydrolytic step, was then studied. The $K_m$ values of hydrolysis by the wild-type ACE, N- and C-domains were 405 ± 74, 602 ± 134, and 106 ± 11 μM, respectively, with $k_{cat}$ values of 6.2 ± 0.3, 2.6 ± 0.9, and 5.8 ± 1.2 $sec^{-1}$. The $k_{cat}/K_m$ value for the BzGSD hydrolysis by the C-domain active site was 15-fold higher than for the N-domain (Table 2). A marked decrease in the $k_{cat}$ value for all forms of ACE was noted.

Therefore, in a first step, the dipeptide KP was efficiently hydrolyzed by the N-domain and in a second step, the final production of BzG resulted from a predominant C-domain hydrolysis. ACE attacked AcSDKP via its C-terminus and cleaved a peptide (BzGSD) with a COOH-terminal dicarboxylic aminoacid (aspartic acid), which was previously considered not possible.

## DISCUSSION

The aim of this study was to map N-domain ACE S3-S1 subsite specificity by using a series of synthetic substrates based on the AcSDKP structure, the natural N-domain-specific substrate.

The effect of the introduction of an aspartic acid residue in P1 (Bz**Asp**-Lys-Pro) and hippuric acid residue in the P2 (Bz**Gly**-**Asp**-Lys-Pro) or the P3 position (Bz**Gly**-**Ser**-**Asp**-Lys-Pro) on the efficacy of the N- versus the C-domain was studied. The presence of an aspartic acid residue in P1 conferred a maximum efficacy to the N-domain for the tetrapeptide hydrolysis. The corresponding pentapeptide

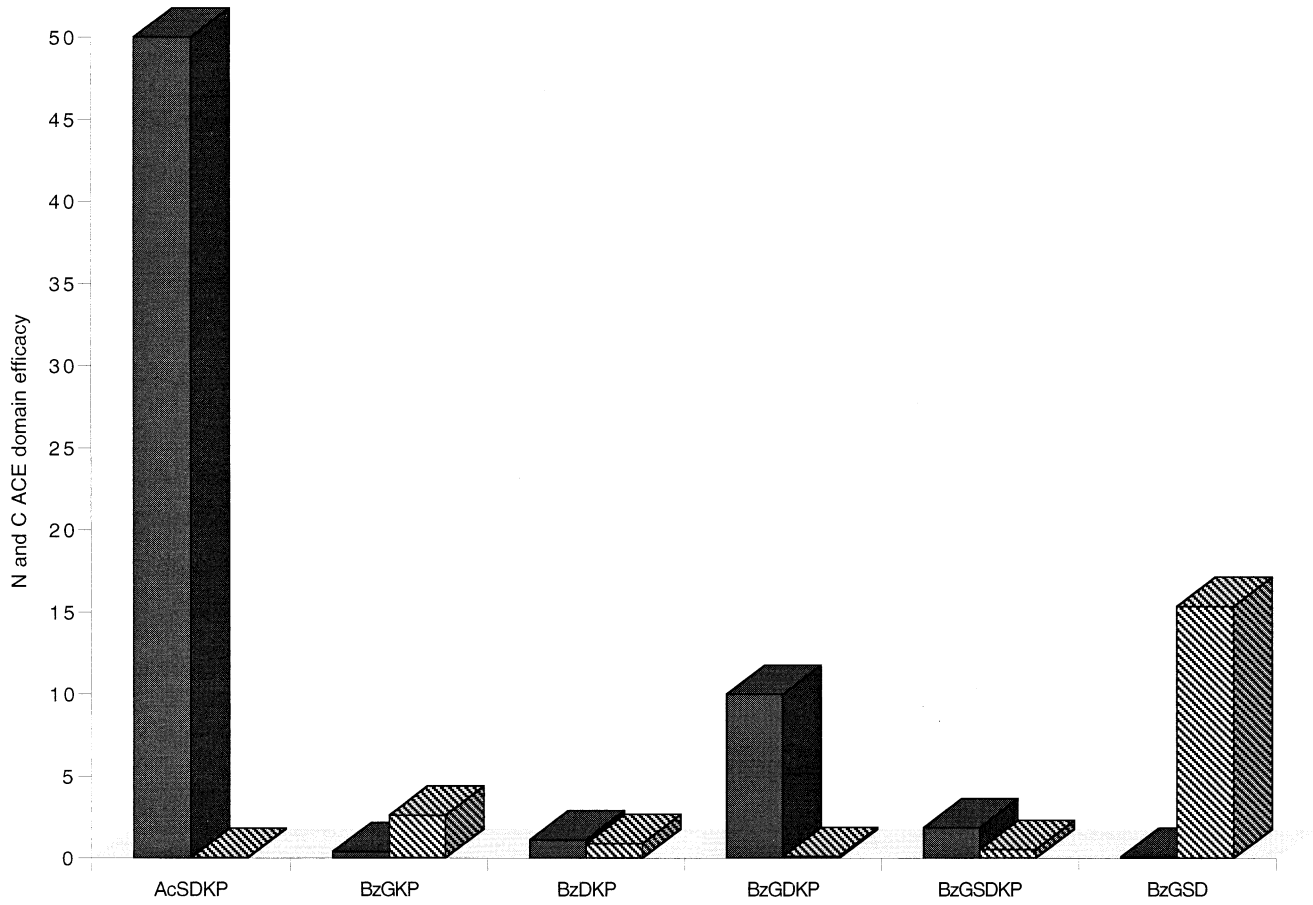



**FIG. 1. Efficacy of the ACE N- and C-domains in the hydrolysis of synthetic substrates homologous to NAcSDKP. ■ Efficacy N/C: ratio of N-domain $k_{cat}/K_m$ versus C-domain $k_{cat}/K_m$. ▨ Efficacy C/N: ratio of C-domain $k_{cat}/K_m$ versus N-domain $k_{cat}/K_m$.**

was less specifically cleaved by the N-domain and was hydrolyzed in two consecutive steps by ACE. No synthetic substrates used in this study were more specifically cleaved by the N-domain than the physiological substrate AcSDKP (Fig. 1).

The change from BzGKP to Bz**D**KP, Bz**GD**KP, and Bz**GSD**KP, respectively, did not affect the binding affinity of these substrates by the three forms of ACE, but rather decreased their catalytic efficiency, especially by lowering the rate of hydrolysis by the C-domain (Fig. 2). It is interesting to note that the catalysis rate was restored for all the different ACEs when pentapeptide hydrolysis was considered. It is possible that an ideal binding geometry is obtained for the two active sites when the peptide length is increased, to the detriment of N- versus C-domain efficacy.

We previously showed that BzGAP and BzGKP are hydrolyzed efficiently by the two ACE active sites with kinetic constants in the physiological range, but none of these substrates are N-domain-specific. The presence of a proline residue in P′2 increases peptide binding to the different ACEs. Proline is preferred in the P′2 position by the N-domain in place of a leucine (as in Hip-His-Leu) or of an aspartic acid residue (as in BzGly-Ser-Asp). It has been suggested that the preference of a proline residue may reside in its rigid conformation, which presents the terminal carboxyl group of the substrate in a favorable alignment for interaction with the putative positively charged residue of the active site of the enzyme. This alignment may be more critical for the N-active site, suggesting that the S′2 subsite of the N-domain active site is more stringent. Comparison of N- versus C-domain selectivity for hydrolysis of BzGly-Ala-Pro and BzGly-Lys-Pro, which differ only in the P′1 position, shows that a lysine residue favors interactions with the N-domain S′1 subsite, whereas the C-domain S′1 subsite accommodates different aminoacids. Interactions with polar groups lining the N-domain S′1 pocket appear to be a prerequisite N-domain selectivity. Our results are in agreement and extend those of radioligand binding inhibitor studies [25] which showed structural differences in the S′1 and S′2 hydrophobic pockets of the N- and C-domain binding sites of ACE: the S′1 and S′2 subsites of the N-domain appear smaller or structurally constrained. It has been hypothesized that a single hydrophobic pocket accommodates the entire C-terminal end of ACE inhibitors [26], instead of two separate pockets (S′2, S′1) in the case of substrates.

The comparison of the hydrolysis of BzGHL and Ang I (DRVYIHPFHL), or of BzGKP and AcSDKP, shows that the C-terminal dipeptide is not sufficient in itself to confer domain substrate specificity. BzGHL is a relatively specific substrate for the C-domain [24], whereas Ang I is cleaved

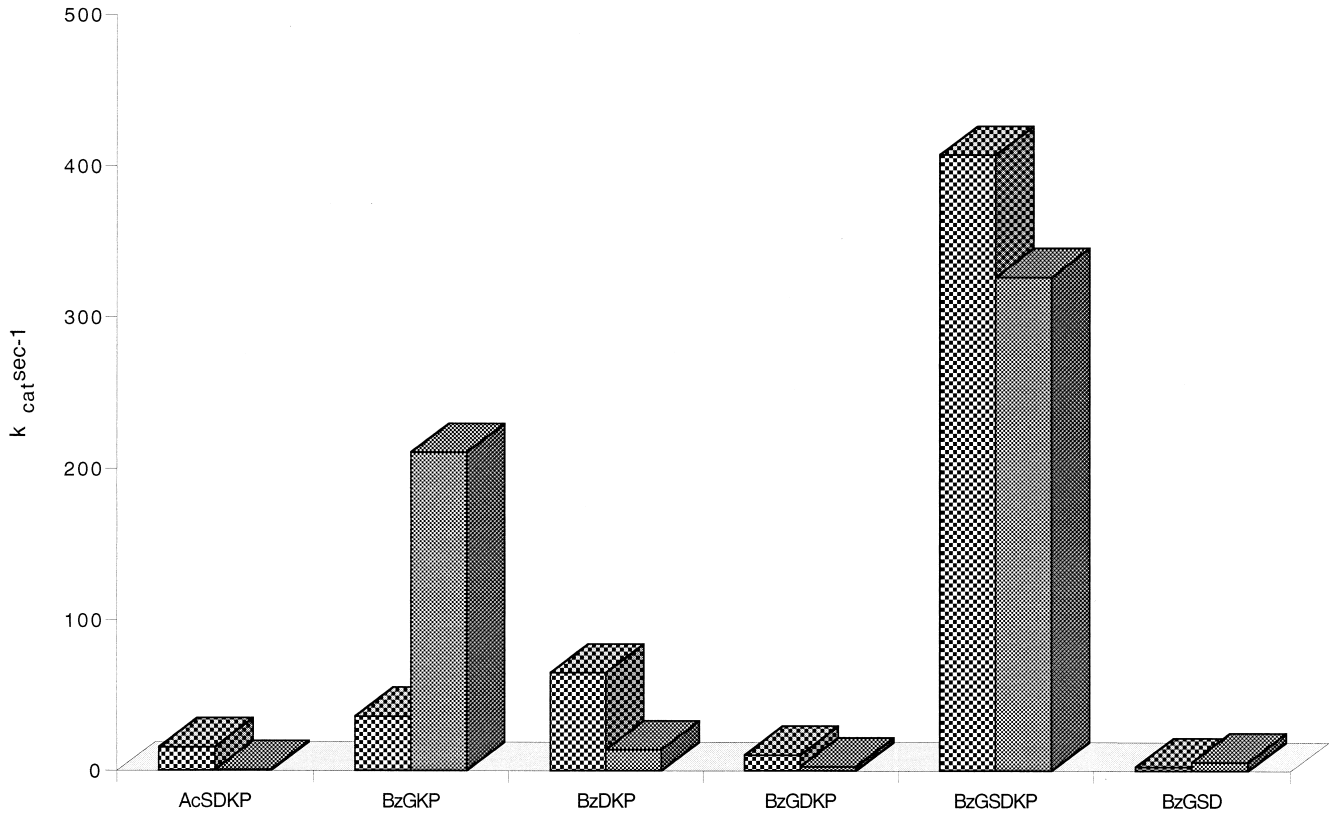



**FIG. 2. Turnover rate of the ACE N- or C-domains according to substrates. ▩ N-domain $k_{cat}$ $sec^{-1}$ and ▨ C-domain $k_{cat}$ $sec^{-1}$.**

by both ACE domains. Conversely, BzGKP is hydrolyzed equally well by the two ACE domains, whereas AcSDKP is an N-domain-specific substrate. It is possible that the presence of a benzoyl-glycine moiety in the P1 position in place of a phenylalanine residue increases the affinity for the C-domain. This hypothesis is supported by the observation that benzyloxycarbonyl Phe-His-Leu is cleaved almost equally well by the two ACE domains [27]. The incorporation of a glycine residue in the substrate P1 position may reduce its interaction with the S1 N-domain subsite. In the present study, we determined the importance of an aspartic acid in the P1 position for N-domain specificity. Most of the observed effects are due to the ionic interactions of the acidic residue in the P1 position with a putative basic side-chain in the S1 N-domain subsite. The present data suggest that the S1 N-domain subsite is formed by more polar groups than the S1 C-domain. Another result from this study is the interference of the P2 and P3 positions in the increasing or decreasing of N-terminal active site selectivity. Figure 1 underlines the preference for a residue with an alcohol function (serine residue) versus a hippuric acid for the S2 N-domain subsite. Interestingly enough, Ang 1–7 (which is more efficiently cleaved by the N-domain than by the C-domain) possesses a tyrosine residue with an alcohol function in P2. Altogether, these results show the importance of the P2 position in the N-active site preference.

The specificity of a substrate towards the N-domain seems to decrease with the length of the peptide, at least in the case of AcSDKP derivative peptides, as illustrated in this study when a hippuric acid is introduced in the P3/P4 position. This result suggests that a distant binding site, away from the catalytic site, is used. This interaction with the S3 subsite is in accordance with the hypothesis that the much higher affinities of bradykinin and Ang I for ACE are due, in large part, to interactions with binding sites distant from the catalytic site [28, 29]. It may be that the specificity of a particular active site decreases with longer peptide substrates. In this case, distal interactions permit accurate substrate alignment and abolish the specific preferences in the P′2-P2 positions responsible for a domain-selective configuration, at the expense of a lower catalytic efficiency. In the case of Ang 1–7, the binding is markedly increased and the N-specificity is followed by an additional inhibitory effect which may be due to peptide aminoacid residues located in distal positions.

Two series of AcSDKP analogs have recently been reported. In one series, the peptide bonds have been replaced by an aminomethylene bond and in the second, the C-terminus of the peptide has been modified by decarboxylation or amidation. Rabbit lung ACE is unable to hydrolyze these compounds, except for decarboxylated AcSDKP [30]. Conversely, AcSDKP hydrolysis is not inhibited by any of these compounds [30], showing the importance of the peptide bonds, even at some distance from the scissile bond.

In conclusion, we have shown that the side-chain preference of N-domain subsites is limited to a tetra- or a pentapeptide. Other combinations of aminoacids in P3-P′2 may also be operational since small structural changes may be important for specificity. A complex interaction of chemical binding forces with separate effects on $k_{cat}$ and $K_m$ plays a great part in this selectivity (additional hydrogen bonds, electrostatic or ionic interactions). The determining factor for N- or C-domain specificity is not only the nature of the aminoacid side-chain present in the neighborhood of the scissile bond but also the size of the substrate molecule. Substrates longer than five residues may involve interdependence between subsites. Indeed, the enzymatic characteristics of substrate hydrolysis are better explained if the entire substrate binding site is taken as an entity rather than several distinct S and S′ subsites. Finally, based on the present experiments, the discovery of highly reactive novel substrates cannot be predicted from single subsite mapping. Instead, combinatorial approaches with multiple aminoacid substitution in different subsites might help to discover new selective ACE domain substrates.

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## References

1. Soubrier F, Alhenc-Gélas F, Hubert C, Allegrini J, John M, Tregear G and Corvol P, Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proc Natl Acad Sci* USA **85:** 9386–9390, 1988.
2. Williams TA, Corvol P and Soubrier F, Identification of two active site residues in human angiotensin I-converting enzyme. *J Biol Chem* **269:** 29430–29434, 1994.
3. Lattion A-L, Soubrier F, Allegrini J, Hubert C, Corvol C and Alhenc-Gélas F, The testicular transcript of the angiotensin I-converting enzyme encodes for the ancestral, non-duplicated form of the enzyme. *FEBS Lett* **252:** 99–104, 1989.
4. Ehlers MRW, Fox EA, Strydom DJ and Riordan JF, Molecular cloning of human testicular angiotensin-converting enzyme: The testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. *Proc Natl Acad Sci* USA **86:** 7741–7745, 1989.
5. Skidgel RA and Erdös EG, Novel activity of human angiotensin I converting enzyme: Release of the $NH_2$– and COOH-terminal tripeptides from the luteinizing hormone-releasing hormone. *Proc Natl Acad Sci* USA **82:** 1025–1029, 1985.
6. Skeggs LT, Kahn JR and Shumway NP, Preparation and function of the hypertension converting enzyme. *J Exp Med* **103:** 295–299, 1956.
7. Yang H-YT, Erdös EG and Levin Y, A dipeptidyl carboxypeptidase that converts angiotensin I and inactivates bradykinin. *Biochim Biophys Acta* **214:** 374–376, 1970.
8. Rousseau A, Michaud A, Chauvet M-T, Lenfant M and Corvol P, The hemoregulatory peptide *N*-acetyl-Ser-Asp-Lys-Pro is a natural and specific substrate of the N-terminal active site of human angiotensin-converting enzyme. *J Biol Chem* **270:** 3656–3661, 1995.
9. Azizi M, Rousseau A, Ezan E, Guyene T-T, Michelet S, Grognet J-M, Lenfant M, Corvol P and Ménard J, Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator *N*-acetyl-seryl-aspartyl-lysyl-proline. *J Clin Invest* **97:** 839–844, 1996.
10. Krege JH, John SWN, Langenbach LL, Hodgin JB, Hagaman JR, Bachman ES, Jennette JC, O'Brien DA and Smithies O, Male–female differences in fertility and blood pressure in ACE-deficient mice. *Nature* **375:** 146–148, 1995.
11. Esther CR, Howard JTE, Marino EM, Goddard JM, Capecchi MR and Bernstein KE, Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology and reduced male fertility. *Lab Invest* **74:** 953–965, 1996.
12. Hagaman JR, Moyer JS, Bachman ES, Sibony M, Magyar PL, Welch JE, Smithies O, Krege JH and O'Brien DA, Angiotensin-converting enzyme and male fertility. *Proc Natl Acad Sci* USA **95:** 2552–2557, 1998.
13. Wei L, Alhenc-Gélas F, Corvol P and Clauser E, The two homologous domains of human angiotensin I-converting enzyme are both catalytically active. *J Biol Chem* **266:** 9002–9008, 1991.
14. Jaspard E, Wei L and Alhenc-Gélas F, Differences in the properties and enzymatic specificities of the two active sites of angiotensin I-converting enzyme (kinase II). Studies with bradykinin and other natural peptides. *J Biol Chem* **268:** 9498–9503, 1993.
15. Deddish PA, Marcic B, Jackman HL, Wang H-Z, Skidgel RA and Erdös EG, N-domain-specific substrate and C-domain inhibitors of angiotensin-converting enzyme: angiotensin-(1–7) and keto-ACE. *Hypertension* **31:** 912–917, 1998.
16. Michaud A, Williams TA, Chauvet M-T and Corvol P, Substrate dependence of angiotensin I-converting enzyme inhibition: captopril displays a partial selectivity for inhibition of *N*-acetyl-seryl-aspartyl-lysyl-proline hydrolysis compared with that of angiotensin I. *Mol Pharmacol* **51:** 1070–1076, 1997.
17. Bünning P, Holmquist B and Riordan J, Specificity and kinetic characteristics of angiotensin converting enzyme. *Biochemistry* **22:** 103–110, 1983.
18. Cheung H-S, Wang F-L, Ondetti M-A, Sabo E and Cushman D, Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. *J Biol Chem* **255:** 401–407, 1980.
19. Pfuetzner R and Chan W-C, Synergistic binding of ligands to angiotensin-converting enzyme. *J Biol Chem* **263:** 4056–4058, 1988.
20. Schechter I and Berger A, On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun* **27:** 157–162, 1967.
21. Beldent V, Michaud A, Wei L, Chauvet MT and Corvol P, Proteolytic release of human angiotensin-converting enzyme: Localization of the cleavage site. *J Biol Chem* **268:** 26428–26434, 1993.
22. Alhenc-Gélas F, Weare JA, Johnson RL and Erdös EG, Measurement of human converting enzyme level by direct radioimmunoassay. *J Lab Clin Med* **101:** 83–96, 1983.
23. Cushman D and Cheung H, Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem Pharmacol* **20:** 1637–1648, 1971.
24. Wei L, Alhenc-Gélas F, Soubrier F, Michaud A, Corvol P and Clauser E, Expression and characterization of recombinant human angiotensin I-converting enzyme: Evidence for a C-terminal transmembrane anchor and for a proteolytic processing of the secreted recombinant and plasma enzymes. *J Biol Chem* **266:** 5540–5546, 1991.
25. Perich RB, Jackson B and Johnston CI, Structural constraints

of inhibitors for binding at two active sites on somatic angiotensin converting enzyme. *Eur J Pharmacol* **266:** 201–211, 1994.
26. Pascard C, Guilhem J, Vincent M, Rémond G, Portevin B and Laubie M, Configuration and preferential solid-state conformations of perindoprilat (S-9780). Comparison with the crystal structures of other ACE inhibitors and conclusions related to structure–activity relationships. *J Med Chem* **34:** 663–669, 1991.
27. Danilov S, Jaspard E, Churakova T, Towbin H, Savoie F, Wei L and Alhenc-Gélas F, Structure–function analysis of angiotensin I-converting enzyme using monoclonal antibodies. Selective inhibition of the amino-terminal active site. *J Biol Chem* **269:** 26806–26814, 1994.
28. Ryan JW, Angiotensin-converting enzyme, dipeptidyl carboxypeptidase I, and its inhibitors. *Methods Enzymol* **163:** 194–210, 1988.
29. Skidgel RA, Defendini R and Erdös EG, Angiotensin I converting enzyme and its role in neuropeptide metabolism. In: *Neuropeptides and Their Peptidases*. (Ed. Turner AJ), pp. 165–182. Ellis Horwood Ltd., Chichester, U.K. 1987.
30. Gaudron S, Adeline M-T, Potier P and Thierry J, NAcSDKP analogues resistant to angiotensin-converting enzyme. *J Med Chem* **40:** 3963–3968, 1997.