Peptidase Specificity Characterization of C- and N-Terminal Catalytic Sites of Angiotensin I-Converting Enzyme†

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ABSTRACT: Quenched fluorescence peptides were used to investigate the substrate specificity requirements for recombinant wild-type angiotensin I-converting enzyme (ACE) and two full-length mutants bearing a single functional active site (N- or C-domain). We assayed two series of bradykinin-related peptides flanked by *o*-aminobenzoic acid (Abz) and *N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp), namely, Abz-GFSPFXQ-EDDnp and Abz-GFSPFRX-EDDnp (X = natural amino acids), in which the fluorescence appeared when Abz/EDDnp are separated by substrate hydrolysis. Abz-GFSPFFQ-EDDnp was preferentially hydrolyzed by the C-domain while Abz-GFSPFQQ-EDDnp exhibits higher N-domain specificity. Internally quenched fluorescent analogues of *N*-acetyl-SDKP-OH were also synthesized and assayed. Abz-SDK(Dnp)P-OH, in which Abz and Dnp (2,4-dinitrophenyl) are the fluorescent donor−acceptor pair, was cleaved at the D−K(Dnp) bond with high specificity by the ACE N-domain (k_{cat}/K_m = 1.1 μM^{-1} s^{-1}) being practically resistant to hydrolysis by the C-domain. The importance of hydroxyl-containing amino acids at the P_2 position for N-domain specificity was shown by performing the kinetics of hydrolysis of Abz-TDK(Dnp)P-OH and Abz-YDK(Dnp)P-OH. The peptides Abz-YRK(Dnp)P-OH and Abz-FRK-(Dnp)P−OH which were hydrolyzed by wild-type ACE with K_m values of 5.1 and 4.0 μM and k_{cat} values of 246 and 210 s^{-1}, respectively, have been shown to be excellent substrates for ACE. The differentiation of the catalytic specificity of the C- and N-domains of ACE seems to depend on very subtle variations on substrate-specific amino acids. The presence of a free C-terminal carboxyl group or an aromatic moiety at the same substrate position determines specific interactions with the ACE active site which is regulated by chloride and seems to distinguish the activities of both domains.

Angiotensin I-converting enzyme (ACE)[1] (peptidyl dipeptidase A, kininase II, EC 3.4.15.1) is a zinc dipeptidyl carboxypeptidase that cleaves the C-terminal dipeptide from angiotensin I to produce the potent vasopressor octapeptide, angiotensin II (*1*), and inactivates bradykinin by the sequential removal of two C-terminal dipeptides (*2*). ACE is expressed as a somatic isoform (150−180 kDa) in endothelial, epithelial, and neuroepithelial cells and as a smaller isoform (90−110 kDa) only in male germinal cells. The somatic form of ACE is composed of two highly homologous domains, called N- and C-domains, each possessing an active site (*3*−*6*). The germinal form of ACE contains a single active site and corresponds to the C-domain of the somatic enzyme (*7*−*9*).

The primary activity of ACE is to cleave free carboxyl group oligopeptides with a wide specificity. Substrates containing Pro at the P_1' position [nomenclature by Schechter and Berger (*10*)] and Asp or Glu at P_2' are resistant to ACE (*11*, *12*). However, ACE can also work as an endopeptidase or a tripeptidyl carboxypeptidase upon certain substrates. The endopeptidase activity of ACE is observed with substrates that have amidated carboxyl groups where the enzyme can cleave a C-terminal dipeptide amide and/or a C-terminal tripeptide amide (*13*, *14*). ACE can cleave luteinizing hormone-releasing hormone (LH-RH) not only at the C-terminal tripeptide amide but also at the N-terminal tripeptide (*15*). We have recently reported the efficient hydrolysis by ACE of the internally quenched fluorogenic bradykinin-related peptide Abz-GFSPFRQ-EDDnp and some of its analogues at the R−Q bond. These peptides have the C-terminal carboxyl group blocked by the quencher EDDnp [*N*-(2,4-dinitrophenyl)ethylenediamine) (*16*).

The C- and N-domains of the somatic form of ACE are functional (*17*) and exhibit similar catalytic activities toward angiotensin I, bradykinin, and substance P (*18*). However, the activity of the C-terminal domain is highly dependent on chloride ion concentration, whereas the N-terminal domain is still active in the absence of chloride and is fully activated at relatively low concentrations of this anion (*17*,

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[1] Abbreviations: ACE, angiotensin I-converting enzyme; AI and AII, angiotensins I and II, respectively; Abz, *o*-aminobenzoic acid; BK, bradykinin; CHO, Chinese hamster ovary; Dnp, 2,4-dinitrophenyl; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; MALDI-TOF, matrix-assisted laser desorption ionization time of flight.

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18). The N active site is preferentially involved in the N-terminal endopeptidase cleavage of LH-RH, however, with low catalytic efficiency (*18*). Captopril and lisinopril display different inhibitory potencies toward the two active sites (*19*, *20*).

In addition to the well-known function of ACE in the renin−angiotensin system, it has been demonstrated that the N active site of the enzyme hydrolyzes the natural circulating tetrapeptide *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP-OH), which is involved in the control of hematopoietic stem cell proliferation (*21*). The demonstration that the physiological functions of ACE are not limited to its cardiovascular role and that the enzyme degrades Ac-SDKP-OH both in vivo and in vitro (*21*−*23*) increased the interest in the studies of each domain specificity and inhibition (*24*−*26*). Another specific substrate for the N-domain is angiotensin 1−7 (Asp-Arg-Val-Tyr-Ile-His-Pro) but at the same time this peptide inhibits the hydrolysis of angiotensin I by the C-domain (*27*).

BK and Ac-SDKP-OH have been elected as model sequences to design internally quenched substrates for the evaluation of ACE peptidase activity. BK was chosen because it is the ACE substrate hydrolyzed with the highest k_{cat}/K_m parameter. In addition, it is converted by both ACE active sites to BK^{1-7} and BK^{1-5}, with similar kinetics, but with K_m at least 30 times lower and k_{cat} 10 times higher than for AI (*18*). The internally quenched analogues of Ac-SDKP-OH, were synthesized in order to investigate the amino acid requirements for N active site specificity.

In the present study, the activities of the wild-type recombinant human ACE and two full-length ACE mutants containing only one functional active site were assayed using internally quenched fluorogenic substrates with blocked (Abz-peptidyl-EDDnp) or free [Abz-peptidyl-K(Dnp)-P-OH] C-terminal carboxyl groups. We reported synthetic fluorescent substrates that distinguish each active site of ACE and can be particularly helpful to study N- and C-domain specificities.

MATERIALS AND METHODS

Substrates. Internally quenched fluorogenic peptides containing the group EDDnp attached to a glutamine residue were synthesized by the solid-phase synthesis method as described elsewhere (*28*) in an automated solid-phase peptide synthesizer, Shimadzu Model PSSM-8. The fluorescent peptides from the series Abz-GFSPFRX-EDDnp containing the EDDnp attached to different amino acids were synthesized by the classic solution method using the general procedure previously described (*28*, *29*). The internally quenched fluorogenic peptides containing the group Dnp incorporated to the ϵ-NH_2 of a Lys residue were synthesized by the solid-phase methodology, using Fmoc-Lys(Dnp)-OH to introduce the quencher group and H-Pro-2-chlorotrityl resin (*30*). All of the peptides obtained were purified by semipreparative HPLC. The molecular weight and purity of synthesized peptides were checked by amino acid analysis and by molecular mass determination with MALDI-TOF mass spectrometry, using a TofSpec E from Micromass (Manchester, U.K.). The stock solutions of Dnp or EDDnp peptides were prepared in DMSO, and the concentrations were measured spectrophotometrically using the molar extinction coefficient $\epsilon_{365} = 17\,300\ M^{-1}\ cm^{-1}$.

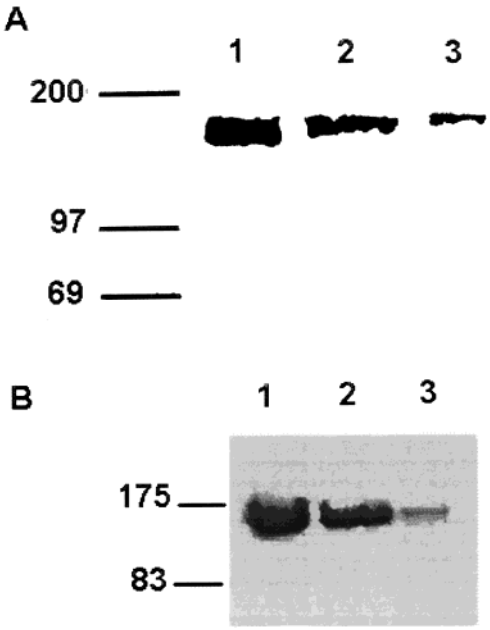


FIGURE 1: SDS−PAGE (7.5%) of recombinant forms of ACE followed by Western blotting analysis with antiserum Y1 (A) or silver staining (B): wild type (lane 1), active C-domain (lane 2), and active N-domain (lane 3). The molecular mass of the standards (in kDa) is indicated to the left.

Enzyme Preparation. The three human recombinant ACE, namely, wild type containing two intact functional domains and the two full-length ACE mutants containing only one intact catalytic site, were kindly supplied by Dr. François Alhenc-Gelas from the Institut National de la Santé et de la Recherche Médicale (Paris, France). The enzymes were obtained through stable expression from CHO cells (*17*), and the ACE mutants with either the N or C catalytic site inactivated by substitution of the two zinc-binding His by Lys are referred as N- or C-domains. The molar concentrations of the enzymes were determined by active site titration with lisinopril and analyzed as previously described (*20*). The enzymes employed were further analyzed by SDS−PAGE, followed by Western blotting, carried out on a 7.5% polyacrylamide gel (*31*). The proteins were stained with silver nitrate or transferred to a polyvinylidine difluoride microporous membrane (Immobilon O, Millipore). The membrane was incubated in 10 mM Tris buffer, pH 8.0, containing 0.15 M NaCl, 0.05% Tween-20, and 3 mg/mL bovine serum albumin for 30 min prior to overnight incubation at 4 °C with antiserum Y1 diluted 1:1000, as previously described (*32*). Figure 1 shows the SDS−PAGE and the Western blot analysis of recombinant ACEs. The N-terminal sequences for the three recombinant ACE, wild type, N, and C active domains were performed in a protein sequencer PPSQ-23 (Shimadzu, Tokyo, Japan). The sequence obtained for the first eight amino acids was Leu, Asn, Pro, Gly, Leu, Gln, Pro, and Gly. The concentrations of enzyme calculated for the cycles of the first three amino acids (Leu, Asn, Pro) were for wild type 50.2 nM (by titration 42.2 nM), C-domain 72.8 nM (by titration 67.2nM), and N-domain 14.2 nM (by titration 12.1 nM).

Enzyme Assay. ACE activity on fluorogenic BK-related substrates flanked by Abz (*o*-aminobenzoic acid) and EDDnp [*N*-(2,4-dinitrophenyl)ethylenediamine] was monitored at 37 °C in 0.1 M sodium phosphate buffer, pH 8.0, containing

0.2 M NaCl (0.35−2.0 mL final volume). The ACE activity on the Abz/EDDnp peptides derived from Ac-SDKP-OH was determined in the same way but in 0.1 M Tris-HCl buffer, pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$. Assays with substrates containing Abz at the N-terminus and Dnp (2,4-dinitrophenyl) attached to Lys were performed at 37 °C in 0.1 M Tris-HCl buffer, pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$ (0.35−2.0 mL final volume). Enzymatic activity of all fluorogenic peptides tested was continuously followed in a Hitachi F-2000 fluorometer by measuring the fluorescence at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 320$ nm. The slope was converted into micromoles of substrate hydrolyzed per minute based on the basis of a calibration curve obtained from complete hydrolysis of each peptide. To correct the inner filter effect, we used an adjusting equation determined experimentally for 0.1−100 μM Abz-FR-OH, used as standard for fluorescence measurements. The equation used is

$$F_c = F_o(100/100 - 10^{0.86\log EDDnp+0.5})$$

where F_c and F_o are the fluorescence corrected and fluorescence observed, respectively.

Determination of Substrate Cleavage Site. The scissile bonds of hydrolyzed peptides were determined by isolation of the fragments in analytical HPLC, and their structures were deduced from amino acid sequencing (protein sequencer PPSQ-23, Shimadzu, Tokyo, Japan) and by MALDI-TOF mass spectrometry. The analytical HPLC was performed as described above.

Optimum pH Determination. The pH dependence of Abz-GFSPFRA-EDDnp hydrolysis by recombinant ACEs (∼0.10 nM) was studied in a substrate concentration of 10 μM over a pH range of 5.0−9.5. Enzymatic activity was measured at 37 °C, using the fluorometric assay described above. The buffers used were as follows: 0.1 M sodium acetate (5.0 < pH < 6.0), 0.1 M sodium phosphate (6.0 < pH < 8.0), and 0.1 M Tris-HCl (pH > 8.0), containing 0.2 M NaCl. Sodium borate buffer (0.4 M) and Tris-HCl (0.1 M) were also used as an alternative to 0.1 M sodium phosphate at pH 8.0. The hydrolysis of 7.5 μM Abz-FRK(Dnp)P-OH by recombinant ACEs (∼0.05 nM) was measured at various pH values over the range 5.0−9.5 at 37 °C in 0.1 M Tris-HCl containing 0.05 M NaCl and 10 μM $ZnCl_2$. Assays were also performed in 0.1 M sodium phosphate at pH 7.0 containing 0.05 M NaCl and 10 μM Zn Cl_2.

Chloride Influence on Catalytic Activity. The influence of chloride ion concentration (0−500 mM) on catalytic activity of recombinant forms of ACE upon Abz-peptidyl-Eddnp compounds was investigated using Abz-GFSPFRQ-EDDnp (10 μM) as substrate at 37 °C in 0.1 M sodium phosphate buffer, pH 8.0. The effect of various chloride concentrations (0−200 mM) on Abz-peptidyl-K(Dnp)-P-OH hydrolysis was studied using Abz-FRK(Dnp)-OH as substrate at 37 °C in 0.1 M Tris-HCl, pH 7.0, containing 10 μM $ZnCl_2$. The hydrolysis was followed by the continuous fluorometric assay described above.

Determination of Kinetic Parameters. Kinetic parameters for hydrolysis of internally quenched fluorogenic peptide substrates flanked by Abz and EDDnp were determined at 37 °C, pH 8.0, in 0.1 M sodium phosphate buffer containing 0.2 M NaCl. The substrates containing Abz and Lys(Dnp) were tested in 0.1 M Tris-HCl, pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$. The inhibition constant (K_i) for captopril was determined under the same conditions after a 30 min preincubation. The kinetic parameters K_m and k_{cat} were calculated by the nonlinear regression data analysis Grafit program (*33*). The standard deviations of the k_{cat} and K_m values were less than 5%. The k_{cat}/K_m values were calculated as the ratio of these two determined parameters. The apparent second-order rate constant k_{cat}/K_m ($*k_{cat}/K_m$) was determined under pseudo-first-order conditions, where $[S] \ll K_m$. These determinations were made in three different concentrations, and the error was less than 7% for any obtained value.

RESULTS

Human Wild-Type Recombinant ACE and Its Functional C- and N-Domain Activities on Abz-peptidyl-EDDnp Substrates. Human wild-type recombinant ACE was able to hydrolyze the internally quenched fluorescent analogue of BK, Abz-RPPGFSPFR-EDDnp, at the F−R bond with a k_{cat}/K_m value of 0.10 μM^{-1} s^{-1}, whereas Abz-RPPGFSPFRQ-EDDnp, which contains the group EDDnp attached to glutamine, was hydrolyzed at the R−Q bond with $k_{cat}/K_m = 0.9$ μM^{-1} s^{-1}. The displacement of the cleavage site suggested that the group EDDnp was acting as a C-terminal residue, occupying the P_2' position. A prolonged incubation produced sequential removal of carboxyl-terminal dipeptides; however, the fluorometer only detects fluorescence of the first cleavage, when the quenching of EDDnp upon Abz is disrupted. The deletion of the N-terminal tripeptide Arg-Pro-Pro from Abz-RPPGFSPFRQ-EDDnp, resulting in Abz-GFSPFRQ-EDDnp, increased its susceptibility to hydrolysis. The k_{cat}/K_m value for Abz-GFSPFRQ-EDDnp hydrolysis by wild-type ACE was 1.3 μM^{-1} s^{-1}. Further removal of the dipeptides Gly-Phe and Ser-Pro decreased the k_{cat}/K_m value for hydrolysis of Abz-SPFRQ-EDDnp to 0.07 μM^{-1} s^{-1} and turned Abz-FRQ-EDDnp resistant to ACE, respectively. These results indicated that a minimum length is required for hydrolysis by ACE of these BK-derived substrates. The peptide Abz-GFSPFRQ-EDDnp was chosen as the model Abz-peptidyl-EDDnp substrate for studies of specificity profiles of wild type and the single functional C- and N-domains of ACE. This peptide was hydrolyzed at the R−Q bond by the three recombinant forms of ACE, and a prolonged incubation produced the sequential removal of the dipeptides F−R and S−P, stopping at Abz-GF.

(A) Effects of pH, Chloride Concentration, and Inhibitors. The effect of pH on the hydrolysis of Abz-GFSPFRA-EDDnp by the three recombinant forms of ACE was determined over the pH range 5.0−9.5 (Figure 2). As this substrate was the more susceptible in the series, it was used in this experiment in order to obtain measurable fluorescence in the acidic pH range, where the ACE activity was very low. The pH profile presented a sigmoid curve, with ACE activity increasing with pH, without reduction at alkaline pH values. Determinations of the kinetic constant were carried out at pH 8.0, which is the typically reported pH for most ACE substrates. The influence of chloride ions on catalytic activity of recombinant ACEs upon Abz-GFSPFRQ-EDDnp was investigated at pH 8.0, as shown in the inset of Figure 2. In the absence of the added chloride the enzymes displayed about 15% of their maximal activity, which was achieved at

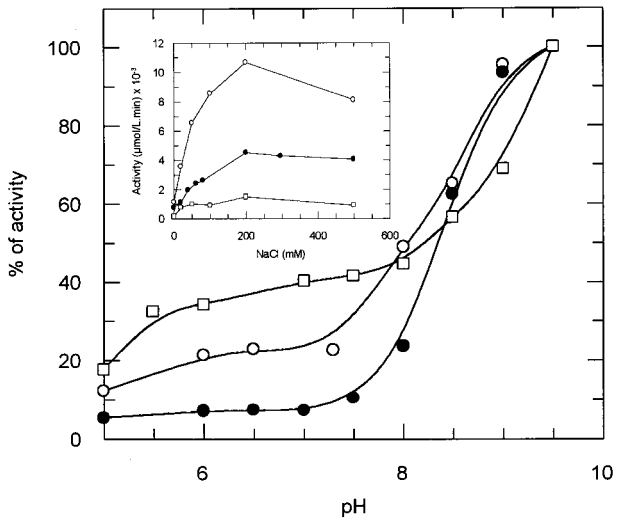


FIGURE 2: pH dependence of Abz-GFSPFRA-EDDnp hydrolysis and NaCl dependence of Abz-GFSPFRQ-EDDnp hydrolysis (inset) by recombinant forms of ACE: wild type (○), active C-domain (●), active N-domain (□). See Materials and Methods section for the hydrolysis conditions.

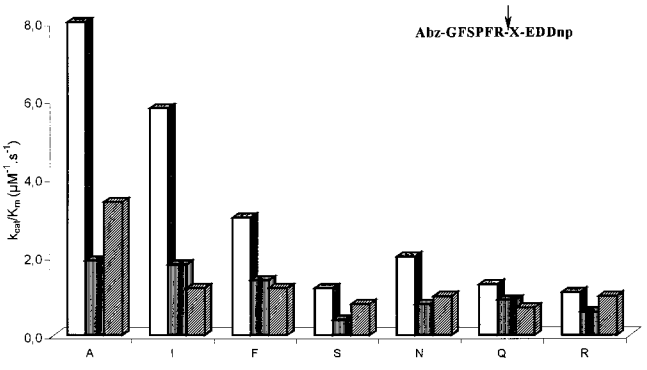
0.2 M NaCl. With concentration higher than 0.2 M, no further increase in activity was observed. The kinetic parameters were obtained in the optimal conditions, i.e., sodium phosphate buffer containing 0.2 M NaCl at pH 8.0. Sodium borate buffer and Tris-HCl at pH 8.0, containing 0.2 M NaCl, were also tested, but no difference was observed on the catalytic activity of the recombinant enzymes (data not shown). The hydrolysis of Abz-GFSPFRQ-EDDnp by the three forms of ACE was completely inhibited by 10 mM EDTA and 1 μM captopril.

(B) Hydrolysis of Abz-GFSPFRX-EDDnp and Abz-GFSPFXQ-EDDnp Peptides. Typical Michaelis−Menten profiles were observed for hydrolysis by wild-type and mutated ACEs of all analogues of Abz-GFSPFRQ-EDDnp. The incubation time was always less than 10 min to avoid the interference on the kinetic measurements of the products of progressive peptide hydrolysis. These initial conditions were checked by the HPLC analysis of the reaction products. All of the peptides of the Abz-GFSPFRX-EDDnp and Abz-GFSPFXQ-EDDnp series were cleaved at the R−X and X−Q bond, respectively.

The kinetic parameters for hydrolysis of Abz-GFSPFRQ-EDDnp with different amino acids at P_1' are presented in Figure 3. A clear preference was observed for hydrophobic residues in this position, with Ala being the more favorable amino acid for the three recombinant enzymes.

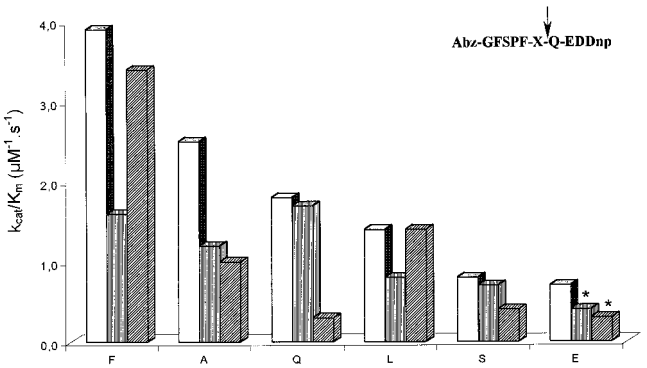
The kinetic data for the hydrolysis of the peptides that resulted from systematic variation at the P_1 position of Abz-GFSPFRQ-EDDnp are presented in Figure 4. A preference of the S_1 subsite for hydrophobic residues was observed, except for the N-domain, which showed higher hydrolytic activity on the glutamine-containing peptide at P_1. This peptide, Abz-GFSPFQQ-EDDnp, but not the analogue containing Asn at the same position, was hydrolyzed five times more efficiently by the N-domain of the enzyme than by the C-domain. The inverse was observed with Abz-GFSPFFQ-EDDnp, which is preferentially hydrolyzed by the C-domain.

The substrates of the Abz-GFSPFRX-EDDnp and Abz-GFSPFXQ-EDDnp series were all hydrolyzed by both C- and N-domains; however, as expected, the wild-type ACE hydrolyzed all of them with higher k_{cat}/K_m values than the two isolated mutated forms.



Peptides	Wild-type		N-domain		C-domain	
X	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
A	1.3	10.4	1.8	3.4	1.4	5.0
I	1.5	8.7	1.4	2.6	2.4	2.9
F	1.4	4.2	0.7	1.0	1.1	1.4
S	0.7	0.9	3.0	1.3	3.0	2.4
N	0.9	1.8	5.0	3.8	3.3	3.3
Q	1.6	2.2	3.0	2.7	6.0	4.2
R	0.9	1.0	1.4	0.9	1.4	1.4

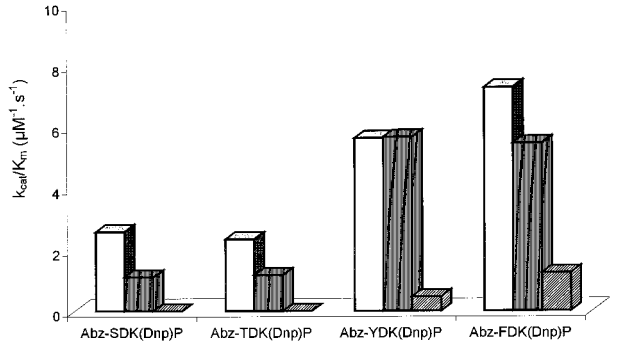
FIGURE 3: Kinetic parameters for hydrolysis by ACE wild type (open box), active N-domain (stripped box), and active C-domain (hatched box) of Abz-GFSPFRX-EDDnp with different amino acids at P_1'. The assays were performed in 0.1 M sodium phosphate buffer containing 0.2 M NaCl, pH 8.0, at 37 °C. The initial rates of hydrolysis were determined fluorometrically (λ_{em} = 420 nm; λ_{ex} = 320 nm). The standard deviations of the k_{cat} and K_m values were less than 5%. The k_{cat}/K_m values (shown as bars in the top) have been calculated as the ratio of these two parameters.



Peptides	Wild-type		N-domain		C-domain	
X	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
F	2.6	10.1	2.4	3.8	3.7	12.6
A	1.3	3.3	1.0	1.2	2.0	2.0
Q	1.1	2.0	0.6	1.0	0.9	0.3
L	2.1	3.7	3.0	2.4	3.4	4.8
S	2.5	2.0	2.3	1.6	2.1	0.8
E	1.6	2.2	-	-	-	-

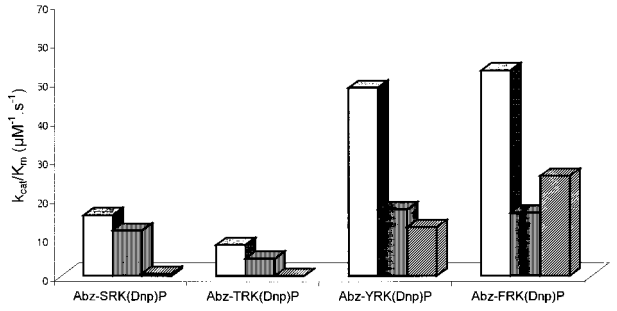
FIGURE 4: Kinetic parameters for the hydrolysis by recombinant forms of ACE, wild type (open box), active N-domain (stripped box), and active C-domain (hatched box), of Abz-GFSPFXQ-EDDnp with different amino acids at P_1. The fluorometric assays were performed at 37 °C in 0.1 M sodium phosphate buffer containing 0.2 M NaCl, pH 8.0. Fluorescense detection: λ_{em} = 420 nm; λ_{ex} = 320 nm. The standard deviations of the k_{cat} and K_m values were less than 5%. The k_{cat}/K_m values have been calculated as the ratio of these two parameters and are shown as bars in the top. The apparent second-order rate constant $*k_{cat}/K_m$ was determined under pseudo-first-order conditions, where [S] ≪ K_m and the error was less than 7%.

Human Wild-Type Recombinant ACE and Its Functional C- and N-Domain Activities on Abz-peptidyl-K(Dnp)P-OH



Peptides	Wild-type		N-domain		C-domain	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
Abz-SDK(Dnp)P	17.0	44.2	22.0	24.6	32.0	0.1
Abz-TDK(Dnp)P	15.4	36.3	15.7	18.7	60.9	0.06
Abz-YDK(Dnp)P	11.1	62.5	5.1	28.9	15.0	7.0
Abz-FDK(Dnp)P	11.0	79.9	18.7	101.7	24.8	31.0

FIGURE 5: Kinetic parameters for hydrolysis by recombinant forms of ACE, wild type (open box), active N-domain (stripped box), and active C-domain (hatched box), of Abz-SDK(Dnp)P-OH with different amino acids at P_2. The experimental conditions were 0.1 M Tris-HCl, pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$, at 37 °C. The rates of hydrolysis were determined fluorometrically (λ_{em} = 420 nm; λ_{ex} = 320 nm). The standard deviations of the k_{cat} and K_m values were less than 5%. The k_{cat}/K_m values have been calculated as the ratio of these two parameters and are shown as bars in the top.



Peptides	Wild-type		N-domain		C-domain	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
Abz-SRK(Dnp)P	14.8	230.9	14.4	167.8	22.9	13.7
Abz-TRK(Dnp)P	10.2	82.0	3.1	13.9	7.4	0.2
Abz-YRK(Dnp)P	5.1	246.3	5.0	85.6	4.7	58.8
Abz-FRK(Dnp)P	4.0	210.4	3.4	54.9	2.7	69.2

FIGURE 6: Kinetic parameters for the hydrolysis by ACE, wild type (open box), active N-domain (stripped box), and active C-domain (hatched box), of Abz-SRK(Dnp)P with different amino acids at P_2. The experimental conditions were 0.1 M Tris-HCl, pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$, 37 °C. The hydrolysis of the fluorogenic substrates was registered continuously at λ_{em} = 420 nm and λ_{ex} = 320 nm. The standard deviations of the k_{cat} and K_m values were less than 5%. The k_{cat}/K_m values have been calculated as the ratio of these two parameters (shown as bars in the top).

Substrates. The peptides Abz-SDK(Dnp)P-OH and Abz-SRK(Dnp)P-OH and their analogues containing Thr, Tyr, and Phe in the position of Ser were cleaved by the three recombinant forms of ACE at the D−K(Dnp) or R−K(Dnp) bond. The kinetic parameters for these activities are shown in Figures 5 and 6. The higher k_{cat}/K_m values were obtained with the hydrolysis of the series derived from Abz-SRK(Dnp)P-OH. The peptides Abz-YRK(Dnp)P-OH and Abz-FRK(Dnp)P-OH which were hydrolyzed by wild-type ACE with K_m values of 5.1 and 4.0 μM and k_{cat} values of 246 and 210 s^{-1}, respectively, have been shown to be excellent substrates for ACE (Figures 6). The series led by Abz-SDK(Dnp)P-OH (Figure 5) resulted in substrates hydrolyzed with lower catalytic efficiency than the Abz-SRK(Dnp)P-OH derivatives due to an increase in K_m and a decrease of k_{cat} values. The peptides Abz-SDK(Dnp)P-OH and Abz-TDK(Dnp)P-OH were barely hydrolyzed by the C-domain, showing a clear N-domain selectivity. Therefore, these synthetic substrates can distinguish the hydrolytic activities of the N-domain from those of C-domain and have the methodological advantage of the internally quenched fluorescent peptides. In addition, we observed that the activity of ACE on the peptides derived from Ac-SDKP-OH depends on the presence of a free carboxyl group, because the sequences Abz-SDKPQ-EDDnp, Abz-SDKP-EDDnp, and Abz-GFSPSDQ-EDDnp, also synthesized and assayed, were completely resistant to hydrolysis by the three forms of ACE (data not shown).

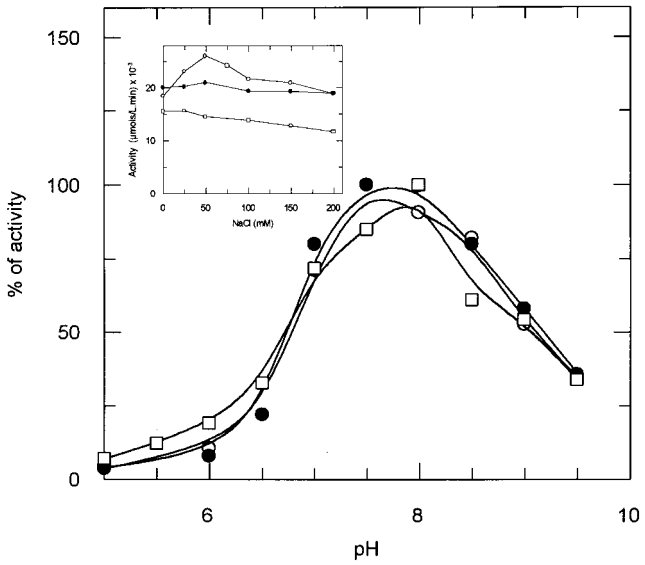


FIGURE 7: pH dependence of Abz-FRK(Dnp)P-OH hydrolysis by recombinant forms of ACE: wild type (○), C-domain (●), and N-domain (□). The effect of NaCl is shown in the inset. See Materials and Methods section for the hydrolysis conditions.

Effect of pH, Chloride, and Inhibitors. The pH effect on hydrolysis of Abz-FRK(Dnp)P-OH by the three recombinant forms of ACE was determined over the pH range 5.0−9.5 (Figure 7). Optimal hydrolysis occurred around pH 7.0, and the curves presented a bell-shaped format, which is typically reported for ACE substrates. No significant influence of chloride anion concentration was detected on the catalytic activity of recombinant ACEs upon this substrate, as shown in the inset of Figure 7. Sodium phosphate at pH 7.0, containing 0.05 M NaCl, presented a 6-fold reduction on the catalytic activity of the recombinant enzymes (data not shown). The hydrolysis of Abz-FRK(Dnp)P-OH and Abz-SDK(Dnp)P-OH by three forms of ACE was completely inhibited by 10 mM EDTA.

Since the potency of ACE inhibitors is substrate dependent (*34*), we determined the effect of captopril on the hydrolysis of Abz-FRK(Dnp)P-OH and Abz-SDK(Dnp)P-OH, which are preferentially hydrolyzed by the C- and N-domains, respectively. The K_i values obtained for Abz-FRK(Dnp)P-OH were 46.0, 25.0, and 16 nM for wild-type, N-domain, and C-domain, respectively. Using Abz-SDK(Dnp)P-OH as substrate, the K_i values were 10.0, 9.5, and 191.0 nM for wild-type, N-domain, and C-domain, respectively. These data

with fluorescent substrates are in accordance with the previously reported observation of captopril inhibition on the hydrolysis of hippuril-His-Leu and Ac-SDKP-OH that are also preferentially hydrolyzed by the C- and N-domains, respectively (*21*).

DISCUSSION

The description of two active sites and the interest in revealing the role of each one on the hydrolysis of various physiological substrates raise the importance of the studies of ACE catalytic mechanism and make apparent the need to improve the assays for ACE substrate specificity. We have previously described a rapid and sensitive method for assaying ACE activity using internally quenched fluorogenic substrates containing Abz as the fluorescent group and EDDnp as the quencher flanking the C-terminal sequence of bradykinin, generating the peptide Abz-GFSPFRQ-EDDnp (*16*). This peptidase activity of human wild-type recombinant ACE and its functional C- and N-domains is very dependent on a minimum size of the substrate. The group EDDnp seems to be recognized as a residue that possibly occupies the enzyme S_2' subsite, since Q-EDDnp and X-EDDnp are removed by ACE from Abz-GFSPFXQ-EDDnp and Abz-GFSPFRX-EDDnp, respectively. Accordingly to this interpretation, we observed that Abz-GFSPFRQ-NH_2 is completely resistant to hydrolysis by wild-type recombinant ACE, suggesting that the aromatic group of EDDnp in these substrates compensates for the absence of a free carboxylate group. This view is supported by the observed sigmoid shape of the curve of pH-dependent hydrolysis of Abz-GFSPFRA-EDDnp, which presented only an ascendant side, in contrast to the bell shape observed for hydrolysis of substrates with a free carboxyl group. This observation suggests that the disruption of an electrostatic interaction between the C-terminal carboxyl group of the substrate and a basic group of the enzyme at pH greater than 8.0 reduces the hydrolysis of substrates containing a free carboxyl group. We speculate that this basic residue interacts with the $\gamma(-)$ electrons of the benzene aromatic ring of EDDnp, allowing a cation$-\pi$ interaction (*35*). Since this group is an amine, the interaction with benzene persists even after deprotonation of the amino group, due to the previously described hydrogen bond acceptor activity of the aromatic ring, particularly from the sp^2 hybridized nitrogen atom (*36*). This effect does not depend on the presence of the NO_2 groups attached to the phenyl group of EDDnp because the peptide Abz-GFSPFRQ-PED [PED = *N*-(phenyl)ethylenediamine], which is devoid of the two NO_2 groups, was hydrolyzed by wild-type ACE with similar efficiency as Abz-GFSPFRQ-EDDnp. Interestingly, the susceptibility to hydrolysis of this class of substrates was very dependent on the chloride ion concentration, contrasting to the almost no chloride requirement for the hydrolysis of substrates with free C-terminal carboxyl groups. This observation incites us to speculate that the interaction of a substrate negative carboxyl group with a specific enzyme subsite induces changes on ACE conformation that can be also caused by chloride ions, which was detected with EDDnp-containing substrates.

The susceptibility of Abz-peptidyl-EDDnp substrates to both ACE active sites seems to be very similar. However, Abz-GFSPFFQ-EDDnp was the peptide hydrolyzed with high preference by the C-domain of ACE. This peptide allocates Phe at S_1, which also occurs with the hydrolysis of angiotensin I, which is also preferentially hydrolyzed by the C-domain (*17*). In this series, the peptide containing glutamine in P_1, but not asparagine, is mainly hydrolyzed by the N-domain. For the peptides of the series Abz-GFSPFXQ-EDDnp with X = F, Q, L, and S, the sum of the k_{cat}/K_m values determined for the N- and C-domains is greater than for wild-type ACE. On the other hand, for Abz-GFSPFAQ-EDDnp and Abz-GFSPFEQ-EDDnp, as well as for all other peptides examined in this work, the sum of the k_{cat}/K_m values determined for the N- and C-domains is similar to that for wild type. On the basis of these observations we speculate that, in wild-type enzyme, the two domains of ACE do not operate independently but may cooperate or coordinate in a manner that depends on substrate size and structure.

The activity of recombinant ACEs upon the internally quenched fluorescent peptides with the general structure Abz-peptidyl-K(Dnp)P-OH was significantly more efficient than the activity described for the Abz-peptidyl-EDDnp analogues of bradykinin. The kinetic parameters presented in Figure 6, for the hydrolysis of Abz-FRK(Dnp)P-OH and Abz-YRK-(Dnp)P-OH by wild-type recombinant ACE, classify these peptides among the best substrates described for ACE, since previously reported k_{cat}/K_m values for hydrolysis of BK, AI, and hippuryl-His-Leu were 61.0, 2.5, and 0.26 μM^{-1} s^{-1}, respectively (*18*, *19*).

The presence of Ser and Thr in the peptides Abz-SRK-(Dnp)P-OH and Abz-TRK(Dnp)P-OH reduces the k_{cat}/K_m values due to an increase of K_m for hydrolysis of both peptides and a decrease of k_{cat} for the hydrolysis of the latter. On the other hand, Abz-SRK(Dnp)P-OH and Abz-TRK-(Dnp)P-OH are hydrolyzed by the N-domain with k_{cat} values 10−70 times higher than those obtained with the C-domain. Abz-SDK(Dnp)P-OH and the analogues obtained by substitution of Ser with Thr, Tyr, and Phe (Figure 5) are hydrolyzed with lower k_{cat}/K_m values than those peptides derived from Abz-FRK(Dnp)P-OH with Arg at P_1. In contrast, the peptides Abz-SDK(Dnp)P-OH and Abz-TDK-(Dnp)P-OH are hydrolyzed by the N-domain with k_{cat} values 250−300 times higher than those obtained with the C-domain. Therefore, Ser and Asp at P_2 and P_1, respectively, have synergic effects on determining the differential specificity of the N-domain, compared to the C-domain. In Abz-SDK(Dnp)P-OH, the presence of Abz instead of an acetyl group and Dnp at the Lys side chain improved the susceptibility of this peptide to wild-type ACE and N-domain activity in comparison to the hydrolysis of the natural peptide, acetyl-SDKP-OH (*21*). The higher dependence to NaCl for the hydrolysis of Abz-peptidyl-EDDnp peptides compared to the activity upon Abz-peptidyl-K(Dnp)P-OH substrates suggested that the absence of negative charge of the C-terminal carboxyl group is compensated by chloride ion. It is noteworthy that the differentiation of hydrolytic activities of the C-domain from that of the N-domain seems to be based on the presence of a free carboxyl group, although the best phosphinic peptide that selectively blocked N-domain activity has an amidated C-terminal carboxyl group (*25*).

In conclusion, the differentiation of the catalytic specificity of the C- and N-domains of ACE seems to depend upon very subtle variations on specific amino acids of the substrates. The presence a C-terminal carboxyl group or an

aromatic moiety at the same substrate position determines specific interactions with the ACE active site which is regulated by chloride and seems to distinguish the activities of both domains. In addition, we described in these paper two highly efficient fluorescent substrates for ACE and two others that can distinguish the hydrolytic activities of the C- and N-domain catalytic sites.

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