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Differences in the Hydrolysis of Enkephalin Congeners by the Two Domains of Angiotensin Converting Enzyme

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ABSTRACT. The hydrolysis of enkephalin (Enk) congeners by the isolated N- (N-ACE) and C-domain of angiotensin I converting enzyme (ACE) and by the two-domain somatic ACE was investigated. Both Leu^5- and Met^5-Enk were cleaved faster by the C-domain than by N-ACE; rates with somatic ACE were 1600 and 2500 nmol/min/nmol enzyme with both active sites being involved. Substitution of Gly^2 by D-Ala^2 reduced the rate to 1/3rd to 1/7th of that of the Enks. N-ACE cleaved Met^5-Enk-Arg^6-Phe^7 faster than the C-domain, probably with the highest turnover number of any naturally occurring ACE substrate (7600 min^{-1}). This heptapeptide is also hydrolyzed in the absence of Cl^-, but the activation by Cl^- is unique; Cl^- enhances the hydrolysis of the heptapeptide by N-ACE but inhibits it by the C-domain, yielding about a 5-fold difference in the turnover number at physiological pH. This difference may result in the predominant role of the N-domain in converting Met^5-Enk-Arg^6-Phe^7 to Enk *in vivo*. BIOCHEM PHARMACOL 53;10:1459–1463, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. kininase II; opioid peptides; ACE N-domain; ACE C-domain; peptidyl dipeptidase; chloride

The widely distributed ACE† has two domains, each with an active center; this form of the enzyme is named somatic ACE [1–4]. The active sites contain zinc as cofactor, and the hydrolysis of most substrates is enhanced by chloride ions, although each to a different degree [5–7]. According to the position of the domains in the NH_2-terminal half or in the COOH-terminal half of the single chain protein, they are named N- or C-domain. The synthesis of ACE is directed by a single gene in the body [2, 3, 8]. The testicular form of the enzyme (germinal ACE) is shorter than the somatic ACE (732 vs 1306 residues) and contains a single C-domain, which is attached to cell membranes [2, 3, 8–12]. The NH_2-terminal 67 amino acids of the human germinal ACE are absent from the somatic enzyme, but the last 665 residues are the same as the COOH-terminal portion of somatic ACE.

We discovered in human ileal fluid, collected after surgery, a naturally occurring, short form of ACE having only the intact N-domain active site, N-ACE. The molecular mass of deglycosylated N-ACE is 68 kDa, while with the attached carbohydrate it has a mass of slightly over 100 kDa [13], suggesting a carbohydrate content of 37%.

Experiments with the naturally occurring N-ACE [13, 14] and with recombinant ACE, where site-directed or deletion mutagenesis left only a single active center functional [15], indicated that, in spite of the high degree of similarity in the amino acid sequence at the active centers of the two domains [2, 3], many of their characteristics may differ.

ACE hydrolyzes a variety of substrates, in addition to the well-known activation of angiotensin I and the inactivation of bradykinin [7, 16], by the release of a COOH-terminal dipeptide. ACE cleaves peptides with a free COOH-terminal group faster than those with a blocked terminus; for example, substance P free acid (Met^{11}-OH) is hydrolyzed faster than substance P (Met-NH_2) [17]. Nevertheless, ACE releases the COOH-terminal tri- or dipeptide of substance P as well as tripeptides from other peptides with protected COOH-termini such as LHRH [18], gastrin, or cholecystokinin [7]. Among the biologically active substrates, the opioid peptides have a higher turnover number than bradykinin [19], but owing to their relatively high K_m, their specificity constant (k_{cat}/K_m) is much lower than that of bradykinin. The short half-lives of Enks and related opioid peptides led to studies of their enzymatic inactivation, almost simultaneously with their discovery [20]. Aminopeptidases cleave the Enks to Tyr^1 and Gly^2-Gly^3-Phe^4-Met^5/Leu^5. Later, to block this effect, derivatives containing D-Ala^2 were synthesized. It was also de-

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† *Abbreviations:* ACE, angiotensin I converting enzyme, kininase II; N-ACE, natural N-domain active site of ACE; LHRH, luteinizing hormone-releasing hormone; Enk, enkephalin; M-Enk-RF, Met^5-enkephalin-Arg^6-Phe^7; TFA, trifluoroacetic acid; and NEP, neutral endopeptidase 24.11, neprilysin.

scribed that ACE on the plasma membrane of cultured endothelial cells or purified ACE cleaves Enks to tri- and dipeptides [21]. The same reaction is catalyzed by a second enzyme called enkephalinase [20], which is identical to NEP.

Another opioid, M-Enk-RF, normally found in brain and adrenal gland, is an eight times more potent analgesic, when given intraventricularly, than Met^5-Enk [22]. Although Enks specifically bind to δ and μ opioid receptors, the COOH-terminally extended peptides have a relatively higher affinity than the pentapeptides to κ receptors [23]. It was also reported that ACE converts M-Enk-RF to Enk, which can be inactivated subsequently by NEP, and that the hydrolysis of this peptide by ACE does not require the presence of chloride ions [24, 25].

However, it was unknown to what degree each of the two active sites on ACE participates in the process and how replacement of some residues in commercial and naturally occurring Enk derivatives can affect the hydrolysis by the two active sites. We report here that the two domains of ACE differed significantly in their efficiency in cleaving Enks and their derivatives. We also found that the N-ACE cleaved M-Enk-RF with the highest turnover rate of any endogenous substrate of ACE, and that the difference in activity of the two domains was influenced profoundly by the difference in Cl^- sensitivity of the respective active centers.

MATERIALS AND METHODS

Materials

Met^5-Enk, Leu^5-Enk, D-Ala^2-Met^5- and Leu^5-Enk, M-Enk-RF, buffers, and reagents were purchased from the Sigma Chemical Co. (St. Louis, MO). M-Enk-RF-amide was purchased from Bachem (King of Prussia, PA).

Enzyme Purification

The purification of N-ACE, C-domain ACE, and somatic ACE has been described [13]. N-ACE was purified from ileal fluid collected after colostomy, somatic ACE from human cadaver kidneys, and germinal ACE from rabbit testicles [10] obtained from the Pel-Freez Co. (Rodgers, AR). Approval for human tissue studies was granted by the Institutional Review Board at the University of Chicago and for animal tissue study by the Animal Care and Usage Committee at the University of Illinois at Chicago.

Protein Assay

The protein concentration of purified enzymes was determined by the method of Bradford [26], using crystalline bovine serum albumin as standard.

Analysis of Hydrolysis Products

The hydrolysis of the various Enk peptides by the ACE enzymes was assayed by HPLC. After incubation of enzymes with a peptide, ice-cold 5% TFA was added to a final concentration of 1.7% to stop the reaction. Peptides and their hydrolysis products were separated on a Waters μBondapak C_{18} reversed phase column with an increasing (5–40%) linear gradient of acetonitrile/0.05% TFA in H_2O/0.05% TFA and detected using a Waters 484 detector at a wavelength of 214 nm [27]. It was established that cleavage of the substrates followed zero order kinetics to the time point of the assay.

The hydrolysis of Enk peptides was assayed either at physiological pH (7.4) or at a pH approaching optimal (pH 7.75). Enk peptides (300 μM) were incubated with 2 nM N-ACE or C-domain ACE or 1.2 nM somatic ACE at 37° in 50 mM HEPES buffer, pH 7.75, containing 100 mM NaCl. The hydrolysis of M-Enk-RF was done in the above buffer in the absence or presence of NaCl in concentrations ranging from 10 to 500 mM. The substrate concentration was 300 μM, and the enzyme concentration was 2 nM. The rates of cleavage of the M-Enk-RF and M-Enk-RF-NH_2 peptides were compared at 37° in 50 mM Tris-maleate buffer, pH 7.4, containing 150 mM NaCl, 300 μM substrate, and 2 nM N-ACE or C-domain ACE. The effect of pH on hydrolysis of M-Enk-RF was assessed in the same buffer at pH values that ranged from 5.4 to 9.4. The kinetics of M-Enk-RF hydrolysis were determined by incubating a 2 nM concentration of enzyme (N-ACE or C-domain ACE) with the peptide in a concentration varying from 25 to 300 μM in 50 mM Tris-maleate, pH 7.4, and 150 mM NaCl at 37°. The amounts of RF and M-Enk formed were estimated from peak areas by comparison to known standards. Kinetic constants were obtained by initial velocity measurements at five substrate concentrations. Data were plotted according to Lineweaver–Burk and the best straight lines calculated by linear regression.

RESULTS

The rates of hydrolysis of Enks by N-ACE and C-domain ACE are summarized in Table 1. The data are expressed per nmol enzyme, and are compared with those obtained with the two-domain somatic ACE. Under the conditions indicated in Table 1, Met^5-Enk and Leu^5-Enk were cleaved by the C-domain faster than by N-ACE. Somatic ACE inactivated Enks at a rate that was about the sum of the rates by the two isolated domains except for D-Ala^2 derivatives.

Interestingly, substitution of Gly^2 with D-Ala^2 as in commercial and in some naturally occurring Enk derivatives [23, 28], which blocks the inactivation by an aminopeptidase [20], also considerably slowed down hydrolysis by the three ACE preparations. The reduction of rate ranged between 70 and 90%, in spite of the fact that the D amino acid was in the P_2 position, one residue removed from the cleavage site (Table 1).

TABLE 1. Hydrolysis of Enks by ACE at pH 7.75 and 100 mM Cl^-

Substrate*	Rate of hydrolysis (nmol/min/nmol enzyme)		
	N-ACE†	C-domain†	Somatic‡
Leu^5-Enk	500 ± 49	1200 ± 187	1600 ± 121
Met^5-Enk	800 ± 58	1700 ± 245	2500 ± 214
D-Ala^2-Leu^5-Enk	160 ± 19	190 ± 16	170 ± 10
D-Ala^2-Met^5-Enk	190 ± 22	400 ± 46	360 ± 22
M-Enk-RF	6200 ± 394	1300 ± 147	7200 ± 291

Results are expressed as means ± SEM, N = 3.
* Substrate concentration = 300 μM.
† Enzyme concentration = 2 nM.
‡ Enzyme concentration = 1.2 nM.

Depending on the pH and chloride concentration, M-Enk-RF was converted to Met^5-Enk 2–5 times faster by the N-ACE between pH values from 7 to 9; at pH 7.4 and in 150 mM Cl^- the N-ACE catalyzed the reaction about 5 times faster than the C-domain (Fig. 1). Somatic ACE cleaved this substrate at a rate that again indicated the participation of both active sites in the hydrolysis (Table 1).

Substitution of Phe^7-OH with Phe^7-NH_2 in M-Enk-RF—thus, instead of the free carboxyl group, the COOH-terminus was an amide—slowed down the cleavage per nmol N-ACE by more than 99% and over 90% by the C-domain ACE.

The K_m of the M-Enk-RF with N-ACE was 83 μM, but lower with the C-domain, 38 μM (Table 2). Because of the lower K_m with the C-domain, the specificity constant (k_{cat}/K_m) of N-ACE was only about twice as high (92 μM^{-1} min^{-1}) as that of C-domain ACE (42 μM^{-1} min^{-1}). Nevertheless, the turnover number of M-Enk-RF with N-ACE is 135 times higher, and with the C-domain ACE it is 13 times higher than that of bradykinin [14].

Although the activity of ACE generally requires the presence of Cl^- in the assay buffers, [5, 7, 8], M-Enk-RF was hydrolyzed even if the buffer lacked Cl^-. In the absence of Cl^-, the two domains cleaved this substrate at about the same rate, but increasing the Cl^- concentration at close to the pH optimum (7.75) enhanced the N-ACE activity and inhibited that of the C-domain, leading to the difference in the hydrolysis rate, as shown in Fig. 2.

TABLE 2. Kinetic parameters of hydrolysis of M-Enk-RF

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
N-ACE	83	7600	92
C-domain	38	1600	42

Values were calculated from Lineweaver–Burk plots of three separate experiments done in duplicate at pH 7.4 in 150 M NaCl.

DISCUSSION

In these studies we used, besides human purified somatic ACE and N-ACE, purified rabbit testicular ACE as the C-domain ACE. This enzyme has 87% identity in the amino acid sequence from residue 73 to 737 with the C-domain (amino acid residues 642–1306) of human somatic ACE [10]. Of the 85 differences in amino acid residues, only nine substitutions can be considered to be non-conservative. In the region of the essential active site residues, 426 to 469 in rabbit testicular and 989 to 1032 in human somatic ACE, the identity is 100%. The homology in the C-domain of the rabbit and human enzymes is considerably higher than between the N- and C-domains of the human enzyme, e.g. the overall identity is 68 and 89% in the active site regions [1]. Thus, the experiments with the rabbit or human enzyme should yield quite similar results. The fact that the rates of hydrolysis of Leu^5-Enk and Met^5-Enk by the human N-ACE and rabbit germinal ACE add up to an overall rate of hydrolysis by human somatic ACE is taken in support of

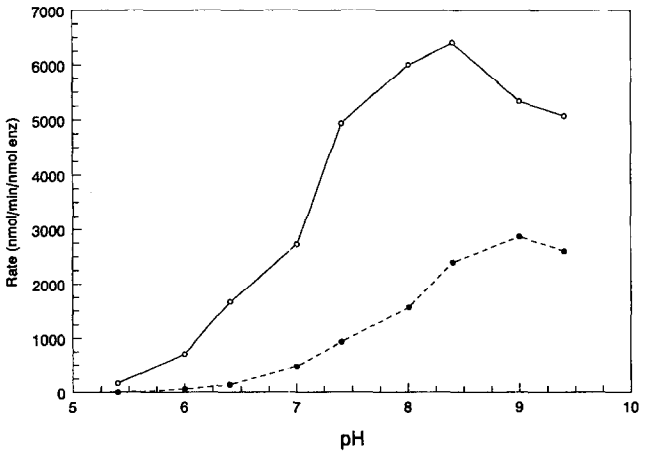

FIG. 1. Effect of pH on the hydrolysis of M-Enk-RF by N-ACE and C-domain ACE. Experiments were done in 50 mM Tris-maleate buffers of 5.4 to 9.4 pH, containing 150 mM NaCl. Substrate concentration = 300 mM; enzyme concentration = 2 nM. Rates are the averages of two experiments done in duplicate. Key: (-○-) N-ACE, and (--●--) C-domain ACE.

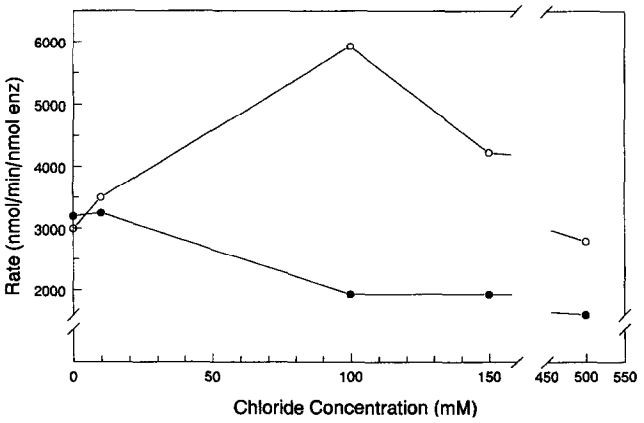

FIG. 2. Effect of Cl^- concentration on the hydrolysis of M-Enk-RF by N-ACE and C-domain ACE. The buffer was 50 mM HEPES, pH 7.75, containing 0 to 500 mM NaCl. Substrate concentration = 300 μM; enzyme concentration = 2 nM. Rates are the averages of two experiments done in duplicate. Key: (-○-) N-ACE, and (--●--) C-domain ACE.

these statements. In contrast, the D-Ala^2 derivatives of Enks may be cleaved only by a single active site on somatic ACE (Table 1).

Cl^- has been considered to be an activator of ACE since Skeggs *et al.* [5] described that its presence is necessary for the conversion of angiotensin I to II. In contrast to these findings, the inactivation of bradykinin can proceed at 30–35% [7] of the optimal rate in the absence of Cl^- [8]. In experiments based on using recombinant mutated ACE having only a single functional active center, or recombinant N- and C-fragments, the N-domain was activated maximally by 20 mM Cl^-, while the C-domain reached optimal activity in over 800 mM Cl^- [29] when the substrate was Hip-His-Leu [15]. Other substrates are hydrolyzed optimally at different Cl^- concentrations [30].

We have shown here that the Cl^- sensitivities of the domains differ in a unique way—the cleavage of M-Enk-RF by N-ACE is enhanced by chloride ions while that by the C-domain is inhibited. The two domains in the absence of chloride cleave this substrate at about an equal rate. When approaching physiological levels of the anion, however, the difference in the turnover is about 5-fold, very likely because Cl^- activates one domain but inhibits the other. Although it is known that the N- and C-domains differ in Cl^- sensitivity, the unique effect of Cl^- on the hydrolysis of M-Enk-RF by the two domains has not been observed with any other substrate. The reason for this phenomenon may be a conformational change induced by the anion. It was suggested, based on optical density measurements, that ACE has a different configuration when in the presence of Cl^- than in its absence [31].

The calculated turnover rate of M-Enk-RF is remarkably fast. It is cleaved 135 times faster than bradykinin by the N-domain [14], while the corresponding ratio for the C-domain is 13. Met^5-Enk was considered to be one of the fastest hydrolyzed substrates of ACE [19], but the turnover of M-Enk-RF was over 12 times higher (Table 1). Taking the specificity constant of bradykinin [19] and adjusting the values according to the ratio of activities of the two domains with this peptide revealed that the k_{cat}/K_m of M-Enk-RF by the N-domain was about the same as that of bradykinin [12, 14, 15]. The hydrolysis of M-Enk-RF by the N-ACE has a turnover number (k_{cat}) that is much higher (7600 min^{-1}) than that determined for the hydrolysis of other substrates by recombinant N-domain ACE, including acetyl-Ser-Asp-Lys-Pro (960 min^{-1}) [32] or angiotensin I (600 min^{-1}) [14]. In addition, the specificity constant (k_{cat}/K_m) for M-Enk-RF was higher (92 min^{-1} μM^{-1}) than that for acetyl-Ser-Asp-Lys-Pro (31 min^{-1} μM^{-1}) or angiotensin I (42 min^{-1} M^{-1}). Thus, M-Enk-RF is likely an important physiological substrate for the N-domain active site of ACE.

The ratio of the activities of the two domains changes when Leu^5-Enk or Met^5-Enk is the substrate; here, the C-domain is over twice as active as the N-domain. These pentapeptides also have a high (1 mM) K_m with somatic ACE [19]. Substitution of Gly^2 with D-Ala slows down the reaction considerably. When, instead of a free COOH group of Phe^7, M-Enk-RF was blocked at the COOH-terminal (Phe^7-NH_2), the decrease in the rate of hydrolysis was even more pronounced. This phenomenon may contribute to the higher potency and stability of the naturally occurring dermorphins and possibly to that of the less active deltorphins. Dermorphins are the ligands for δ and deltorphins for μ receptors [23, 28], and generally contain D-Ala^2 and a $CONH_2$ carboxyl terminus.

In conclusion, both active sites on the somatic ACE participate in the hydrolysis of Enks and M-Enk-RF. This cleavage results in the conversion of a heptapeptide to Enk or in the inactivation of Enks. The activities of the individual active site domains, nevertheless, differ with the Enk peptide substrates, and Cl^- concentration can greatly influence the relative contributions of each domain to the hydrolysis of M-Enk-RF *in vitro* and very likely *in vivo* as well. It remains to be seen whether any therapeutically employed ACE inhibitor could affect this reaction, and if such an inhibition would have an analgesic effect as in animal experiments [33].

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