

# 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

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## SUMMARY

Angiotensin I-converting enzyme (ACE) is composed of two highly similar domains (referred to here as the N and C domains) that play a central role in blood pressure regulation; ACE inhibitors are widely used in the treatment of hypertension. However, the negative regulator of hematopoiesis, *N*-acetyl-seryl-aspartyl-lysyl-prolyl (AcSDKP), is a specific substrate of the N domain-active site; thus, in addition to the cardiovascular function of ACE, the enzyme may be involved in hematopoietic stem cell regulation, raising the interest of designing N domain-specific ACE inhibitors. We analyzed the inhibition of angiotensin I and AcSDKP hydrolysis as well as that of three synthetic ACE substrates by wild-type ACE and the N and C domains by

using a range of specific ACE inhibitors. We demonstrate that captopril, lisinopril, and fosinoprilat are potent inhibitors of AcSDKP hydrolysis by wild-type ACE, with  $K_i$  values in the subnanomolar range. However, of the inhibitors tested, captopril is the only compound able to differentiate to some degree between AcSDKP and angiotensin I inhibition of hydrolysis by wild-type ACE: the  $K_i$  value with AcSDKP as substrate was 16-fold lower than that with angiotensin I as substrate. This raises the possibility of using captopril to enhance plasma AcSDKP levels with the aim of normal hematopoietic stem cell protection during chemotherapy and a limited effect on the cardiovascular function of ACE.

ACE (peptidyl dipeptidase A; EC 3.4.15.1) is a zinc metalloenzyme anchored to the plasma membrane, with the bulk of its mass, including its active sites, exposed at the extracellular surface of the cell. The enzyme displays a wide tissue distribution in that it is expressed at the surface of endothelial, epithelial, and neuroepithelial cells (1). Two distinct isoforms of ACE are expressed in mammalian tissues: the predominant isoform (referred to here as somatic ACE) is composed of two highly similar domains (called N and C domains) with a molecular mass of 170 kDa (2). The other isoform is expressed exclusively in mature spermatids (germinal ACE); it is composed of a single catalytic domain with a molecular mass of 100 kDa and is identical to the C domain of somatic ACE with the exception of a short amino-terminal germinal ACE-specific sequence (3).

The primary specificity of the enzyme is to remove carboxyl-terminal dipeptides from the carboxyl terminus of an oli-

gopeptide substrate, although other, atypical cleavages have been described (4, 5). The classic physiological substrates of ACE are Ang I, which is converted to the vasopressor Ang II by the removal of a single carboxyl-terminal dipeptide (6), and the vasodilatory peptide bradykinin, which is inactivated by the sequential removal of two carboxyl-terminal dipeptides (7). Thus, ACE plays a central role in blood pressure homeostasis, and a large number of highly potent and specific ACE inhibitors have been developed that are used as orally active drugs in the treatment of hypertension and congestive heart failure. The current generation of ACE inhibitors exert their action by coordinating the catalytically essential active site zinc ion and were designed on the basis of the known active site of another zinc metalloenzyme, carboxypeptidase A. However, a new generation of ACE inhibitors has been developed that have been synthesized with the aim of inhibiting not only ACE but also other zinc metallopeptidases involved in cardiovascular regulation. Such mixed inhibitors have been designed successfully for ACE-

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**ABBREVIATIONS:** ACE, angiotensin I-converting enzyme; Ang I, angiotensin I; Ang II, angiotensin II; AcSDKP, *N*-acetyl-seryl-aspartyl-lysyl-prolyl; Hip, hippuryl-(benzoyl-glycyl); HPLC, high performance liquid chromatography; CHO, Chinese hamster ovary; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; N domain, amino-terminal extracellular part of the protein; C domain, carboxyl-terminal extracellular part of the protein.

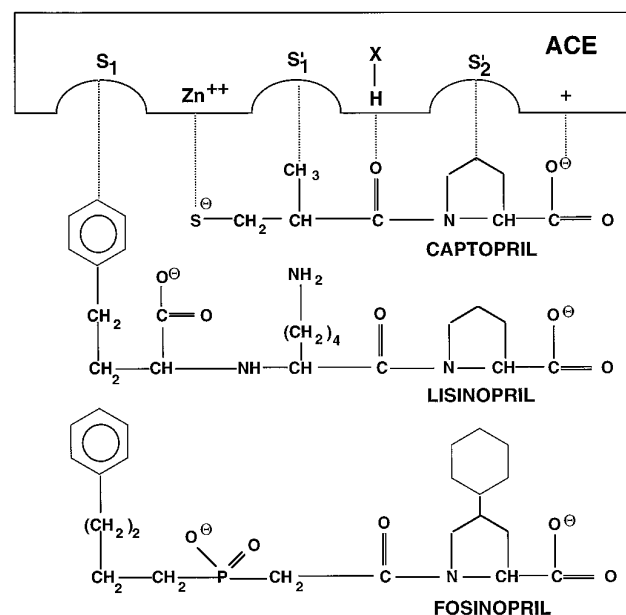
neutral endopeptidase (8) and for ACE-thromboxane synthase (9).

In addition to the well-established roles of ACE in Ang I and bradykinin metabolism, ACE has recently been demonstrated to be involved both *in vitro* (10) and *in vivo* (11) in the hydrolysis of AcSDKP, a regulatory factor of hematopoiesis. A competitive ACE inhibitor, captopril, has been shown to inhibit angiogenesis by being able to block neovascularization *in vivo* (12). Therefore, the physiological functions of ACE are not limited to its cardiovascular role, and ACE may be involved in hematopoietic stem cell regulation by constantly degrading AcSDKP, a natural circulating inhibitor of cell entry into S phase. Interestingly, AcSDKP is hydrolyzed 50-fold faster by the N domain-active site compared with the C domain and constitutes the only natural and specific substrate identified to date of the N domain of somatic ACE. This newly discovered function of ACE reveals that ACE inhibitors, which inhibit both the N and C domain-active sites of somatic ACE, can act on peptides involved in functions unrelated to blood pressure and water and salt homeostasis.

A series of ACE mutants containing only one intact domain were used to demonstrate that the N and C domains exhibit similar catalytic activities toward Ang I, bradykinin, and substance P (13). However, the N and C domains exhibit some interesting functional differences: (a) the two active sites are activated differently by chloride ions; in particular, the C domain-active site is far more sensitive to chloride concentration for the hydrolysis of some substrates, (b) the ACE N domain catalyzes both *in vitro* and *in vivo* AcSDKP degradation, and (c) the two domains of ACE interact differently with competitive inhibitors, and the chloride concentration has a more marked effect on inhibitor interaction with the C domain than with the N domain (14). These functional differences indicate that despite the high level of primary sequence homology between the N and C domains, structural differences do exist between the two active sites of somatic ACE, raising the possibility of designing or screening for ACE domain-specific inhibitors. The development of active site-specific inhibitors is of considerable interest: a specific inhibitor of the N domain would increase plasma AcSDKP levels without altering Ang I and bradykinin metabolism by the C domain. Such an inhibitor would be of potential value during chemotherapy in the protection of hematopoietic stem cells without affecting cardiovascular function.

To identify an ACE inhibitor that displays some selectivity for the inhibition of AcSDKP hydrolysis compared with that of Ang I, we used a range of specific ACE inhibitors to analyze the inhibition of hydrolysis of these two substrates by somatic ACE and two full-length ACE mutants that contain a single functional domain. The ACE inhibitors used in this study were chosen on the basis of their interaction with the inhibitor binding subsites of ACE as well as the coordination of the zinc ion or ions in the ACE active site or sites: captopril and fosinopril coordinate the active site zinc ion via their sulfhydryl and phosphinyl groups, respectively, whereas lisinopril binds the zinc ion via its carboxyl group (Fig. 1). In addition, with a view to the large-scale screening of preexisting compounds for a N domain-specific inhibitor, we tested the inhibition (by using the same ACE inhibitors described above) of wild-type somatic ACE and the N and C domains using three synthetic substrates: (a) Hip-His-Leu, which

## INHIBITORS



ACE:	S <sub>1</sub>	S <sub>1</sub> '	S <sub>2</sub>
<b>Synthetic substrates</b>			
Bz-Gly	-	His	-
Bz-Gly	-	Lys	-
Bz-Gly	-	Ala	-
<b>Natural substrates</b>			
AngI- - -Phe <sub>8</sub>	-	His <sub>9</sub>	-
AcSDKP - Asp <sub>2</sub>	-	Lys <sub>3</sub>	-

Fig. 1. Schematic representation of the binding of inhibitors and substrates to the active site of ACE.

mimics the carboxydiptide of Ang I; this peptide is preferentially cleaved by the ACE C domain and has been routinely used for ACE inhibitor screening, (b) Hip-Lys-Pro, which mimics the carboxydiptide of AcSDKP and as such is a potential specific synthetic substrate of the N domain, and (c) Hip-Ala-Pro, which mimics the dipeptide of captopril and is an example of a substrate whose hydrolysis is not chloride dependent (15).

## Experimental Procedures

### Materials

**Enzymes.** Wild-type somatic ACE and the two ACE mutants containing only one functional active site were obtained through stable expression from CHO cells transfected with the appropriate ACE cDNA. 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 by lysyl residues (ACE<sub>K361,365</sub> and ACE<sub>K959,963</sub>). These mutants are hereafter referred to as the N and C domains, indicating the sole catalytically active domain. The construction of the ACE cDNAs and their expression in CHO cells have been previously described (16). The purification of the corresponding membrane-bound proteins was performed according to Beldent *et al.* (17), except that the chromatographic steps were replaced by ultrafiltration on an Ultrafree-MC membrane, nominal molecular mass limit, 100,000 D (Millipore, Bedford, MA).

The enzymatic activities of the recombinant enzymes were determined using Hip-His-Leu as substrate according to Cushman and

Cheung (18), and the released Hip was quantified by HPLC as described previously (19). ACE concentrations were quantified by direct radioimmunoassay (20) or deduced from their enzymatic activities. A relative molecular mass of 170,000 for ACE was used for the calculation of  $k_{\text{cat}}$  values.

**Peptides.** Hip-His-Leu, His-Leu, AcSDKP, Lys-Pro, Ala-Pro, and Ang I were purchased from Bachem (Bubendorf, Switzerland). Ang II and hippuric acid (Hip) were from Sigma Chemical (Poole, Dorset, UK). Hip-Lys-Pro and Hip-Ala-Pro were synthesized by Neosystem (Strasbourg, France). The purity of these last two peptides was greater than 90%.

**Inhibitors.** Captopril and fosinoprilat were a gift from Bristol-Myers Squibb (Princeton, NJ) and lisinopril was donated by Merck Sharp and Dohme (Paris, France).

## Methods

Kinetic and inhibition studies of wild-type ACE and ACE mutants were performed using Hip-His-Leu, Hip-Lys-Pro, Hip-Ala-Pro, Ang I, and AcSDKP as substrates in the presence of different ACE inhibitors. For each enzyme and substrate, the optimal  $\text{Cl}^-$  concentration was first determined as described below.

The rate of hydrolysis of all substrates used was quantified by HPLC on a Waters (Milford, MA) apparatus consisting of 600 controllers, 486 absorbance detectors and 717 or 712 autosamplers WISP directed by a millennium chromatography manager.

**Hip-Lys-Pro and Hip-Ala-Pro hydrolysis.** Reactions were performed using  $0.1\text{--}0.5 \times 10^{-9}$  M enzyme in 100 mM potassium phosphate, pH 8.0, 10  $\mu\text{M}$   $\text{ZnSO}_4$ , 20 mM or 100 mM NaCl, and 1 mg/ml BSA. Reactions were initiated by the addition of Hip-Lys-Pro or Hip-Ala-Pro in a total volume of 250  $\mu\text{l}$  and incubated for 10 or 15 min at 37° to produce 5% substrate hydrolysis. Reactions were terminated by the addition of 12%  $\text{H}_3\text{PO}_4$  (50  $\mu\text{l}$ ). Hip and Hip-Lys-Pro were resolved and quantified by isocratic reverse-phase HPLC on a 10- $\mu\text{m}$  Nucleosil C18 column in 24% (v/v) acetonitrile, and 1 mM potassium phosphate, pH 3.0, at a flow rate of 1.0 ml/min. Retention time for Hip and Hip-Lys-Pro was 6.7 and 4.94 min, respectively, with a detection limit of 0.1 nmol at 228 nm. Hip and Hip-Ala-Pro were resolved and quantified as for Hip and Hip-Lys-Pro but in 15% (v/v) acetonitrile and 1 mM potassium phosphate, pH 3.0. Retention time for Hip and Hip-Ala-Pro was 8.8 and 12.0 min, respectively, with a detection limit of 0.1 nmol at 228 nm.

The pH dependence of Hip-Lys-Pro or Hip-Ala-Pro hydrolysis was determined in 100 mM Tris-maleate buffer over a pH range of 5.0–9.2. Assays were also performed using 100 mM HEPES, pH 6.8–8.0, or potassium phosphate, pH 5.5–8.0, buffers. Chloride activation was determined in 100 mM potassium phosphate, pH 8.0, and 0–600 mM NaCl.

After the determination of optimal conditions for substrate hydrolysis, the kinetic parameters for Hip-Lys-Pro (pH 8.0 and 20 mM NaCl) and Hip-Ala-Pro (pH 8.0 and 100 mM NaCl) hydrolysis by the three different enzymes were calculated from Lineweaver-Burk plots. Initial velocities were measured over a substrate concentration range of 0.02–1.5 mM.

**Ang I hydrolysis.** Reactions were performed using  $0.1\text{--}0.2 \times 10^{-9}$  M enzyme in 50 mM HEPES, pH 7.5, 50 mM NaCl, 10  $\mu\text{M}$   $\text{ZnSO}_4$ , and 1 mg/ml BSA and stopped by the addition of 2%  $\text{H}_3\text{PO}_4$  (final concentration). Ang II and Ang I were resolved and quantified by isocratic reverse-phase HPLC in 18% (v/v) acetonitrile and 86 mM triethylammonium phosphate, pH 3.0, at a flow rate of 1 ml/min on a 3- $\mu\text{m}$  Hypersil C18 column at 45°. Retention time of Ang II and Ang I was 3.4 and 7.0 min, respectively, with a detection limit of 0.5 nmol at 200 nm.

**AcSDKP hydrolysis.** Reactions were performed using  $0.2\text{--}2 \times 10^{-9}$  M enzyme in 100 mM HEPES, pH 7.0, 50 mM NaCl, 10  $\mu\text{M}$   $\text{ZnSO}_4$ , and 1 mg/ml BSA and terminated by the addition of 0.1% trifluoroacetic acid (final concentration). The rate of hydrolysis of AcSDKP was determined by measuring the production of Lys-Pro, which was resolved and quantified under isocratic conditions [water/

acetonitrile (98:2), 0.1% trifluoroacetic acid]. Unhydrolyzed AcSDKP was eluted and quantified by increasing the concentration of acetonitrile to 10%. The products were resolved by reverse-phase HPLC on a 5- $\mu\text{m}$  Puresil C18 column at a flow rate of 1 ml/min. Retention time of Lys-Pro and AcSDKP was 4.7 and 13.2 min, respectively, with a detection limit of 0.2 nmol at 200 nm.

**Enzyme inhibition assays.** The potency of each inhibitor toward each form of ACE was determined by establishing dose-dependent inhibition curves at equilibrium. Because all the compounds tested are competitive slow-tight binding inhibitors, the preincubation time required to produce steady state equilibrium between enzyme and inhibitor was first determined. Preincubations were performed using  $0.1 \times 10^{-9}$  M enzyme and  $0.25\text{--}1 \times 10^{-9}$  M inhibitor at 37° for 0–120 min, and reactions were initiated by the addition of substrate. A preincubation of 1 hr was required for fosinoprilat, whereas the inhibition by captopril and lisinopril was unchanged for a 0.5–2-hr preincubation. Enzyme activities in the absence of inhibitor did not change during control incubations of 3 hr, demonstrating enzyme stability for  $\geq 3$  hr at 37°.

The optimal chloride concentration for each substrate hydrolysis was determined and was 50 mM NaCl for Ang I and AcSDKP, 20 mM NaCl for Hip-Lys-Pro, 100 mM NaCl for Hip-Ala-Pro, and 300 mM for Hip-His-Leu. For inhibition assays, a range of inhibitor concentrations was used at two different substrate concentrations ( $0.5 \times K_m$  and  $3 \times K_m$ ) at a fixed concentration of enzyme. Inhibition of Ang I, Hip-Ala-Pro, and Hip-Lys-Pro hydrolysis was determined using  $0.1 \times 10^{-9}$  M wild-type ACE and  $0.2 \times 10^{-9}$  M N and C domains; for the inhibition of Hip-His-Leu hydrolysis,  $0.1 \times 10^{-9}$  M concentration of both wild-type and C domain and  $1 \times 10^{-9}$  M N domain were used; and  $0.2 \times 10^{-9}$  M wild-type,  $0.4 \times 10^{-9}$  M N domain, and  $2 \times 10^{-9}$  M C domain were used for the inhibition of AcSDKP hydrolysis. Enzymes were preincubated in the absence or presence of inhibitor (five inhibitor concentrations in the range of  $1 \times 10^{-11}$  to  $5 \times 10^{-8}$  M) at 37° for 1 hr, and reactions were initiated by substrate addition at two different concentrations. Reactions were performed for 5, 10, 30, and 60 min. Inhibitor potency was determined by calculation of apparent  $K_i$  values derived from Henderson plots: for each fixed substrate concentration,  $[I_i]/(1 - v_i/v_0)$  was plotted versus  $v_0/v_i$ , where  $[I_i]$  is the total concentration of inhibitor, and  $v_0$  and  $v_i$  are initial velocities in the absence and presence of inhibitor, respectively. The slope of each line represents the apparent  $K_i$  value and a plot of apparent  $K_i$  versus  $[S]$  (substrate concentration) gives the  $K_i$  value by extrapolation of the apparent  $K_i$  to  $[S] = 0$ . Results were confirmed using ENZFITTER software (21).

The potential inhibition of ACE by the hydrolysis products Ala-Pro and Lys-Pro was investigated using Ang I as substrate after preincubation (30 min) of ACE with different concentrations of Ala-Pro and Lys-Pro ( $1 \times 10^{-9}$  to  $1 \times 10^{-5}$  M). The concentration of each form of ACE corresponded to that used for the determination of catalytic constants as described above. The potential inhibition of ACE by the hydrolysis product His-Leu was investigated as discussed above using Hip-Ala-Pro as substrate.

## Results

Kinetic studies of wild-type ACE and the ACE mutants were performed using Hip-His-Leu, Hip-Lys-Pro, Hip-Ala-Pro, Ang I and AcSDKP, as substrates. We tested the potential inhibitory action of the dipeptide products generated from the hydrolysis of the five substrates: Lys-Pro, Ala-Pro, and His-Leu. Inhibition of ACE activity was detected only at high concentrations of either Lys-Pro or Ala-Pro using Ang I as substrate: Lys-Pro and Ala-Pro displayed apparent  $K_i$  values of 0.5–3 mM and 0.9–5 mM, respectively (Table 1). His-Leu displayed an apparent  $K_i$  value of 0.2 mM for the inhibition of Hip-Ala-Pro hydrolysis by wild-type ACE and the C domain and an apparent  $K_i$  value of 0.7 mM for the



TABLE 1  
Inhibitory potency of dipeptides generated after hydrolysis of the five substrates by the three ACE forms

ACE	$K_{app}$				
	Lys-Pro <sup>a</sup>		Ala-Pro <sup>a</sup>	His-Leu <sup>b</sup>	
	<i>mM</i>				
Wild-type	1.37 ± 1.32 <sup>c1</sup>	1.17 ± 1.03 <sup>c2</sup>	0.87 ± 0.63 <sup>c3</sup>	0.17 ± 0.07 <sup>c4</sup>	0.17 ± 0.07 <sup>c5</sup>
N domain	0.52 ± 0.51 <sup>c1</sup>	2.36 ± 0.26 <sup>c2</sup>	5.20 ± 2.50 <sup>c3</sup>	0.65 ± 0.25 <sup>c4</sup>	0.13 ± 0.06 <sup>c5</sup>
C domain	3.30 ± 0.20 <sup>c1</sup>	1.19 ± 0.61 <sup>c2</sup>	3.11 ± 0.08 <sup>c3</sup>	0.15 ± 0.05 <sup>c4</sup>	0.17 ± 0.07 <sup>c5</sup>

Values are mean  $\pm$  standard error from two independent determinations with ENZFITTER software for five peptide concentrations.

<sup>a</sup> Ang I as substrate.

<sup>b</sup> Hip-Ala-Pro as substrate.

<sup>c</sup> Concentration of each form of ACE was equal to that used for the hydrolysis of <sup>c1</sup>Hip-Lys-Pro, <sup>c2</sup>AcSDKP, <sup>c3</sup>Hip-Ala-Pro, <sup>c4</sup>Hip-His-Leu, or <sup>c5</sup>Ang I, as described in Experimental Procedures.

inhibition of hydrolysis by the N domain with enzyme concentration equal to that used for the hydrolysis of Hip-His-Leu (Table 1). These high apparent  $K_i$  values of the hydrolysis products indicate a negligible effect on the determination of inhibition constants.

#### Characterization of Two ACE Synthetic Substrates: Hip-Lys-Pro and Hip-Ala-Pro

To identify a hippuryl-dipeptide substrate more specific for the N domain-active site than the C domain-active site, Hip-Lys-Pro, a novel ACE substrate, and Hip-Ala-Pro were incubated with wild-type ACE and the N or C domain active forms of ACE, and the rate of hydrolysis of the two substrates was calculated from Hip production.

**pH dependence and chloride activation of Hip-Lys-Pro and Hip-Ala-Pro hydrolysis.** Wild-type ACE and the N and C domains displayed an optimal cleavage of Hip-Lys-Pro at pH 8 (Fig. 2A, *inset*). In addition, the optimum chloride concentration for the hydrolysis of this substrate was  $\sim 20$  mM for all three enzymes, with an inhibition of substrate hydrolysis observed at supraoptimal chloride concentrations ( $>200$  mM, Fig. 2A). In the absence of chloride ions, the N domain displayed 80% of optimal activity compared with 10% optimal activity for the C domain.

The pH profile for the hydrolysis of Hip-Ala-Pro was determined over the same pH range as for Hip-Lys-Pro (Fig. 2B, *inset*). Wild-type somatic ACE and the N and C domains displayed a pH optimum of pH 8.0 for Hip-Ala-Pro hydrolysis, and this hydrolysis was markedly reduced at pH  $< 7.5$  and pH  $> 8.5$ . All forms of ACE exhibited an optimal activity at 100 mM chloride at pH 8.0. Sixty percent optimal activity was observed in the absence of chloride for the N domain compared with 0% for the C domain (Fig. 2B).

**Kinetic parameters of Hip-Lys-Pro and Hip-Ala-Pro hydrolysis.** The kinetic parameters of Hip-Lys-Pro hydrolysis were determined under optimal conditions of 20 mM NaCl, pH 8.0. The  $K_m$  values calculated from Lineweaver-Burk plots of Hip-Lys-Pro hydrolysis by wild-type ACE and the N and C domains were 112, 74, and 169  $\mu\text{M}$ , respectively, with  $k_{cat}$  values of 167, 36, and 211  $\text{sec}^{-1}$  (mean of three experiments). As shown in Table 2, wild-type ACE and the C domain exhibit a similar  $k_{cat}/K_m$  value, which is 3-fold higher than that for the N domain.

The kinetic parameters of Hip-Ala-Pro hydrolysis were determined in the presence of 100 mM NaCl, pH 8.0. The  $K_m$  values of Hip-Ala-Pro hydrolysis by wild-type ACE and the N and C domains were 50, 21, and 66  $\mu\text{M}$ , respectively, with  $k_{cat}$

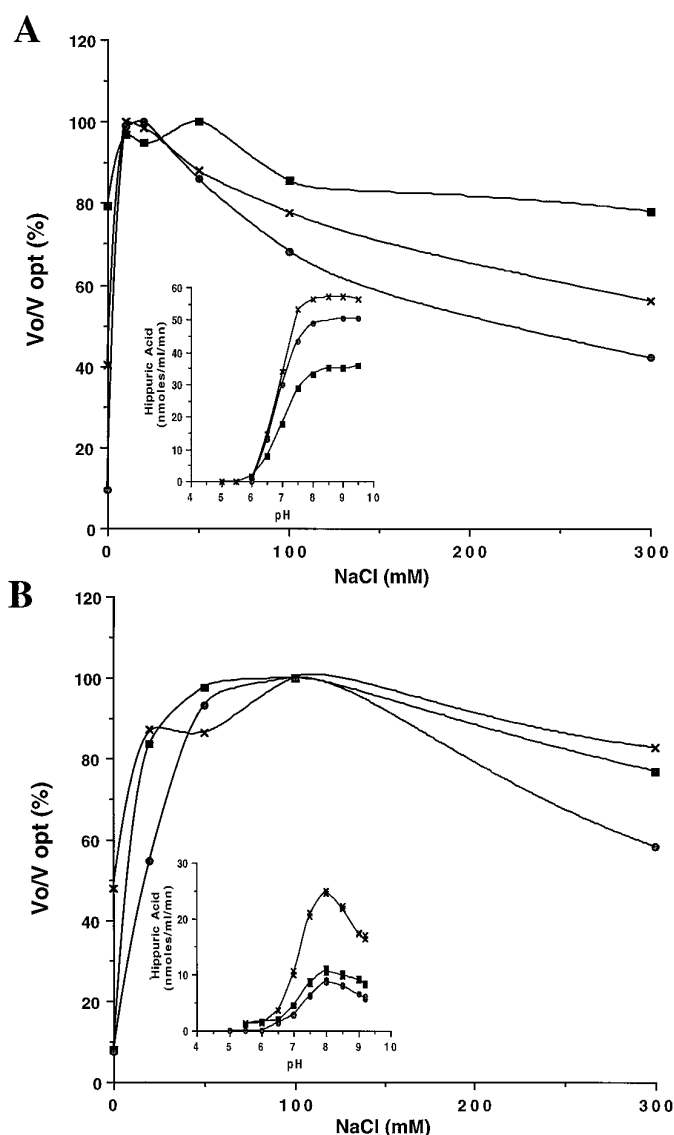


Fig. 2. pH and chloride dependence of (A) Hip-Lys-Pro and (B) Hip-Ala-Pro hydrolysis by recombinant forms of ACE:  $\times$ , wild-type ACE;  $\bullet$ , C domain; and  $\blacksquare$ , N domain. *Inset*, pH dependence: Hip production (nmol/ml/min) was quantified as described in Experimental Procedures and plotted against pH value. For the chloride dependence of enzyme activity, the velocity ( $v_0$ ) was measured at optimal pH over a range of NaCl concentration (0–600 mM), and the optimal velocity was calculated for each point. The ratio  $v_0/v_{opt}$  was plotted against the NaCl concentration.

TABLE 2

Kinetics parameters ( $k_{cat}/K_m$ ) of recombinant forms of ACE acting on synthetic and natural substrates

ACE	Hip-Lys-Pro <sup>a</sup>	Hip-Ala-Pro <sup>a</sup>	Hip-His-Leu <sup>c</sup>	AcSDKP <sup>b</sup>	Ang I <sup>c</sup>
	$\mu\text{M}/\text{SEC}$				
Wild-type	1.49 $\pm$ 0.26	5.28 $\pm$ 2.19	0.26	0.30	2.50
N domain	0.48 $\pm$ 0.04	1.20 $\pm$ 0.10	0.02	0.50	0.73
C domain	1.25 $\pm$ 0.24	4.68 $\pm$ 1.81	0.23	0.01	1.88
Optimal Cl <sup>-</sup> concentration (mM)	20	100	300	50	50

<sup>a</sup> Values are mean  $\pm$  standard error from two or three independent experiments.<sup>b</sup> Values are from Rousseau et al. (10).<sup>c</sup> Values are from Wei et al. (16).

values of 264, 25, and 305 sec<sup>-1</sup>. The  $k_{cat}/K_m$  value for Hip-Ala-Pro hydrolysis by the C domain active site is 4-fold higher than that for the N domain (Table 2).

### Comparison of ACE Inhibitor Potency for the Hydrolysis of Synthetic and Natural Substrates by Wild-Type ACE

The  $K_i$  values for the inhibition of Hip-His-Leu or Ang I hydrolysis are similar for each inhibitor but otherwise vary markedly according to the substrate used; for example, lisinopril has a  $K_i$  value of 3.9 nM for the inhibition of Hip-Lys-Pro hydrolysis, whereas the same inhibitor displays a  $K_i$  value of 0.39 nM with Hip-His-Leu as substrate. Similarly, the  $K_i$  value of captopril for the inhibition of AcSDKP hydrolysis is 17-fold lower than that with Hip-Lys-Pro as substrate (Table 3). For the inhibition of physiological substrate hydrolyses, captopril is 16-fold more potent for the inhibition of AcSDKP hydrolysis compared with the inhibition of Ang I hydrolysis. In contrast, lisinopril is equally potent for the inhibition of these two substrate hydrolyses, whereas fosinoprilat is 6-fold more efficient for inhibition of AcSDKP hydrolysis compared with Ang I hydrolysis.

### Comparison of ACE Inhibitor Potency for the Hydrolysis of Different Substrates by the ACE N and C Domains

The inhibitory potency of ACE inhibitors toward the ACE N and C domains was determined using synthetic and natural substrates. For wild-type ACE, the potency of the three inhibitors on the N or C domain is related to the substrate used; for example, captopril displays  $K_i$  values of 0.1–17 nM (Table 4). Captopril displays a similar potency for the inhibition of the N and C domain-active sites, except for the inhibition of Hip-His-Leu and AcSDKP hydrolysis, for which captopril is 5- and 10-fold more effective, respectively, for inhibition of the N domain than of the C domain. A similar potency of lisinopril for inhibition of the N and C domains is observed toward Hip-Lys-Pro or AcSDKP, whereas lisinopril

TABLE 3

 $K_i$  values of three ACE inhibitors (wild-type ACE acting on synthetic or natural substrates)

Substrate	Captopril	Lisinopril	Fosinoprilat
	nM		
Hip-Lys-Pro	4.12 $\pm$ 0.37	3.90 $\pm$ 0.75	4.62 $\pm$ 0.53
Hip-Ala-Pro	1.08 $\pm$ 0.74	1.73 $\pm$ 0.22	0.74 $\pm$ 0.34
Hip-His-Leu	3.42 $\pm$ 0.33	0.39 $\pm$ 0.07	0.29 $\pm$ 0.11
AcSDKP	0.24 $\pm$ 0.01	0.24 $\pm$ 0.15	0.06 $\pm$ 0.05
Ang I	3.98 $\pm$ 1.70	0.36 $\pm$ 0.13	0.37 $\pm$ 0.24

Values are mean  $\pm$  standard error from two or three independent determinations with ENZFITTER software for five inhibitor concentrations in the presence of optimal Cl<sup>-</sup> concentrations for each substrate as described in Experimental Procedures.

TABLE 4

 $K_i$  values of three ACE inhibitors (N and C domains of ACE acting on synthetic and natural substrates)

Substrate	Captopril	Lisinopril	Fosinoprilat
	nM		
Hip-Lys-Pro			
N domain	17.35 $\pm$ 0.65	3.90 $\pm$ 0.44	2.86 $\pm$ 0.52
C domain	16.72 $\pm$ 0.57	7.26 $\pm$ 1.40	1.21 $\pm$ 0.08
Hip-Ala-Pro			
N domain	4.37 $\pm$ 0.02	1.72 $\pm$ 0.16	1.00 $\pm$ 0.21
C domain	3.58 $\pm$ 0.29	0.47 $\pm$ 0.31	0.16 $\pm$ 0.02
Hip-His-Leu			
N domain	0.41 $\pm$ 0.21	6.56 $\pm$ 2.49	2.80 $\pm$ 1.60
C domain	2.07 $\pm$ 0.19	0.29 $\pm$ 0.01	1.25 $\pm$ 0.15
AcSDKP			
N domain	0.08 $\pm$ 0.01	0.53 $\pm$ 0.25	0.02 $\pm$ 0.01
C domain	1.14 $\pm$ 0.09	0.27 $\pm$ 0.10	0.07 $\pm$ 0.03
Ang I			
N domain	13.25 $\pm$ 1.35	2.05 $\pm$ 0.99	2.78 $\pm$ 0.87
C domain	14.36 $\pm$ 5.74	1.96 $\pm$ 1.15	0.48 $\pm$ 0.18

Values are mean  $\pm$  standard error from two or three independent determinations with ENZFITTER software for five inhibitor concentrations in the presence of optimal Cl<sup>-</sup> concentrations for each substrate as described in Experimental Procedures.

is 20-fold more effective for C domain inhibition of Hip-His-Leu hydrolysis than for N domain inhibition. For the inhibition of Ang I hydrolysis, captopril inhibits the N and C domains equally efficiently, as does lisinopril; however, fosinoprilat is almost 6-fold more effective at C domain inhibition than at N domain inhibition.

## Discussion

The tetrapeptide AcSDKP inhibits hematopoietic stem cell proliferation (22); the peptide also appears to block S phase entry of other cell types, such as hepatocytes, lymphocytes, and several continuous cell lines (23, 24). Other inhibitors of hematopoiesis have been identified, such as macrophage inflammatory protein-1 $\alpha$ , interferon, and tumor necrosis factor; however, the latter two display a complex action, and their hematopoietic effects are likely to be indirect and non-specific (25). The action of AcSDKP on the inhibition of hematopoietic cell cycling is specific for normal hematopoietic stem cells and has no effect on leukemic cells; thus, AcSDKP was proposed as a therapeutic agent for the protection of normal bone marrow progenitors during chemotherapy (26, 27). In fact, the coadministration of AcSDKP with cytotoxic drugs to mice *in vivo* increases survival (28).

The hydrolysis of AcSDKP *in vitro* is inhibited by specific ACE inhibitors, such as captopril and lisinopril (10, 29). Rousseau et al. (10) demonstrated that AcSDKP is hydrolyzed *in vitro* by ACE and that AcSDKP is a specific substrate

of the ACE N domain-active site. Recently, Azizi *et al.* (11) showed that a single oral dose of captopril (50 mg) administered to healthy subjects induced a 5.5-fold increase in plasma levels of AcSDKP and a 90–99% inhibition of *in vitro* [<sup>3</sup>H]AcSDKP hydrolysis. This study demonstrated clearly that AcSDKP is the first natural peptide hydrolyzed specifically by the N domain of ACE not only *in vitro* but *in vivo*. The physiological role of the ACE N domain-active site in the degradation of AcSDKP highlights the interest of designing ACE domain-specific inhibitors. Specific inhibition of the ACE N domain could lead to the maintenance of normal undifferentiated stem cells in the quiescent phase during chemotherapy, thus providing protection against cytotoxic drugs while enabling the C domain to perform its established role of ACE in Ang I production. The inhibition of hematopoietic stem cell proliferation in response to the specific ACE inhibitor enalapril has been reported recently (30); these authors showed that the oral administration of enalapril (20 mg/day) over a 15-day period to healthy subjects significantly decreased the granulocyte colony-forming and erythroid burst-forming units, demonstrating the inhibition of hematopoietic stem cell differentiation; this observation was accompanied by a significant increase in undifferentiated cells (granulocyte, erythroid, macrophage, and megakaryocyte colony-forming units). The use of an ACE inhibitor to raise plasma AcSDKP levels is preferable to the administration of exogenous AcSDKP because this peptide requires continuous infusion to be effective due to its constant production and degradation, displaying a half-life *in vivo* of 4.5 min (31). In contrast, a single oral dose of captopril (50 mg) produces a 5.5-fold longer-lasting increase in plasma AcSDKP (11).

In the current study, we determined the potency of commonly used ACE inhibitors for the hydrolysis of the physiological ACE substrates Ang I and AcSDKP by wild-type ACE and the ACE N and C domains. Such a study could aid the identification or eventual design of ACE inhibitors that exhibit preferential inhibition of the N domain for the hydrolysis of the natural N domain-specific substrate, AcSDKP. In addition, we tested the inhibition of wild-type ACE and the N and C domains using three different synthetic substrates to develop an optimized protocol to allow the large-scale screening of active compounds as an alternative approach for the identification of domain-specific inhibitors.

The synthetic peptide Hip-Lys-Pro was synthesized because it mimics the carboxydipeptide Lys-Pro of AcSDKP and as such could constitute a specific synthetic substrate of the N domain of ACE. However, together with Hip-Ala-Pro, it exhibits a strong affinity for both the N and C domains of ACE, with  $K_m$  values close to those of ACE physiological substrates. In addition, both synthetic peptides are hydrolyzed efficiently by all three forms of ACE, with  $k_{cat}$  values comparable to that for Ang I hydrolysis. Therefore, the synthetic substrates used in the current study cannot replace AcSDKP as a specific N domain substrate, but we demonstrate that Hip-Lys-Pro and Hip-Ala-Pro are hydrolyzed efficiently by ACE with kinetic constants in the physiological range. In fact, Hip-Ala-Pro is the best synthetic substrate of this series and could serve as a substitute for Ang I to measure ACE activity; it offers the advantage of being protected against carboxypeptidase hydrolysis by its carboxyl-terminal proline residue. This would be useful in, for exam-

ple, analysis of ACE activity in tissue samples and biological fluids that contain carboxypeptidases.

The amino acid requirement for a peptide to serve as a substrate for ACE seems to be confined to the residues composing the carboxyl-terminal tripeptide. The presence of a proline residue in P'2 (carboxyl-terminal position) increases peptide binding to the different forms of ACE, whereas a histidine residue in P'1 (penultimate carboxyl-terminal position) seems to confer a specificity for the C domain. Comparison of Hip-His-Leu and Ang I or of Hip-Lys-Pro and AcSDKP shows that the carboxyl-terminal dipeptide is not sufficient to confer domain substrate specificity. Hip-His-Leu is a relatively specific substrate for the C domain, whereas Ang I is cleaved by both ACE domains. Conversely, Hip-Lys-Pro is hydrolyzed equally well by the two ACE domains, and AcSDKP is a N domain-specific substrate. It is possible that the presence of a benzoyl-glycine moiety (hippuryl) in the P position (antepenultimate carboxyl-terminal position) in place of a phenylalanine residue increases the affinity for the C domain. This hypothesis is supported by the observation that Z-Phe-His-Leu is cleaved almost equally well by the two ACE domains (32). It is possible that the presence of an aspartic acid residue in the P position is imperative for N domain specificity or that the minimum length required for achieving N domain specificity is a tetrapeptide like AcSDKP.

In agreement with previous observations (33), the efficacy of an ACE inhibitor is clearly substrate dependent, with the lowest  $K_i$  value observed when each inhibitor was tested with AcSDKP and the highest  $K_i$  value observed with Hip-Lys-Pro as substrate. Fosinoprilat is the most potent inhibitor of AcSDKP hydrolysis by the N domain; however, captopril displays the greatest selectivity for the inhibition of AcSDKP and Ang I hydrolysis by wild-type ACE, being 16-fold more potent with AcSDKP as substrate compared with Ang I. This finding is of therapeutic relevance in that captopril is the most efficient ACE inhibitor for blocking AcSDKP metabolism, with a relatively lesser effect on the inhibition of Ang I hydrolysis. These *in vitro* data will have to be confirmed by *in vivo* studies in which captopril is administered to enhance plasma AcSDKP with the aim of protecting hematopoietic stem cells during chemotherapy for acute myeloblastic leukemia.<sup>1</sup> Interestingly, for the inhibition of Ang I hydrolysis, captopril is a more potent inhibitor of wild-type ACE compared with the N and C domains. This may be due to the interaction of captopril at one active site of wild-type ACE impeding substrate interaction with the other active site, an effect that is observed with the larger peptide Ang I compared with the other substrates used and only with captopril, which is the weakest competitive inhibitor for wild-type ACE used in this study. Thus, each domain of somatic ACE may not be inhibited independently of the other domain as reported previously (14), but rather a certain degree of cooperativity exists with respect to inhibitor binding.

In conclusion, we demonstrated that the potency of an ACE inhibitor is substrate dependent and that captopril is the most appropriate compound, as identified in this study, for clinical use in the protection of hematopoietic stem cells from cytotoxic drug therapy.

<sup>1</sup> M. Azizi, personal communication.



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