

## Substrate Specificity and Kinetic Characteristics of Angiotensin Converting Enzyme<sup>†</sup>

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**ABSTRACT:** Furanacryloyl-Phe-Gly-Gly has been shown to be a convenient substrate for angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1). A detailed kinetic analysis of the hydrolysis of this substrate indicates normal Michaelis-Menten behavior with  $k_{\text{cat}} = 19000 \text{ min}^{-1}$  and  $K_M = 3.0 \times 10^{-4} \text{ M}$  determined at pH 7.5, 25 °C. The enzyme is inhibited by phosphate and activated by chloride; maximal activity is observed with 300 mM NaCl. In the absence of added zinc, activity is lost rapidly below pH 7.5 due to spontaneous dissociation of the metal, but in the presence of

zinc, the enzyme remains fully active to about pH 6. The pH-rate profile indicates two groups on the enzyme with apparent pK values of 5.6 and 8.4. The substrate specificity of the enzyme has been examined in terms of the fundamental specificity quantity  $k_{\text{cat}}/K_M$  as well as the separate constants by using a series of furanacryloyl-tripeptides. The activity toward furanacryloyl-Phe-Gly-Gly has been compared with that toward the physiological substrates angiotensin I and bradykinin.

Angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE)<sup>1</sup> catalyzes the hydrolytic release of dipeptides from the carboxyl terminus of oligopeptide substrates. Although the enzyme has broad specificity, its best known physiological function is to convert the decapeptide angiotensin I into the active octapeptide angiotensin II by the removal of the C-terminal dipeptide His-Leu (Skeggs et al., 1954, 1956a,b). However, the enzyme also catalyzes the inactivation of bradykinin (Yang et al., 1970) and the Leu- and Met-enkephalins by hydrolysis of their C-terminal dipeptides (Erdős et al., 1978) and, in general, acts on a wide range of peptide substrates (Beckner & Caprioli, 1980).

Although extensive effort has gone into the investigation of methods for monitoring ACE activity (Cushman & Cheung, 1971; Stevens et al., 1972; Bakhle, 1974; Carmel & Yaron, 1978; Persson et al., 1978), only limited work has been carried out on its substrate specificity and even less on the details of the kinetics and mechanism of the enzyme. Most of the assay procedures that have been described have limitations with respect to substrate variability while remaining compatible with a rapid, continuous spectral readout. Recent interest in the design of inhibitors of ACE as pharmacological agents for controlling hypertension in humans has underscored the need for improving these assays. In addition, little is known about the mode of action of chloride, which is a potent stimulator of ACE activity. Because of these reasons, we recently examined the suitability of furanacryloyl-blocked tripeptides as ACE substrates (Holmquist et al., 1979). Their hydrolysis can be monitored by following the change in the near-UV region of the substrate absorption spectrum that occurs on release of the C-terminal dipeptide. Among the various FA-tripeptides that were studied, FA-Phe-Gly-Gly was found to offer the best characteristics for routine assays of ACE. We have now carried out an extended study of the kinetics of its hydrolysis and have established optimal conditions to assess the effects of chloride and hydrogen ion concentration on

catalysis. The substrate specificity of the enzyme has been defined in terms of the  $k_{\text{cat}}/K_M$  values of a series of FA-tripeptides, and these have been compared with the physiological substrates angiotensin I and bradykinin.

### Materials and Methods

Rabbit lung acetone powder was purchased from Pel-Freez Biologicals, Inc. (Rogers, AR). Hydroxylapatite, Bio-Beads SM-2, and Chelex 100 were obtained from Bio-Rad Laboratories (Richmond, CA), DEAE-Sephadex A-50 and Sepharose 6B from Pharmacia Fine Chemicals (Piscataway, NJ), XM-50 ultrafiltration membranes from Amicon Corp. (Lexington, MA), 2.5–27% polyacrylamide gradient gels from Isolab, Inc. (Akron, OH), molecular weight marker mixtures from BDH Chemicals Ltd. (Poole, England), and angiotensin I (human) and bradykinin from Peninsula Laboratories, Inc. (San Carlos, CA). The dipeptides, which were used for inhibition studies and calibration of the amino acid analyzer, glycylglycine, L-alanylglycine, L-leucylglycine, L-alanyl-L-phenylalanine, L-histidyl-L-leucine, and L-phenylalanyl-L-arginine were obtained from Vega Biochemicals (Tucson, AZ). Solutions of  $\text{Zn}^{2+}$  were prepared from the chloride salt (ultrapure grade; Ventron Corp., Danvers, MA). All other chemicals were reagent grade or of the highest purity available. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

**Purification of ACE.** ACE was purified from rabbit lung acetone powder. All purification steps were carried out at 4 °C. The acetone powder (50 g) was suspended in 400 mL of 5 mM Tris-HCl buffer, pH 7.0. The suspension was stirred for 25 min and centrifuged at 20000g for 20 min. The precipitate was extracted a second time with the same buffer. This procedure removed a large amount of protein but almost no ACE activity from the acetone powder. Subsequently, the enzyme was solubilized from the acetone powder by four extractions, each with 450 mL of 5 mM Tris-HCl buffer, pH 8.5, containing 0.5% Triton X-100. The combined extracts were dialyzed against three 30-L changes of 13 mM potassium

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<sup>1</sup> Abbreviations: ACE, angiotensin converting enzyme; ammediol, 2-amino-2-methyl-1,3-propanediol; *N*-Cbz, *N*-carbobenzyloxy; DCC, *N,N'*-dicyclohexylcarbodiimide; FA, 2-furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ONSu, succinimidooxy; Tris, tris(hydroxymethyl)aminomethane.

phosphate buffer, pH 7.0, and passed through a hydroxylapatite column (5 × 25 cm) equilibrated with the same buffer. While most of the protein was retarded on the column, the enzyme activity was found almost entirely in the breakthrough fractions. The combined active fractions were stirred for 45 min with 40 g of washed Bio-Beads SM-2 to remove the Triton X-100 (Holloway, 1973). The suspension was filtered through paper on a Büchner funnel. The filtrate was again subjected to the Bio-Beads procedure, and it was then dialyzed against three changes of 5 mM Tris-HCl buffer, pH 7.5. The dialysate was added to 750 mL of DEAE-Sephadex A-50 (swollen in 5 mM Tris-HCl buffer, pH 7.5) and, after being stirred for 12 h, the resin was packed into a column of 5-cm diameter. The column was washed with 2 L of 5 mM Tris-HCl buffer, pH 7.5, and the enzyme was eluted with a 2-L linear gradient from 0 to 1 M sodium chloride in the equilibration buffer. The active fractions were concentrated to about 4 mL by ultrafiltration using an XM-50 membrane. The concentrate was applied to a Sepharose 6B column (2.5 × 90 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 200 mM sodium chloride. The active fractions from the column were concentrated to 0.5 mL by XM-50 ultrafiltration and applied to a second Sepharose 6B column (1 × 90 cm) equilibrated with 200 mM sodium chloride in 50 mM Hepes buffer, pH 7.5. After rechromatography on Sepharose 6B, the enzyme preparation appeared homogeneous in gradient gel electrophoresis and sodium dodecyl sulfate gel electrophoresis. The specific activity was 21.6  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  as determined by the standard assay procedure using FA-Phe-Gly-Gly.

Gradient gel electrophoresis was carried out according to the method of Rodbard et al. (1971). Gels with a polyacrylamide gradient from 2.5 to 27% were run in a Pharmacia Model GE-4 gel electrophoresis apparatus. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber et al. (1972). Slab gels of 5% polyacrylamide were run in a Bio-Rad Model 220 apparatus. All gels were stained with Coomassie brilliant blue. A molecular weight marker mixture consisting of five proteins with molecular weights from 53 000 to 265 000 was used for calibration.

In the course of the purification procedure of ACE, protein concentrations were determined according to Lowry et al. (1951). Concentrations of homogeneous ACE preparations were determined by measuring the absorbance at 280 nm. To obtain the molar absorptivity, we monitored the spectrum of ACE on a Varian Model 219 spectrophotometer. Prior to analysis, the protein was dialyzed against 20 mM Hepes buffer, pH 7.5, and the dialysis buffer was used as a reference. The protein concentration was determined by amino acid analysis. On the basis of a molecular weight of 130 000, a molar absorptivity of 204 000  $\text{M}^{-1} \text{cm}^{-1}$  was determined.

Zinc was determined with a Perkin-Elmer atomic absorption Model 5000 spectrophotometer. Prior to analysis, the protein was dialyzed extensively against metal-free 20 mM Hepes buffer, pH 7.5.

Adventitious metal ions were removed from all buffers and substrate solutions by extraction with fresh solutions of dithizone (0.01%) in carbon tetrachloride or by passage through a Chelex 100 column.

**Furanacryloyl-Tripeptide Hydrolysis.** Standard conditions for enzyme assays are 25 °C at  $5 \times 10^{-5}$  M FA-Phe-Gly-Gly in 50 mM Hepes buffer and 300 mM NaCl, pH 7.5. Under these conditions, hydrolysis is first order in substrate ( $[S] < K_M$ ). Activities expressed as  $V_0/[E]$  in units of reciprocal minutes were obtained either from initial velocities or from

half-lives after complete hydrolysis according to

$$V_0/[E] = 0.693[S]/([E]t_{1/2})$$

where  $V_0$  = the initial velocity,  $[E]$  = the total enzyme concentration in the reaction mixture,  $[S]$  = the initial substrate concentration, and  $t_{1/2}$  = the half-life of the reaction.

Kinetic parameters for the hydrolysis of FA-tripeptides were obtained from Lineweaver-Burk plots. Initial velocities were measured over a substrate concentration range from  $2 \times 10^{-5}$  to  $2 \times 10^{-3}$  M as described by Holmquist et al. (1979). Values for  $k_{\text{cat}}$  are based on a molecular weight for ACE of 130 000.

**Angiotensin I and Bradykinin Assays.** Hydrolyses of angiotensin I and bradykinin were followed by measuring the appearance of the products His-Leu and Phe-Arg, respectively, with the amino acid analyzer. Kinetic parameters for angiotensin I and bradykinin were obtained from Lineweaver-Burk plots. Initial velocities were measured during the first 20% of substrate hydrolysis. Enzyme assays were carried out at 25 °C over a substrate concentration range from  $2 \times 10^{-5}$  to  $3 \times 10^{-4}$  M in 0.8 mL of 50 mM Hepes buffer, pH 7.5, containing 300 mM sodium chloride. Hydrolyses were initiated by the addition of enzyme to the assay mixture. After 2 and 5 min, 90- $\mu\text{L}$  aliquots were withdrawn from the assay mixture and quenched with 30  $\mu\text{L}$  of 0.8 M citrate buffer, pH 2.2. From this mixture, 60  $\mu\text{L}$  was applied to the D500 amino acid analyzer and analyzed by the standard ninhydrin procedure for amino acid hydrolysates according to the manufacturer's instructions. The products of angiotensin I and bradykinin hydrolyses, His-Leu and Phe-Arg, respectively, were identified and quantified with authentic dipeptide samples. They were eluted from the amino acid analyzer with retention times of 68 min for His-Leu in a position between Phe and His and 96 min for Phe-Arg in a position close to Arg. The detection limit of the method is 0.05 nmol for both dipeptides.

**Furanacryloyl Intermediates and Substrates.** Di- and tripeptides were either purchased from Vega Biochemicals (Tucson, AZ) or Sigma Chemical Corp. (St. Louis, MO) or synthesized by standard procedures employing *N*-carbobenzoyloxy-blocked amino acids, coupling with DCC, and catalytic hydrogenolysis using 10% Pd/charcoal at atmospheric pressure.

The 2-furanacryloyl-blocked tripeptides and intermediates FA-Ala-Ala-Ala, FA-Gly-Ala-Gly, FA-Gly-Leu-Gly, FA-Gly-Leu-Ala, FA-Gly-Leu-Phe, FA-Phe-Leu-Gly, FA-ONSu, and FA-Gly-ONSu (Blumberg & Vallee, 1975) and FA-Phe-Gly-Gly, FA-Phe, and FA-Phe-ONSu (Holmquist et al., 1979) were from earlier studies. The syntheses of other intermediates and substrates not previously described are given in the supplementary material (see paragraph at end of paper regarding supplementary material).

## Results

**Purification and Properties of ACE from Rabbit Lungs.** ACE was isolated from rabbit lung acetone powder by extraction with Triton X-100 and purified by hydroxylapatite chromatography, ion-exchange chromatography, and gel filtration (Table I). The overall purification of the enzyme was 114-fold with a 40% yield. The enzyme preparation has a specific activity of 21.6  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  with FA-Phe-Gly-Gly as substrate under standard assay conditions.

The enzyme preparation appeared homogeneous on gradient and sodium dodecyl sulfate gel electrophoresis. The molecular weight of the enzyme was determined to be 130 000 by gradient gel electrophoresis and comparison with appropriate marker proteins. The same molecular weight was found by

Table I: Purification of ACE from Rabbit Lungs<sup>a</sup>

purification step	volume (mL)	total activity (mol/min)	sp act. (mol min <sup>-1</sup> mg <sup>-1</sup> )	purification (x-fold)	yield (%)
extraction	1720	620	0.19	1	100
hydroxylapatite	1900	475	0.63	3.3	77
DEAE-Sephadex A-50	675	390	8.2	43	63
Sepharose 6B	70	320	20	105	52
Sepharose 6B	10	250	22	114	40

<sup>a</sup> Assay conditions: 5 × 10<sup>-5</sup> M FA-Phe-Gly-Gly and 50 mM Hepes, pH 7.5, containing 300 mM NaCl, 25 °C.

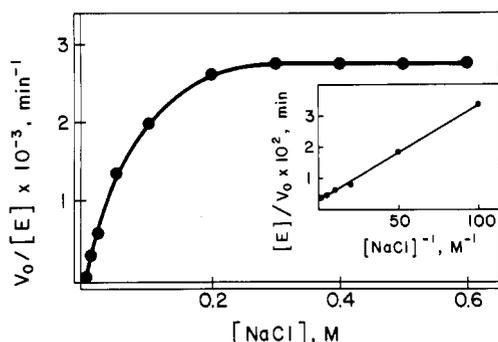


FIGURE 1: Effect of sodium chloride concentration on the activity of ACE measured with 5 × 10<sup>-5</sup> M FA-Phe-Gly-Gly as substrate in 50 mM Hepes buffer, pH 7.5, at 25 °C. Inset: An apparent *K<sub>A</sub>* of 100 mM for the chloride activation was obtained from a double-reciprocal plot of activity vs. chloride concentration.

sodium dodecyl sulfate gel electrophoresis under reducing conditions, demonstrating that the enzyme consists of a single polypeptide chain. The molar absorptivity at 280 nm in 20 mM Hepes buffer, pH 7.5, is 204 000 M<sup>-1</sup> cm<sup>-1</sup>. The metal content of the enzyme determined by atomic absorption spectrometry was 1.05 ± 0.05 mol of zinc per mol. The amino acid composition of this enzyme preparation is almost identical with that previously reported by Das & Soffer (1975).

**FA-Phe-Gly-Gly Hydrolysis.** From a variety of FA-tripeptides tested, FA-Phe-Gly-Gly was shown to be the most suitable ACE substrate and has been recommended for routine assays (Holmquist et al., 1979). Since a detailed kinetic investigation of the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly has not been reported previously, this substrate was chosen for further analysis.

The Lineweaver-Burk plot for the hydrolysis of FA-Phe-Gly-Gly is linear over the concentration range from 5 × 10<sup>-5</sup> to 2 × 10<sup>-3</sup> M. The kinetic parameters, obtained in the presence of 300 mM NaCl, are *K<sub>M</sub>* = 3 × 10<sup>-4</sup> M and *k<sub>cat</sub>* = 19 000 min<sup>-1</sup>. Thus, this substrate shows satisfactory kinetic behavior from about 10-fold above to 10-fold below the *K<sub>M</sub>*.

**FA-Phe-Gly-Gly Hydrolysis: Chloride Dependence.** One characteristic property of ACE catalysis is an almost absolute requirement for monovalent anions, with chloride being the most effective activator. Therefore, the influence of chloride on the hydrolysis of FA-Phe-Gly-Gly was investigated at pH 7.5 and 5 × 10<sup>-5</sup> M substrate concentration, i.e., under first-order reaction conditions (Figure 1). In the absence of added chloride, the enzyme possesses less than 0.5% of its maximal activity. With increasing chloride concentrations, the enzyme activity, expressed as a turnover number, increases and reaches a maximum of 2750 min<sup>-1</sup> at 300 mM chloride. With chloride concentrations higher than 300 mM, no further increase in activity is observed. A double-reciprocal plot of activity vs. chloride concentration yields an apparent *K<sub>A</sub>* for activation of 100 mM (Figure 1, inset). Thus, the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly exhibits the chloride dependence characteristic of most other ACE substrates.

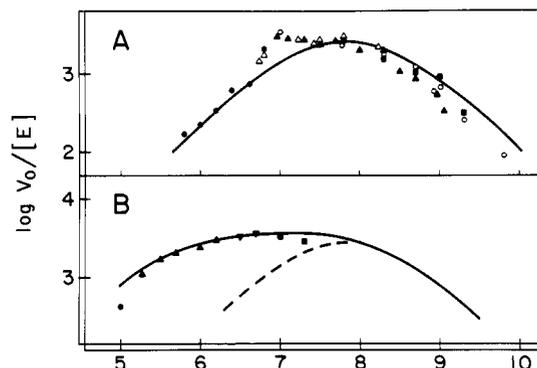


FIGURE 2: (A) pH dependence of the ACE-catalyzed hydrolysis of 5 × 10<sup>-5</sup> M FA-Phe-Gly-Gly determined at 25 °C in 300 mM sodium chloride in either 50 mM Mes (●), Hepes (Δ), Tris (▲), ammediol (■), or borate (○) buffers. The solid line describes a theoretical curve for the pH dependence of a diprotic system with apparent *pK<sub>a</sub>* values of 7.2 and 8.4 and a maximal turnover rate of 4000 min<sup>-1</sup>. (B) Effect of zinc on the pH dependence of ACE activity. Conditions of assays were the same as those in Figure 2A, except that 10<sup>-2</sup> (●), 10<sup>-3</sup> (▲), 10<sup>-4</sup> (▼), or 10<sup>-5</sup> M (■) Zn<sup>2+</sup> was added to the assays. The solid line describes a theoretical curve for the pH dependence of a diprotic system with apparent *pK<sub>a</sub>* values of 5.6 and 8.4 and a maximal turnover rate of 4000 min<sup>-1</sup>, and for comparison, the curve from Figure 2A is included as a broken line.

Table II: Effect of Buffer Concentration on the ACE-Catalyzed Hydrolysis of FA-Phe-Gly-Gly

buffer	<i>pK<sub>a</sub></i>	activity (% control) <sup>a</sup>
Mes	6.1	87
Hepes	7.5	87
Tris	8.1	64
ammediol	8.8	60
borate	9.2	88

<sup>a</sup> Activity was measured in 100 mM buffer relative to that in 10 mM buffer. All assays were carried out at a pH equal to the *pK<sub>a</sub>* of the buffer employed. Other assay conditions were 5 × 10<sup>-5</sup> M FA-Phe-Gly-Gly, 300 mM NaCl, 25 °C, and 10 and 100 mM buffer.

**FA-Phe-Gly-Gly Hydrolysis: Buffer and pH Dependence.** The variation of log (*V<sub>0</sub>*/[E]) for FA-Phe-Gly-Gly hydrolysis as a function of pH and buffer is shown over the pH range from 5 to 10 (Figure 2A). Throughout this range, the hydrolysis of FA-Phe-Gly-Gly at a concentration of 5 × 10<sup>-5</sup> M follows first-order kinetics. The assays were carried out in 300 mM chloride, which is the optimal chloride concentration at pH 7.5 (Figure 1). The pH-rate profile exhibits an optimum centered at pH 7.7 and falls off markedly when the pH is decreased below 7.0.

For most of the buffers examined, there appears to be only a small effect of buffer concentration on the rate of FA-Phe-Gly-Gly hydrolysis (Table II). The amine buffers Tris and ammediol are somewhat more inhibitory than the anionic buffers Mes, Hepes, and borate.

The decrease in activity below pH 7 is due, in part, to dissociation of zinc from the enzyme. Loss of metal can be

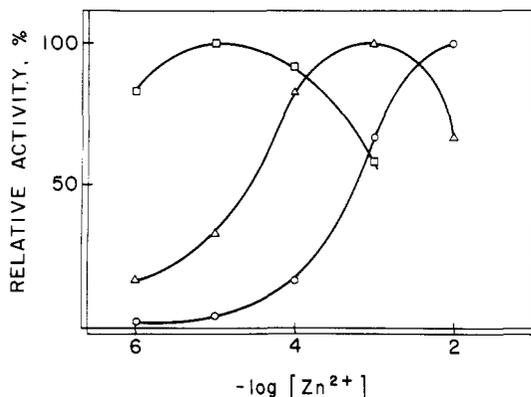


FIGURE 3: Effect of zinc concentration on the relative ACE activity at pH 5 (O), pH 6 ( $\Delta$ ), and pH 7 ( $\square$ ) obtained under conditions as in Figure 2A. Activity is expressed as percent of the maximum for each pH curve.

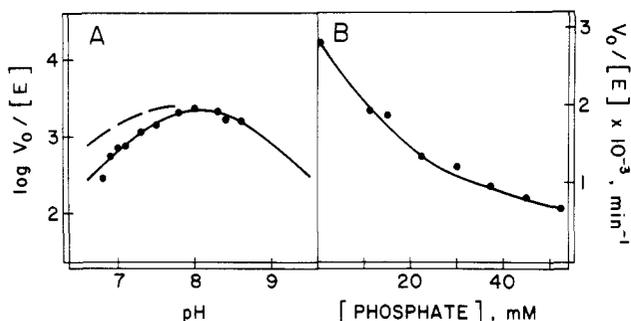


FIGURE 4: (A) Effect of phosphate concentration on the pH dependence of ACE activity. Conditions of assays were the same as those in Figure 2A except that 50 mM potassium phosphate was added to the assay. The solid line describes a theoretical curve for the pH dependence of a diprotic system with apparent pK values of 7.8 and 8.4 and a maximal turnover rate of 4000  $\text{min}^{-1}$ , and for comparison, the curve from Figure 2A is included as a broken line. (B) Effect of phosphate concentration on ACE activity at pH 7.0, obtained under assay conditions as in Figure 2A.

overcome by addition of zinc to the assay mixture which increases ACE activity in the acidic pH region (Figure 2B). The concentration of zinc that gives maximal activity differs for each pH and was determined by activity titrations with zinc at each pH value studied. At pH 7.0, a zinc concentration of  $10^{-5}$  M is sufficient for full enzyme activity, whereas at pHs 6 and 5,  $10^{-3}$  and  $10^{-2}$  M zinc, respectively, are required (Figure 3). Above pH 7.0, addition of zinc does not result in activation. Using these optimal zinc concentrations for determining the  $\log (V_0/[E])$  vs. pH profile, it is evident that zinc shifts the acidic apparent pK from 7.2 to 5.6 (Figure 2B). Thus, under the appropriate assay conditions, the pH-rate profile for the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly has apparent pKs for the free enzyme (Dixon, 1953) of 5.6 and 8.4.

FA-Phe-Gly-Gly hydrolysis is altered significantly by the presence of 50 mM phosphate in the buffer solutions (Figure 4). ACE activity is markedly reduced by phosphate, a commonly employed buffer, in the acidic pH region but is almost unchanged at alkaline pH values. Thus, at pH 7.0, in 50 mM phosphate, the activity of ACE is reduced to about 25% of the control, but at pH 8 and above, the activities with and without phosphate are identical. Phosphate inhibition increases with decreasing pH as indicated in Figure 4A. This inhibition is not observed with Hepes buffer.

**Hydrolysis of Other FA-Tripeptides.** Various other FA-tripeptides were synthesized in order to investigate the kinetics of their hydrolyses and, thereby, obtain information on the

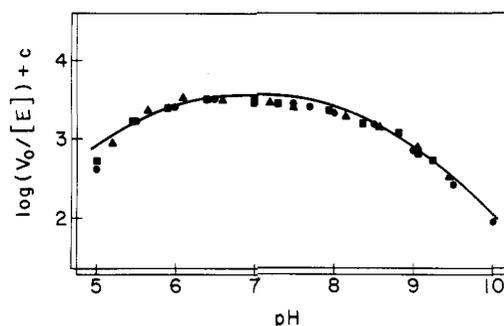


FIGURE 5: pH dependence of the ACE-catalyzed hydrolysis of  $3 \times 10^{-5}$  M FA-Phe-Ala-Phe ( $\blacksquare$ ,  $c = 0.14$ ),  $3 \times 10^{-5}$  M FA-Phe-Leu-Gly ( $\blacktriangle$ ,  $c = 0.81$ ), and  $5 \times 10^{-5}$  M FA-Phe-Gly-Gly ( $\bullet$ ,  $c = 0$ ). Conditions of the assay were the same as those in Figure 2B. The solid line was derived as in Figure 2B.

substrate specificity of ACE. The pH dependence of the hydrolysis of two such FA-tripeptides, FA-Phe-Ala-Phe and FA-Phe-Leu-Gly, was examined over the range from pH 5 to 10 and compared to the pH-rate profile for FA-Phe-Gly-Gly hydrolysis. The optimal chloride concentration at pH 7.5 for the action of ACE toward these substrates is 300 mM; hence, the pH-rate profiles were again studied in the presence of 300 mM sodium chloride. The substrate concentrations employed were well below the  $K_M$ . The hydrolyses of FA-Phe-Ala-Phe and FA-Phe-Leu-Gly are also activated by zinc in the acidic pH region in the same manner as was already observed for the hydrolysis of FA-Phe-Gly-Gly (Figure 2B). Therefore, at acidic pH values, zinc was added to the assay mixtures at the concentrations found optimal for the hydrolysis of FA-Phe-Gly-Gly (Figure 3). For comparison of the pH dependences of the hydrolyses of these three peptides, the profiles of pH vs.  $\log (V_0/[E])$  have been normalized to the turnover number of FA-Phe-Gly-Gly at pH 7.5 (Figure 5). The pH-rate profiles for all three substrates are characterized by the ionization of two groups on the free enzyme with apparent pKs of about 5.6 and 8.4.

The kinetic parameters of a number of additional FA-tripeptides were determined in 50 mM Hepes buffer, pH 7.5, containing 300 mM sodium chloride (Table III). These parameters were obtained from Lineweaver-Burk plots all of which were linear over a substrate concentration range from  $2 \times 10^{-5}$  to  $2 \times 10^{-3}$  M. Activities, expressed as  $k_{\text{cat}}/K_M$  in  $\text{min}^{-1} \text{M}^{-1}$ , are highest for the FA-tripeptides of the series FA-Phe-X-Y, which also includes FA-Phe-His-Leu, an analogue of the physiological substrate angiotensin I. The highest  $k_{\text{cat}}/K_M$  values are obtained for FA-Phe-Ala-Phe, followed by FA-Phe-Gly-Gly and FA-Phe-His-Leu. The highest  $k_{\text{cat}}$  values were displayed by FA-Phe-Ala-Gly and FA-Phe-Gly-Gly, 20 700 and 19 000  $\text{min}^{-1}$ , respectively. The lowest  $K_M$ ,  $6.8 \times 10^{-5}$  M, was observed for FA-Phe-His-Leu. FA-tripeptides of the series FA-Leu-X-Y and FA-Ala-X-Y exhibit lower activities, and those activities of the FA-Gly-X-Y type are all well below 10% of that for FA-Phe-Gly-Gly. In general, within this series, peptides of the FA-Phe-X-Y group display the highest activities while the type of amino acid residue in the penultimate and ultimate positions seems to have a smaller effect on the rate of hydrolysis. Those with a leucyl residue in the penultimate position display low  $k_{\text{cat}}$  values while those with a phenylalanyl residue in the terminal position exhibit low  $K_M$  values. FA-dipeptides and FA-Leu-Gly-Gly-OEt are not hydrolyzed by ACE in accordance with its specificity as a dipeptidyl carboxypeptidase.

**Product Inhibition.** The X-Y dipeptides that are released from FA-tripeptides of the series FA-Phe-X-Y as hydrolysis

Table III: Kinetic Parameters of ACE-Catalyzed Hydrolysis of Various Furanacryloyl-Tripeptides<sup>a</sup>

peptide	$k_{cat}$ (min <sup>-1</sup> )	$K_M$ (M)	$k_{cat}/K_M \times 10^{-6}$ (min <sup>-1</sup> M <sup>-1</sup> )
FA-Phe-His-Leu	4100	$6.8 \times 10^{-5}$	60
FA-Phe-Leu-Gly	3800	$2.5 \times 10^{-4}$	15
FA-Phe-Ala-Phe	7700	$9.3 \times 10^{-5}$	83
FA-Phe-Ala-Gly	20700	$5.0 \times 10^{-4}$	41
FA-Phe-Gly-Gly	19000	$3.0 \times 10^{-4}$	63
FA-Leu-Leu-Gly	5200	$4.9 \times 10^{-4}$	11
FA-Leu-Ala-Gly	17300	$4.1 \times 10^{-4}$	42
FA-Leu-Gly-Gly	32600	$1.5 \times 10^{-3}$	22
FA-Ala-Leu-Ala	1900	$3.8 \times 10^{-4}$	5
FA-Ala-Ala-Ala	12400	$5.2 \times 10^{-4}$	24
FA-Ala-Leu-Gly	9500	$2.7 \times 10^{-3}$	4
FA-Gly-Leu-Phe	3100	$8.1 \times 10^{-4}$	4
FA-Gly-Leu-Ala	410	$2.4 \times 10^{-3}$	0.2
FA-Gly-Leu-Gly	950	$3.3 \times 10^{-3}$	0.3
FA-Gly-Ala-Gly	3200	$4.1 \times 10^{-3}$	0.8
FA-Gly-Gly-Phe	7500	$2.0 \times 10^{-3}$	3.8
FA-Gly-Gly-Leu	2100	$3.7 \times 10^{-3}$	0.6
FA-Gly-Gly-Gly	2900	$1.6 \times 10^{-2}$	0.2
FA-Phe-Ala		NH <sup>b</sup>	
FA-Leu-Gly		NH	
FA-Gly-Gly		NH	
FA-Leu-Gly-Gly-OEt		NH	

<sup>a</sup> Assay conditions: 50 mM Hepes buffer, pH 7.5, containing 300 mM NaCl, 25 °C. <sup>b</sup> NH, not hydrolyzed.

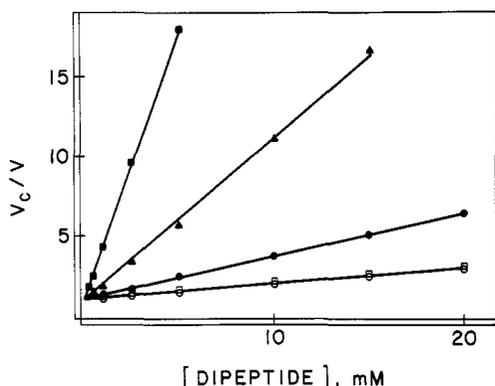


FIGURE 6: Inhibition of ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly by Ala-Phe (■), His-Leu (▲), Ala-Gly (●), Leu-Gly (□), and Gly-Gly (○). Assays were carried out in 300 mM sodium chloride in 50 mM Hepes buffer, pH 7.5, and  $5 \times 10^{-5}$  M substrate concentration at 25 °C. Activities are expressed as the ratio of that in the absence,  $V_c$ , to that in the presence,  $V$ , of dipeptide.

products were examined for their effects on FA-Phe-Gly-Gly hydrolysis. Under first-order reaction conditions ( $[S] < K_M$ ),  $K_I$  values for their inhibition are readily determined from plots of  $V_c/V$  vs. inhibitor concentration according to the relation  $K_I = [I]/[V_c/V - 1]$ , where  $V_c$  and  $V$  are the initial velocities in the absence and presence of inhibitor, respectively (Figure 6). Under these conditions, competitive inhibition is indistinguishable from noncompetitive inhibition. The lowest  $K_I$  values were obtained for Ala-Phe and His-Leu, 0.3 and 1.0 mM, respectively. These two dipeptides are the hydrolysis products of FA-Phe-Ala-Phe and FA-Phe-His-Leu, substrates displaying the lowest  $K_M$  values. Ala-Gly, Gly-Gly, and Leu-Gly show considerable inhibition only at higher concentrations, their  $K_I$  values being 3.8, 10, and 10 mM, respectively. Because of product inhibition, FA-Phe-Ala-Phe and FA-Phe-His-Leu are less favorable substrates, especially when employed at high substrate concentration ( $[S] \gg K_M$ ), and make FA-Phe-Gly-Gly the substrate of choice.

Table IV: Kinetic Parameters of ACE-Catalyzed Hydrolysis of Angiotensin I and Bradykinin<sup>a</sup>

substrate	$k_{cat}$ (min <sup>-1</sup> )	$K_M$ (M)	$k_{cat}/K_M \times 10^{-6}$ (min <sup>-1</sup> M <sup>-1</sup> )
angiotensin I	660	$7 \times 10^{-5}$	9
bradykinin	145	$<3 \times 10^{-6}$	>48

<sup>a</sup> Assay conditions: 50 mM Hepes buffer, pH 7.5, containing 300 mM NaCl, 25 °C.

**Hydrolysis of Angiotensin I and Bradykinin.** Kinetic parameters for FA-Phe-Gly-Gly and FA-Phe-His-Leu (Table III) are compared to those for the physiological substrates of ACE, angiotensin I and bradykinin (Table IV). Among these substrates, FA-Phe-Gly-Gly displays the highest  $k_{cat}$  and  $K_M$  values. For FA-Phe-His-Leu, both parameters are lower, but the  $k_{cat}/K_M$  value is almost identical with that for FA-Phe-Gly-Gly. The physiological substrate angiotensin I displays the same  $K_M$  as its analogue FA-Phe-His-Leu, but the  $k_{cat}$  is almost an order of magnitude smaller, and that of bradykinin is lower yet. However, bradykinin has a very low  $K_M$  value. Due to limitations of the assay method, a precise number could not be determined, but it is at least an order of magnitude smaller than the  $K_M$  for angiotensin I.

Discussion

Recent efforts to isolate and purify ACE generally have employed lung tissue as the starting material since the endothelial lining of the pulmonary vasculature has been shown to be a major locus of ACE activity (Ryan & Smith, 1973). The enzyme is associated with endothelial cell membranes (Ryan et al., 1975; Caldwell et al., 1976), and, hence, the most successful preparations appear to be those that start by extracting with a nonionic detergent solution such as Nonidet P40 (Das & Soffer, 1975) or Triton X-100 (Stevens et al., 1972). The isolation scheme followed in the present study is similar to those of Oshima et al. (1974) and Cheung et al. (1980). It begins with an acetone powder prepared from rabbit lung, a common, convenient source of ACE. Non-membrane-bound proteins are first removed by extracting the powder twice with Tris-HCl buffer, pH 7.0. A large amount of protein but only a very small fraction of the total ACE activity is lost during this step. Subsequently, the enzyme is solubilized by four successive extractions of the pellet with Tris-HCl buffer, pH 8.5, containing 0.5% Triton X-100. Chromatography on hydroxylapatite, DEAE-Sephadex, and Sepharose 6B results in an overall 114-fold purification of the enzyme with a recovery of 40% from the detergent extract.

The physicochemical properties of the enzyme prepared by this procedure are essentially the same as those reported for enzyme obtained by other methods. Its molecular weight, amino acid composition, and metal content are close to those cited for the preparation of Das & Soffer (1975). In addition, it has a specific activity of 21.6 units/mg when assayed according to our standard procedure employing FA-Phe-Gly-Gly. It was also assayed by the method of Cushman & Cheung (1971) by using hippuryl-His-Leu as substrate and had a specific activity of 30.9 units/mg. These values are based on a protein concentration determined by amino acid analysis. We have found that ACE preparations do not exhibit a linear concentration dependence when measured by the procedure of Lowry et al. (1951). Hence, values for the specific activity based on this protein determination may be misleading. Similarly, the spectrophotometric method of Warburg & Christian (1941) is not sufficiently accurate to provide a valid

measure of ACE concentration. Since the concentrations of virtually all preparations of converting enzyme reported in the literature are calculated by using one or the other of these methods, a legitimate comparison of the specific activities cited for various preparations is not possible. However, we note that our preparation has about the same specific activity toward hippuryl-His-Leu as does that of Cheung et al. (1980). In addition, using angiotensin I as substrate and assaying at 25 °C, we obtained a  $K_M$  of 70  $\mu\text{M}$  and a  $k_{\text{cat}}$  of 660  $\text{min}^{-1}$ . These values are quite comparable to those of Das & Soffer (1975), who carried out their assays with this substrate at 37 °C and found the same  $K_M$  and a  $k_{\text{cat}}$  of 792  $\text{min}^{-1}$ .

We have recently shown that furanacryloyl-substituted tripeptides are excellent substrates for the angiotensin converting enzyme (Holmquist et al., 1979). FA substrates have already found acceptance for assays and mechanistic investigations of other metalloproteases (Feder & Schuck, 1970; Holmquist & Vallee, 1973; Holmquist, 1977). These peptides exhibit a maximal absorbance at 306 nm ( $\epsilon = 25\,000\ \text{M}^{-1}\ \text{cm}^{-1}$ ) that shifts to 303 nm on hydrolysis to the FA-blocked amino acid plus a dipeptide. Although this change is relatively small, it is sufficient to allow hydrolysis to be monitored continuously over a wide range of substrate concentrations. The maximum in the difference spectrum between substrate and products occurs at 328 nm ( $\Delta\epsilon = -2300\ \text{M}^{-1}\ \text{cm}^{-1}$ ), the wavelength selected for standard assays. Thus, a change of 0.115 absorbance unit accompanies the complete hydrolysis of a  $5 \times 10^{-5}\ \text{M}$  solution of a FA-tripeptide.

Among the FA-tripeptides studied, FA-Phe-Gly-Gly was found to be the most suitable for assaying ACE (Holmquist et al., 1979). Not only does it have a high rate of turnover but also it is stable, easy to prepare, and has adequate solubility for kinetic studies over a broad concentration range. Most importantly, it provides a direct, continuous assay for optimal convenience. In addition, the product, Gly-Gly, is a rather poor inhibitor of ACE ( $K_I = 10\ \text{mM}$ ), thus obviating a potential complication in any kinetic interpretations. In view of these many advantages over other peptides that have been suggested, we proposed FA-Phe-Gly-Gly as a convenient substrate for routine assays of ACE.

The kinetics of hydrolysis of FA-Phe-Gly-Gly appear to be devoid of kinetic anomalies. Over a concentration range from about 10-fold below to about 10-fold above the  $K_M$ , the double-reciprocal plot is linear with no evidence of either substrate activation or substrate inhibition. The parameters calculated from the slope and intercepts of the line are  $K_M = 3 \times 10^{-4}\ \text{M}$  and  $k_{\text{cat}} = 19\,000\ \text{min}^{-1}$ , comparable with those for the most commonly employed ACE substrate, hippuryl-His-Leu, which are  $2.4 \times 10^{-3}\ \text{M}$  and  $15\,600\ \text{min}^{-1}$ , respectively.

As in the case of most other substrates of ACE, hydrolysis of FA-Phe-Gly-Gly requires the presence of chloride ions: in the absence of chloride, the enzyme appears to be almost completely inactive toward this substrate. Maximal hydrolysis occurs at a chloride concentration of 300 nM, similar to that seen for hippuryl-His-Leu. In a separate study, we have shown that chloride activation is due to an effect on the  $K_M$  for substrate binding (Riordan et al., 1980; Bünnig & Riordan, 1983) and is the same for a number of FA-substituted tripeptides carrying hydrophobic side chains. The kinetics of chloride activation of the FA-Phe-Gly-Gly hydrolysis imply an ordered, bireactant mechanism in which the anion must bind to the enzyme prior to the substrate. Direct evidence for such a mechanism has been obtained by the use of radiationless energy transfer studies using a fluorescently labeled substrate (Bünnig & Riordan, 1981). However, on the other hand,

Rohrbach et al. (1981) have obtained evidence that chloride serves as a nonessential activator in the hydrolysis of hippuryl-His-Leu. Recent work by Cheung et al. (1980) has also indicated that some substrates of ACE are hydrolyzed in the absence of chloride. In particular, the hydrolysis of bradykinin is only affected minimally while that of hippuryl-Ala-Pro is actually decreased by the presence of NaCl. In yet other cases, chloride has been reported to increase the  $V_{\text{max}}$  (Dorer et al., 1976). The results obtained with the FA-tripeptides studied here, however, indicate that, in general, they all require activation of ACE by chloride in order to be hydrolyzed.

There seems to be little effect of buffer ions on ACE activity under the conditions employed (Table II), but with buffer concentrations of 100 mM, some inhibition was observed with both Tris and ammediol. Although the present results do not rule out buffer ion effects that might pertain at concentrations below 10 mM, other studies indicate that Hepes shows minimal inhibition or activation between 0.5 and 10 mM (R. Shapiro, unpublished experiments). For this reason, routine activity determinations are carried out in Hepes buffer. Phosphate is still widely used as a buffer in ACE assays despite reports that it inhibits the activity of the enzyme toward benzoyl-Phe-Phe-Gly and hippuryl-Gly-Gly (Yang et al., 1971; Dorer et al., 1976). We have found that it inhibits activity toward FA-Phe-Gly-Gly as well. There is minimal effect of phosphate above pH 8, but as the pH is decreased below this value, progressively greater inhibition is observed (Figure 4), indicating that the dibasic form of the buffer is responsible for inhibition.

The rate of turnover of FA-Phe-Gly-Gly varies markedly as a function of pH (Figure 2). Without added zinc but in the presence of 300 mM chloride, optimal hydrolysis occurs between pH 7.0 and 8.0, which is the range typically reported for most ACE substrates. Compared to the pH-rate profile for another zinc metalloenzyme, carboxypeptidase A, this range for ACE would seem to be rather narrow; it falls off markedly below pH 7 and somewhat less so above pH 8. However, the ACE pH profile determined in the absence of zinc does not represent solely the ionization of groups on the free enzyme. It also reflects spontaneous dissociation of the active-site zinc that occurs at pH values below 7.0. Under the circumstances employed here, adventitious zinc ions would have been removed from the assay mixture by the extraction of all buffer and substrate solutions with 0.01% dithizone in  $\text{CCl}_4$ . This procedure reduces the concentration of zinc and other heavy metals to less than 0.1  $\mu\text{M}$ . Above pH 7, it is unnecessary to add zinc to the assay system. In fact, at pH 7.5, zinc ACE is inhibited by concentrations of zinc greater than 10  $\mu\text{M}$ . However, below pH 7, zinc readily dissociates from the enzyme, and therefore it is essential to add zinc to the assay solution in order to ensure maximal activity. The amount of zinc required depends on the pH; at pH 6, the optimal concentration of zinc is  $10^{-3}\ \text{M}$  while at pH 5 it is  $10^{-2}\ \text{M}$ . After the pH-rate profile is corrected for loss of zinc (Figure 2B), the data points can best be fit on the basis of the ionization of two groups with apparent pK values of 5.6 and 8.4, much closer to those observed for carboxypeptidase A (Auld & Vallee, 1970).

An investigation of the kinetic parameters of a series of FA-tripeptide substrates has provided some insight to the specificity of ACE. Activities, expressed as  $k_{\text{cat}}/K_M$ , are highest and substrate binding tightest for the series FA-Phe-X-Y. Clearly, among the substrates tested, the enzyme shows preference for those with an aromatic residue in the antepenultimate position, designated  $P_1$  in the terminology of

Schechter & Berger (1967), adjacent to the scissile bond. The  $k_{\text{cat}}$  values for FA-Phe-Gly-Gly and FA-Phe-Ala-Gly are the largest in this series, perhaps reflecting facile product dissociation. Activities seem to be largely determined by the residue at  $P_1$  with Phe > Leu > Ala > Gly, and to a lesser extent by that at  $P_1'$  or  $P_2'$ . Substrates with a leucyl residue at  $P_1'$  have low  $k_{\text{cat}}$  values while those with a phenylalanyl residue at  $P_2'$  have low  $K_M$  values.

The influence of the  $P_2'$  residue on  $K_M$  is also seen in the inhibition by dipeptides. The lowest  $K_I$  values were obtained with dipeptides having a carboxyl-terminal Phe or Leu whereas those with Gly in that position were about an order of magnitude weaker. All of the dipeptides studied were strictly competitive inhibitors. Cheung et al. (1980) have examined an extensive series of dipeptides to determine the concentrations that inhibit 50% of the activity of ACE toward 5 mM hippuryl-His-Leu. Their results were similar to those reported here, with the most favorable carboxyl-terminal amino acids being the aromatic acids, tryptophan, tyrosine, and phenylalanine. Importantly, Gly-Gly, the product of hydrolysis of FA-Phe-Gly-Gly, is one of the poorest inhibitors tested. This virtual absence of product inhibition makes the parent peptide a much better substrate for studying the kinetic characteristics of ACE or simply for routine assays than either FA-Phe-Ala-Phe or FA-Phe-His-Leu even though the latter two have similarly high  $k_{\text{cat}}/K_M$  values.

The activity of ACE toward FA-Phe-Gly-Gly has been compared with that toward two physiological substrates, angiotensin I and bradykinin (Tables III and IV). The synthetic N-substituted tripeptide is hydrolyzed much more rapidly than either of the natural peptides, its  $k_{\text{cat}}$  value being  $19\,000\text{ min}^{-1}$  vs. 660 and  $145\text{ min}^{-1}$  for the other two, respectively. On the other hand, the natural peptides bind more tightly to the enzyme, their  $K_M$  values being 70 and  $<3\ \mu\text{M}$ , respectively, compared to  $300\ \mu\text{M}$  for FA-Phe-Gly-Gly. The method employed in these studies lacks sufficient sensitivity to determine a more accurate value of the  $K_M$  for bradykinin. However, Dorer et al. (1974) have reported a  $K_M$  of  $0.85\ \mu\text{M}$  measured under somewhat different conditions, and this would give a  $k_{\text{cat}}/K_M$  value for bradykinin of  $168 \times 10^6\text{ min}^{-1}\text{ M}^{-1}$ , compared to  $63 \times 10^6\text{ min}^{-1}\text{ M}^{-1}$  for FA-Phe-Gly-Gly and  $9.4 \times 10^6\text{ min}^{-1}\text{ M}^{-1}$  for angiotensin I. The availability of a synthetic tripeptide substrate that is hydrolyzed by ACE with a catalytic efficiency ( $k_{\text{cat}}/K_M$ ) comparable to those of physiological substrates and yet provides for a rapid, convenient, and continuous spectrophotometric assay should greatly facilitate the physicochemical and mechanistic characterization of the enzyme.

One of the physiological roles of ACE is to generate the octapeptide angiotensin II, which through its powerful vasoconstricting action and the release of aldosterone is an important factor in the control of blood pressure. Recently, it has been shown that a number of potent inhibitors of ACE are useful in the treatment of hypertension, and their effectiveness is thought to relate to their binding specificity (Cheung et al., 1980). These studies with FA-Phe-Gly-Gly and related substrates are intended to help increase understanding of the structural basis for the specificity of ACE and thus aid in the development of inhibitory agents with improved antihypertensive properties. Moreover, the assay system itself is a convenient means to assess the mode of action of these inhibitors and should be useful for monitoring their concentrations in blood as well as the kinetics of drug action.

#### Acknowledgments

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#### Supplementary Material Available

A description of the syntheses of 15 FA-peptides (5 pages). Ordering information is given on any current masthead page.

**Registry No.** ACE, 9015-82-1; bradykinin, 58-82-2; angiotensin I, 484-42-4; FA-Phe-His-Leu, 71067-14-6; FA-Phe-Leu-Gly, 56186-51-7; FA-Phe-Ala-Phe, 71067-11-3; FA-Phe-Ala-Gly, 71067-12-4; FA-Phe-Gly-Gly, 64967-39-1; FA-Leu-Leu-Gly, 83803-10-5; FA-Leu-Ala-Gly, 71067-13-5; FA-Leu-Gly-Gly, 83803-11-6; FA-Ala-Leu-Ala, 83803-12-7; FA-Ala-Ala-Ala, 56186-47-1; FA-Ala-Leu-Gly, 83803-13-8; FA-Gly-Leu-Phe, 56186-49-3; FA-Gly-Leu-Ala, 56186-50-6; FA-Gly-Leu-Gly, 56186-48-2; FA-Gly-Ala-Gly, 56186-46-0; FA-Gly-Gly-Phe, 83803-14-9; FA-Gly-Gly-Leu, 83803-15-0; FA-Gly-Gly-Gly, 83803-16-1; FA-Phe-Ala, 83803-17-2; FA-Leu-Gly, 83803-18-3; FA-Gly-Gly, 83803-19-4; FA-Leu-Gly-Gly-OEt, 83803-20-7.

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## Activation of Angiotensin Converting Enzyme by Monovalent Anions<sup>†</sup>

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**ABSTRACT:** The angiotensin converting enzyme catalyzed hydrolysis of furanacryloyl-Phe-Gly-Gly is activated by monovalent anions in the order  $\text{Cl}^- > \text{Br}^- > \text{F}^- > \text{NO}_3^- > \text{CH}_3\text{COO}^-$ . In the alkaline pH region, increasing anion concentrations decrease the  $K_M$  but do not change the  $k_{\text{cat}}$ . This behavior is characteristic of an ordered bireactant mechanism in which the anion binds to the enzyme prior to the substrate. At acidic pH values, however, the anion activation is a result of both a decrease in  $K_M$  and an increase in  $k_{\text{cat}}$ , implying a bireactant mechanism in which anion and substrate bind randomly. For both the ordered and the bireactant mecha-

nisms the anion serves as an essential activator. The effect of chloride on enzyme activity was studied over the pH range 5-10 under  $k_{\text{cat}}/K_M$  conditions and demonstrates that the apparent chloride binding constant increases from 3.3 mM at pH 6.0 to 190 mM at pH 9.0. The  $k_{\text{cat}}$  vs. pH profile exhibits two pK values of 5.6 and 9.6, while the variation of  $K_M$  with pH is characterized by a pK of 8.9 and a 2-fold increase between pH 6.5 and 7.5. The chloride activation of the hydrolysis of furanacryloyl-Phe-Gly-Gly is compared with that of the physiological substrates angiotensin I and bradykinin.

**A**ngiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE)<sup>1</sup> catalyzes the hydrolytic release of dipeptides from the carboxyl terminus of oligopeptide substrates. Although the enzyme has broad specificity, its best known physiological functions are the conversion of the decapeptide angiotensin I into the vasoactive octapeptide angiotensin II (Skeggs et al., 1954, 1956a,b) and the inactivation of bradykinin (Yang et al., 1970).

One of the earliest properties of ACE to be recognized was its requirement for monovalent anions, notably chloride, for catalytic activity toward angiotensin I (Skeggs et al., 1954). Later it was demonstrated that the activity toward bradykinin is also enhanced by chloride but the presence of the anion did not seem essential for the hydrolysis of this substrate (Dorer et al., 1974). Moreover, chloride activation has been demonstrated for the hydrolysis of a number of synthetic oligopeptide substrates (Piquilloud et al., 1970; Cushman & Cheung, 1971; Dorer et al., 1976). Although the anion activation of ACE has been known for a long time, knowledge about the kinetic and structural basis of this phenomenon is still rather fragmentary. In part, this has been due to the lack

of a rapid spectrophotometric assay of the enzyme. We have recently introduced FA-tripeptides as chromophoric substrates for ACE which allow continuous spectrophotometric monitoring of their hydrolysis (Holmquist et al., 1979). Utilizing this assay procedure, we have now investigated the effects of anions on the hydrogen ion and substrate concentration dependence of FA-Phe-Gly-Gly hydrolysis and have compared them to the hydrolysis of the physiological substrates angiotensin I and bradykinin. A preliminary account of these studies has been reported (Riordan et al., 1980).

### Materials and Methods

Angiotensin I (human) and bradykinin were purchased from Peninsula Laboratories Inc. (San Carlos, CA). Solutions of  $\text{Zn}^{2+}$  were prepared from the chloride salt (ultrapure grade; Ventron Corp., Danvers, MA). All other chemicals were reagent grade or of the highest purity available.

ACE was purified to homogeneity from rabbit lung acetone powder (Pel-Freez Biologicals Inc., Rogers, AR) as previously described (Bünning et al., 1983). Concentrations of ACE were determined by measuring the absorbance at 280 nm and expressed in molar concentrations by using a molar absorptivity of  $204\,000\ \text{M}^{-1}\ \text{cm}^{-1}$ . Adventitious metal ions were removed from all buffers and substrate solutions by extraction with

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<sup>1</sup> Abbreviations: ACE, angiotensin converting enzyme; Bz, benzoyl; FA, 2-furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.