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NOVEL SUBSTRATES FOR ANGIOTENSIN I CONVERTING ENZYME

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Homogenous human angiotensin converting enzyme (EC 3.4.15.1) cleaves dipeptides from the C-terminus of substrates containing a free carboxyl group. In this study we demonstrate that peptides containing a C-terminal nitrobenzylamine are also cleaved by the enzyme. The hydrolysis of these substrates is inhibited by the specific converting enzyme inhibitors captopril and MK421 as well as by anti-converting enzyme antibody. Sodium chloride accelerates the rate of hydrolysis forty-fold. The product of the reaction, an amino acid nitrobenzylamide, was identified by thin layer chromatography and high performance liquid chromatography. These results suggest that the carboxyl group is not an absolute requirement for substrate hydrolysis.

Human angiotensin I converting enzyme or kininase II is a peptidyl dipeptidase (EC 3.4.15.1) that cleaves C-terminal dipeptides of diverse substrates such as angiotensin I (1), bradykinin (1), enkephalin (2), and a series of N-terminal blocked synthetic tripeptides (1,2). Specific inhibitors of CE were synthetized (3) in the hope that they will gain wide acceptance in the treatment of high blood pressure and congestive heart failure(4,5). The substrates and specific inhibitors of CE contain a C-terminal amino acid with a free carboxyl group. This group is essential for attachment to a postively charged group, probably to an arginine residue, on the enzyme (6). The aim of the present study was to determine whether a C-terminal carboxyl group is an

Abbreviations - CE - angiotensin I converting enzyme; Abz-2-aminobenzoyl, Nba-4-nitrobenzylamine.

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absolute requirement or whether substrates not having this component could also be effectively hydrolyzed by homogeneous human CE. In order to establish this we tested four fluorogenic substrates, which contained a 4-nitrobenzylamine in place of the C-terminal amino acid and a 2-aminobenzoyl group on the N-terminal amino acid.

The fluorescent 2-aminobenzoyl group in these substrates is quenched by the 4-nitrobenzyl group. Cleavage at any peptide bond leads to an increase in fluorescence which can be easily detected. Previously reported quench CE substrates include Abz-Gly-Phe(NO_2)-Pro (7) and Z(NO_2)-Gly-Trp-Gly (8). However both of these substrates contain a free C-terminal amino acid.

Methods

Human kidney CE was purified as previously described (9) to a specific activity of 80 μmol/min per mg using α-N-Benzoyl-Gly-His-Leu as a substrate. (10) Antibody to converting enzyme was obtained in rabbits.

The fluorogenic substrates used in this study were prepared and assayed as described in detail elsewhere (Rush, Mitas, Powers, Tanaka, and Hersh, submitted for publication). The hydrolysis of these substrates was measured in 0.25 ml reaction mixtures containing 100 mM HEPES buffer, pH 8.0, 100 mM sodium chloride, 1% dimethylformamide, substrate and enzyme. In routine assays the substrate concentration was 50 μM. The increase in fluorescence was monitored at an excitation wavelength of 340 nm and an emission wavelength of 415 nm.

The preparation of glycyl-4-nitrobenzylamide and leucyl-alanyl-glycyl-4-nitrobenzylamide was given previously (Rush, Mitas, Powers, Tanaka, and Hersh, submitted for publication).

The split products of the reaction with the substrates listed in Table I were analyzed by thin layer and by a high pressure liquid chromatography system. Thin layer chromatography was carried out on silica gel thin layer plates using chloroform:methanol (2:8) as a solvent. High performance liquid chromatographic analyses were conducted in an automated Waters gradient system with a μ-Bondapak C-18 column. Products were eluted with a 20 min linear gradient of 0-70% acetonitrile containing 0.02% trifluoroacetic acid and were detected at 254 nm.

Results and Discussion

CE activity is believed to depend on the presence of a free carboxyl group at the C-terminal amino acid of its substrates, as stated above. It was anticipated that the fluorogenic peptides containing a 4-nitrobenzylamide in place of the C-terminal amino acid would not be hydrolyzed by CE. However, as summarized in Table 1, CE cleaved all four fluorogenic substrates tested. Although the fluorogenic peptides had a considerably lower kcat than the synthetic substrate α-N-Benzoyl-Gly-His-Leu (6,800 min^{-1}; 10), the rates of hydrolysis are more comparable to the rate of hydrolysis of larger peptide

Table 1 Kinetics of Hydrolysis of Fluorogenic Substrates by Human Kidney CE

	Substrate	K_m (μM)	V_{max} μmol (min-mg)	k_{cat} s^{-1}	k_{cat}/K_m M^{-1} s^{-1}
1.	Abz-Ala-Ala-Leu-Ala-Gly-Nba	170	0.55	1.83	0.8×10^4
2.	Abz-Ala-Tyr-Leu-Ala-Gly-Nba	180	1.32	3.30	1.8×10^4
3.	Abz-Ala-Ala-Tyr-Leu-Ala-Gly-Nba	140	1.01	2.53	1.8×10^4
4.	Abz-Val-Tyr-Leu-Ala-Gly-Nba	60	0.34	0.85	1.4×10^4

substrates such as angiotensin I or bradykinin which exhibited a kcat of ∿8 s^{-1} (10).

The possibility that the hydrolysis of the fluorogenic substrates was due to a contaminant in the CE preparation was ruled out by the use of specific inhibitors for CE. As shown in Table 2, the hydrolysis of 4-aminobenzoyl-Val-Tyr-Leu-Ala-Gly-4-nitrobenzylamide was inhibited by captopril (3), MK 421 (11), o-phenanthroline, (2) and antibody to human CE (9,10). In addition, chloride ions activated the enzyme as expected since chloride accelerates the hydrolysis of most substrates by CE (2).

Human kidney "enkephalinase" (Gafford, Skidgel, Erdös, and Hersh submitted for publication), and rat brain "enkephalinase" (Rush, Mitas, Powers, Tanaka, and Hersh, submitted for publication), neutral Zn proteases, cleave the fluorogenic substrates listed in Table 1, at the amino side of leucine. When we tested human CE, thin layer chromatography analysis revealed that it

Table 2: Effect of Inhibitors and Chloride Ion on the Hydrolysis of Abz-Val-Tyr-Leu-Ala-Gly-Nba

	Agent	Concentration	Inhibition	Activation
1.	o-Phenanthroline	1×10^{-4}M	60	
2.	MK 421 (diacid)	2×10^{-8}M	50	
3.	Captopril	2×10^{-8}M	50	
4.	Rabbit antiserum to human CE	1:100 dilution (v/v)	50	
5.	Sodium chloride	1×10^{-1}M	-	40-fold

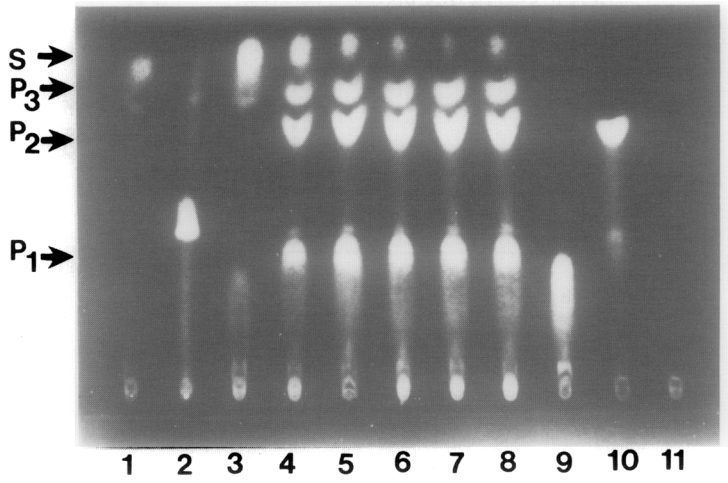

Figure 1. Thin layer chromatography of the products of hydrolysis of Abz-Ala-Tyr-Leu-Ala-Gly-Nba by human converting enzyme.

Reaction mixtures contained 100 mM HEPES buffer, pH 8.0, 10 mM sodium chloride, 1 mM substrate, and 0.75 µg of human kidney CE in a final volume of 150 µl. At the time points noted a 25 µl aliquot was withdrawn and added to 5 µl of 60 mM EDTA to stop the reaction. Fifteen µl of each sample was spotted on thin layer plates and chromatographed as described in methods. From left to right the samples are:

1. Substrate alone; 2. Leu-Ala-Gly-4-nitrobenzylamide 3-8; incubation times of 0,10,20,30,40 and 60 min respectively; 9. Gly-4-nitrobenzylamide, 10.Leu-Ala; 11. enzyme alone.

released as products glycyl-4-nitrobenzylamide and the dipeptide leucylalanine. Fig. 1 shows the results obtained with substrate 2 of Table I. The formation of glycyl-4-nitrobenzylamide was confirmed by high performance liquid chromatography analysis of the reaction products (Fig. 2). It is noteworthy to point out that at all time periods measured both leucylalanine and glycyl-4-nitrobenzylamide were observed. This suggests that CE liberates glycyl-4-nitrobenzylamide first which is followed by a rapid sequential release of leucylalanine. Since leucylalanine does not absorb at 254 nm, the wavelength employed in high performance liquid chromatography, the second peak in Figure 2 with a slightly longer retention time than glycyl-4-nitrobenzylamide was due to the appearance of the N-terminal fragment of the substrate.

The cleavage pattern observed suggests that the nitro group may serve to mimic a free carboxyl group because human CE cleaved glycyl-nitrobenzylamide as a putative dipeptide. All the specific inhibitors and substrates of CE

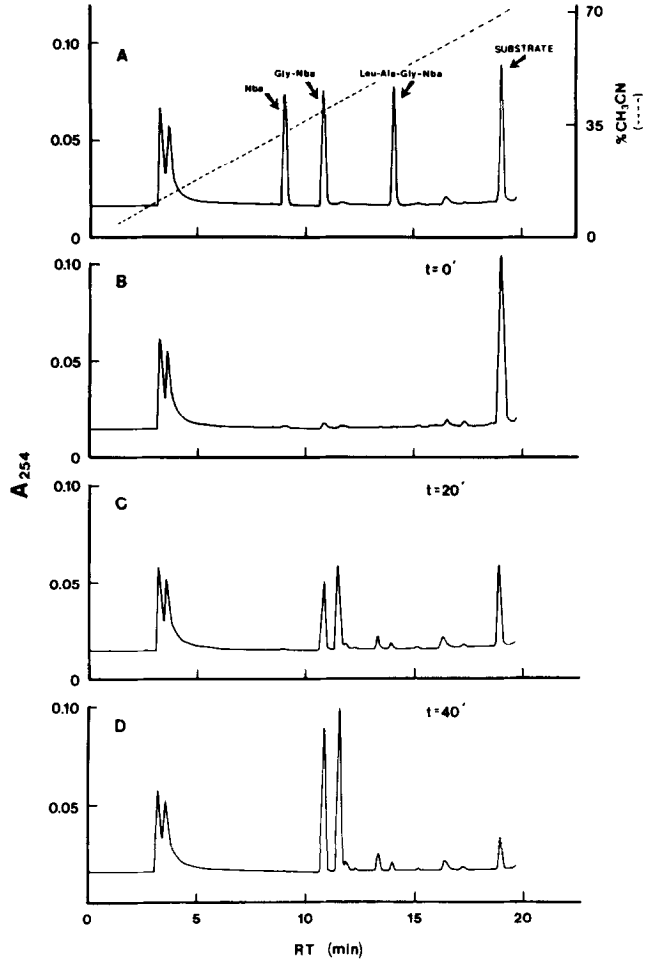

Figure 2. Separation of the products of hydrolysis of 2-aminobenzoyl Ala-Tyr-Leu-Ala-Gly-4-nitrobenzylamide by human converting enzyme in high performance liquid chromatography.

Samples derived as described in figure 1 were diluted to 50 ul with water. Aliquots of 20 µl were analyzed by high performance liquid chromatography as described in Methods.

Panel A standards: 4-Nitrobenzylamine (6 nmol); Gly-4-nitrobenzylamide (6 nmol); Leu-Ala-Gly-4-nitrobenzylamide (6 nmol); Substrate (6 nmol); Panel B, 0 time reaction; Panel C, after 20 min reaction time; Panel D, after 40 min reaction time; Abscissa: retention time in min. Ordinates : absorbance at 254 nm and acetonitrile gradient.

used until now contain a C-terminal amino acid with a free carboxyl group. Alternatively these fluorogenic substrates may bind to CE with the nitrophenyl group bound in the hydrophobic S'_2 pocket. In this binding mode the nitrophenyl group mimics the side chain of the P'_2 amino acid and not the terminal carboxyl group.

It has not been determined yet which amino acid in the penultimate position and adjacently would be optimal for the hydrolysis of this type of substrate by CE. However, these fluorogenic substrates, in addition to being convenient tools for measuring CE activity, widen the scope of compounds with affinity to the active center of CE.

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