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ESTERASE ACTIVITY OF RABBIT PULMONARY ANGIOTENSIN CONVERTING ENZYME

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SUMMARY: A series of depsipeptides have been synthesized and used to demonstrate the esterase activity of rabbit pulmonary angiotensin converting enzyme. Among the esters studied, Bz-Phe-OPhe-Ala was found to have the highest k_{cat}/K_m which is about 1/5 that of its exact peptide analog, Bz-Phe-Phe-Ala. Esters such as Bz-Gly-OGly-Phe, Bz-Gly-OPhe-Phe and Bz-Gly-OLeu-Ala were also hydrolyzed but at much lower rates. Normal Michaelis-Menten behavior is observed and the kinetic parameters obtained indicate that the esters and their peptide analogs bind to the enzyme equally well, but that peptides are hydrolyzed at much higher rates. Studies on the pH-rate profiles, chloride ion effect, inhibition and chemical modifications detect no mechanistic differences between ester and peptide hydrolysis.

INTRODUCTION: Angiotensin converting enzyme (dipeptidyl carboxypeptidase, E.C. 3.4.15.1) (ACE) is a dipeptide releasing carboxypeptidase with activity towards a broad range of oligopeptide substrates (1). The best known physiological function of this enzyme is to maintain blood pressure via production of the pressor peptide, angiotensin II, and the destruction of a depressor peptide, bradykinin (2-4). The clinical importance of one or both of these processes in hypertensive disease has been clarified by

ABBREVIATIONS: ACE, angiotensin converting enzyme; Bz, benzoyl; OLeu, 2-hydroxyisocaproic acid; OPhe, 2-phenyllactic acid; OGly, glycolic acid; hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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the finding that specific inhibitors of converting enzyme are potent drugs for the control of hypertension in man (5).

ACE contains a catalytically essential zinc atom and, therefore, has been thought to possess mechanistic characteristics similar to the more extensively studied zinc proteases, bovine carboxypeptidase A and thermolysin (6). Most studies on the mechanism of action of carboxypeptidase A have been guided by the dual specificity (peptidase and esterase) of this enzyme as altered either by replacement of the metal at the active site or by chemical modification of amino acid side chains (7). Although thermolysin has been shown to catalyze the hydrolysis of ester substrates, the mechanistic features of this activity are quite different from those of carboxypeptidase A (8). Esterase activity of ACE has not previously been demonstrated. We have found that rabbit pulmonary ACE catalyses the hydrolysis of a series of depsipeptide substrates. We have also attempted to differentiate the esterase and peptidase activities of ACE by studying the effect of pH, chloride ion concentration, inhibition and chemical modification on rates of hydrolysis of the ester and peptide substrates.

MATERIALS AND METHODS: ACE was purified from rabbit lung acetone powder (Pel-Freez Bioanimals, Inc., Rogers, Arkansas) by a variation of the method of Oshima, et al (9). Protein concentrations were determined by absorbance at 280 nm using a molar absorptivity of 2.04×10^5 M^{-1} cm^{-1} for the pure enzyme (10). Depsipeptides Bz-Phe-OPhe-Ala, Bz-Gly-OPhe-Ala, Bz-Gly-OPhe-Phe, Bz-Gly-O-Gly-Phe, Bz-Gly-OLeu-Ala and their exact peptide analogs were prepared by methods previously described (8). All kinetic measurements were made at 25° ± 0.1°C in 50 mM hepes buffer, pH 7.5 containing 0.3 M NaCl unless otherwise indicated. Hydrolysis of N-benzoyl blocked substrates was followed spectrophotometrically by continuous monitoring of the increase in absorbance at 257 nm (8). Chemical modifications were carried out as described in respective standard procedures or in the text.

RESULTS: The activity of ACE, expressed as k_{cat}/K_m, of a series of depsipeptides and their exact peptide analogs is given in

Table I. The results clearly indicate that the rabbit pulmonary enzyme has considerable esterase activity. Thus, Bz-Phe-OPhe-Ala is hydrolysed with a k_{cat}/K_m of 1.7 x 10^7 M^{-1} min^{-1}, approximately one-fifth that obtained for its peptide analog, Bz-Phe-Ala-Phe. The rates of hydrolysis of the other esters studied are two to eight hundred fold lower than that of the Bz-Phe-OPhe-Ala. Without exception the esters are hydrolyzed at slower rates than the corresponding peptides.

The kinetic parameters, k_{cat} and K_m (Table II) of the substrate pairs: Bz-Phe-Phe-Ala, Bz-Phe-OPhe-Ala and

TABLE I

Ester and Peptide Substrates for Angiotensin Converting Enzyme[a]

	k_{cat}/K_m ($\times 10^{-4}$ M^{-1} min^{-1})
Bz-Phe-OPhe-Ala	1700[c]
Bz-Phe-Phe-Ala	8300[c]
Bz-Gly-OPhe-Ala	3.9[b]
Bz-Gly-Phe-Ala	660[b]
Bz-Gly-OPhe-Phe	7.9[b]
Bz-Gly-Phe-Phe	650[b]
Bz-Gly-OGly-Phe	44[c]
Bz-Gly-Gly-Phe	1480[c]
Bz-Gly-OLeu-Ala	1.9[b]
Bz-Gly-Leu-Ala	160[b]

[a]Measured at pH 7.5 in 0.05 M hepes buffer containing 0.3 M NaCl.
[b]k_{cat}/K_m were calculated from the first order rate constants for substrate hydrolysis measured under the condition that [S] << K_m.
[c]Calculated from data obtained by analysis of Lineweaver-Burk plots.

TABLE II

Kinetic Parameters for Two Ester-Peptide Substrate Pairs with ACE Determined from Lineweaver-Burk Plots[a]

Substrate	$k_{cat}(min^{-1})$	$K_m(M)$
Bz-Phe-Phe-Ala	5.4×10^{3}	6.5×10^{-5}
Bz-Phe-OPhe-Ala	1.1×10^{3}	6.5×10^{-5}
Bz-Gly-Gly-Phe	3.7×10^{4}	2.5×10^{-3}
Bz-Gly-OGly-Phe	1.1×10^{3}	2.5×10^{-3}

Bz-Gly-Gly-Phe, Bz-Gly-OGly-Phe were determined in 50 mM hepes buffer, pH 7.5 containing 0.3 M NaCl from Lineweaver-Burk plots which were linear in all cases. The values for both pairs of substrates studied indicate that depsipeptides and their exact peptide analogs bind to the enzyme equally well but that the peptides are turned over at much faster rates.

The dipeptide Val-Trp has been shown to competively inhibit the peptidase activity of ACE (11) when examined with Hip-His-Leu. In the present study, Val-Trp inhibits the hydrolysis of both Bz-Phe-Phe-Ala and Bz-Phe-OPhe-Ala competitively and with a K_i of 1.8×10^{-6} M, identical for both substrates (Figure 1).

The rates of hydrolysis of the substrate pair, Bz-Gly-Gly-Phe and Bz-Gly-OGly-Phe were studied over the pH range from 6 to 9 where the hydrolyses of these substrates, at a concentration of 5×10^{-5} M, follow first order kinetics. The variation of log $[k_{cat}/K_m]$ with pH for the ACE-catalysed hydrolysis of this ester-peptide pair in the absence of added

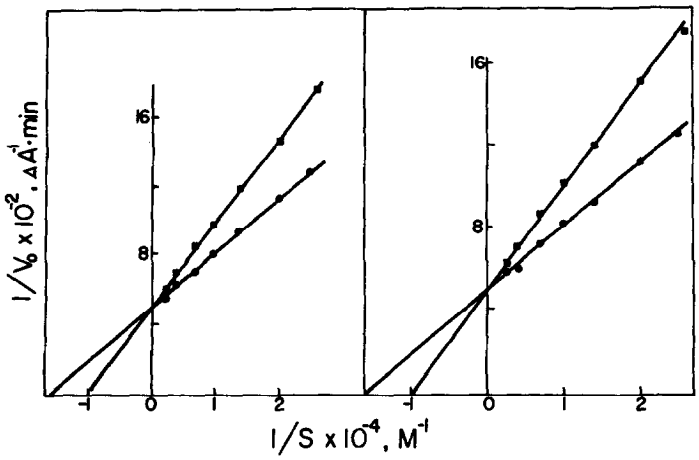

Figure 1 Lineweaver-Burk plots of the ACE catalysed hydrolysis of the ester-peptide substrate pairs Bz-Phe-Phe-Ala (left) and Bz-Phe-OPhe-Ala (right) in the absence (●) and presence (■) of Val-Trp (1 μM). Hydrolysis was carried out in 50 mM hepes buffer, pH 7.5, containing 0.3 M NaCl.

zinc is shown in Figure 2. Over the entire pH range, the activity profiles for the two substrates are superimposable with activity toward the peptide being approximately thirty fold greater than that of the ester.

The same peptide-ester pair was used to study the effects of chloride ion concentration on the peptidase and esterase activities of ACE. In the absence of chloride, ACE exhibits

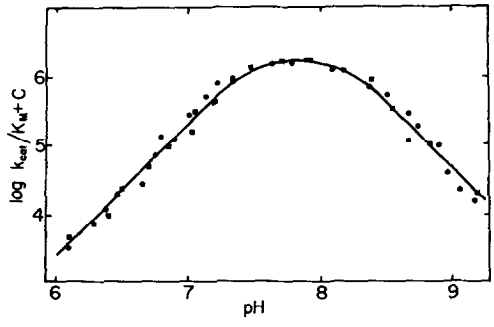

Figure 2 pH-rate profiles for the ACF-catalysed hydrolysis of the peptide, Bz-Gly-Gly-Phe (■) and the ester, Bz-Gly-OGly-Phe (●); both 5 x 10^{-5} M, in 50 mM hepes buffer containing 0.3 M NaCl. C = 0.33 for esterase activity and 0 for peptidase activity.

essentially no activity towards either the ester or the peptide. The peptidase and esterase activities of ACE increase rapidly and concomitantly as the chloride concentration in the assay medium increases. Half maximal activity for both is attained at 40 mM NaCl and maximal activity at 0.3 M NaCl, Table III.

Arginine modification of ACE with 2,3-butanedione was carried out in two media, i.e. 50 mM borate buffer, pH 8.3 and 60 mM hepes buffer, pH 8.3, for 4 hours. Both the esterase and peptidase activities of the enzyme were inactivated to the same extent by 20 mM butanedione (Table III). Acetylation of tyrosyl residues with N-acetylimidazole has also been studied. Within 2 hours, 1 mM N-acetylimidazole abolishes both the peptidase and esterase activity of ACE when assayed with Bz-Phe-Phe-Ala and Bz-Phe-OPhe-Ala (Table III).

DISCUSSION: Recently, it has been demonstrated that ACE is a zinc metalloenzyme with mechanistic properties similar to the more

TABLE III

Effect of Chloride, Butanedione, and N-Acetylimidazole on the Esterase and Peptidase Activity of ACE[a]

Agent	Activity[b], % Esterase	Peptidase
Control[b]	100	100
40 mM $Cl^{\ominus}$	50	50
2,3-butanedione	45	45
2,3-butanedione + borate	3	4
N-Acetylimidazole	<2	<2

[a]Ester; Bz-Phe-OPhe-Ala, peptide; Bz-Phe-Phe-Ala

[b]Relative activity determined in 50 mM hepes, pH 7.5, 0.3 M NaCl

extensively studied proteolytic zinc enzymes, bovine carboxypeptidase A and thermolysin (10). In addition to a catalytically essential zinc atom, chemical modification studies have shown that both carboxypeptidase A and ACE have functional tyrosyl, arginyl and carboxyl residues (12). The esterolytic activity of carboxypeptidase has been known for many years (7) and recently, the hydrolysis of ester substrates by zinc neutral proteases has been documented (8). Therefore, the finding that ACE also catalyses the hydrolysis of esters is not unexpected. The ratios of esterase to peptidase activities of ACE are unusually low and are primarily due to low turnover numbers for the ester substrates (Table II).

Like the peptidase activity, the esterase activity of ACE is dependent on chloride concentration. Since it has been shown that chloride ion is essential for substrate binding (13), the fact that peptidase and esterase activities increase in parallel with increasing NaCl concentration suggests that peptides and esters bind to the enzyme in a similar mode. The finding that the inhibitor Val-Trp competes equally with peptide and ester substrates also suggests that esters and peptides bind to identical sites on the enzyme.

Identical amino acid residues seem to be essential to the catalysis of both esters and peptides as indicated by the identity of their pH-rate profiles. Furthermore, chemical modification of arginyl and tyrosyl residues with butanedione and N-acetylimidazole, respectively, leads to concommitant loss of both the esterase and peptidase activity. Based on these results, it would appear that in contrast to carboxypeptidase A (14) but like thermolysin the hydrolysis of peptides and esters by ACE proceeds by identical binding and catalytic mechanisms

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