Fujikawa, K., Titani, K., and Davie, E. W. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3359.
Furie, B., Gottlieb, A. J., and Williams, W. J. (1970), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 29,* 709.
Furie, B. C., and Furie, B. (1975), *J. Biol. Chem. 250,* 601.
Gibbons, R. A. (1966), in Glycoproteins, Their Composition, Structure and Function, Vol. 5, Gottschalk, A., Ed., Amsterdam, Elsevier, p 61.
Glossmann, H., and Neville, D. M. (1971), *J. Biol. Chem. 246,* 6339.
Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11,* 4493.
Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 59.
Hugli, T. E., and Moore, S. (1972), *J. Biol. Chem. 247,* 2828.
Jackson, C. M., Gordon, J. G., and Hanahan, D. J. (1971), *Biochim. Biophys. Acta 252,* 255.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41,* 410.
Kisiel, W., Ericsson, L. H., and Davie, E. W. (1976), *Biochemistry,* preceding paper in this issue.
Lee, J. C., and Timasheff, S. N. (1974), *Arch. Biochem. Biophys. 665,* 268.
Lindquist, P., Fujikawa, K., and Davie, E. W. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1353.
Macfarlane, R. G., and Barnett, B. (1934), *Lancet 2,* 985.
Markwardt, F., and Walsmann, P. (1962), *Thromb. Diath. Haemorrh. 7,* 86.
McPhie, P. (1971), *Methods Enzymol. 22,* 23.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.
Roffman, S., Schmaka, U., and Troll, W. (1970), *Anal. Biochem. 36,* 11.
Schiffman, S., Theodor, I., and Rapaport, S. I. (1969), *Biochemistry 8,* 1397.
Segrest, J. P., and Jackson, R. L. (1972), *Methods Enzymol. 28,* 54.
Shapiro, S. S., and Waugh, D. F. (1966), *Thromb. Diath. Haemorrh. 16,* 469.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Spiro, R. G. (1966), *Methods Enzymol. 8,* 3.
Svendsen, L., Blomback, B., Blomback, M., and Olsson, P. I. (1972), *Thromb. Res. 1,* 267.
Teller, D. C. (1973), *Methods Enzymol. 27,* 346.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Williams, W. J., and Esnouf, M. P. (1962), *Biochem. J. 84,* 52.
Wright, I. (1959), *J. Am. Med. Assoc. 170,* 325.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

Substrate Binding Properties of Converting Enzyme Using a Series of *p*-Nitrophenylalanyl Derivatives of Angiotensin I†

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ABSTRACT: The binding properties of angiotensin I for the active site of rabbit lung converting enzyme (CE) have been investigated. A series of angiotensin I like substrates, all containing the C-terminal tripeptide, (NO_2)Phe-His-Leu, were synthesized by increasing the length of the peptide at the N-terminal end. A total of eight peptides were studied, the largest being [Asn^1,(NO_2)Phe^8]angiotensin I. As determined by thin-layer chromatography, all substrates were hydrolyzed only at the (NO_2)Phe-His bond by purified converting enzyme, with the release of the dipeptide, His-Leu. By using an absorbance increase upon hydrolysis, the Michaelis constants and velocity maxima were determined and used to estimate those amino acids in the angiotensin I molecule that contribute significantly to binding to converting enzyme. It was hypothesized that, upon addition or substitution of one or more amino acids to the N-terminal end, a proportional decrease in both K_M and V_m is needed in order to conclude that the substrate actually increases its affinity for the enzyme. A test of the proportionality for the variation of K_M and V_m was found to be positive for all the substrates, except the N-terminal carbobenzoxy-blocked tripeptide, Z(NO_2)Phe-His-Leu. Substitutions near the bond that is hydrolyzed (e.g., proline for the carbobenzoxy group) appear to alter the catalytic properties of CE, while additions far removed from the site of hydrolysis (e.g., the N-terminal tripeptide Asn-Arg-Val) may enhance binding affinity.

Angiotensin I converting enzyme (CE)[1] (EC 3.4.15.1) catalyzes the hydrolysis of the decapeptide, angiotensin I, to the octapeptide, angiotensin II, and the dipeptide, histidyl-leucine (Page and Bumpus, 1961). The enzyme also inactivates bradykinin by hydrolysis of the C-terminal dipeptide (Yang et al., 1970). The enzyme was originally partially purified from plasma by Skeggs et al., 1956 and later by Yang and Erdös, 1967; Lee et al., 1971a,b. Preparations of purified CE have also been obtained from lung (Lee et al., 1971c; Cushman and Cheung, 1972; Nakajima et al., 1973; Dorer et al., 1972; Igic et al., 1972; Stevens et al., 1972; Lanzillo and Fanburg, 1974; Massey and Micalizzi, 1974), which contains a particularly high concentration of converting enzyme (DePierre and Roth, 1972).

Assays of CE have employed analysis of the hydrolysis

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[1] Abbreviations used are: CE, rabbit lung converting enzyme; Boc, *tert*-butoxycarbonyl; DMF, dimethylformamide; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; TLC, thin-layer chromatography; uv, ultraviolet.

products of angiotensin I by ion-exchange chromatography with a subsequent bioassay for angiotensin II (Boucher et al., 1970), high-voltage electrophoresis (Lee et al., 1971a,b), radioimmunoassay for angiotensin II (Page et al., 1969), and quantitation of a fluorometric species from the hydrolysis product His-Leu and the reagent *o*-phthaldialdehyde (Piquilloud et al., 1970). Use of small tripeptide substrates, which are more easily synthesized than angiotensin I, has made possible simpler chemical analysis of the hydrolysis products. Hydrolysis of Hip-His-Leu was assayed by extraction of hippuric acid into an organic solvent (Cushman and Cheung, 1971). A fluorometric assay for His-Leu was employed by Piquilloud et al. (1970) with the substrates, Z-Pro-Phe-His-Leu and Z-Phe-His-Leu. A continuous spectrophotometric assay of the hydrolytic activity of converting enzyme has been reported with Boc-(NO_2)Phe-Phe-Gly (Yang et al., 1970) and Z-(NO_2)Phe-His-Leu, Z-(NO_2)Phe-Gly-Gly, (NO_2)Hip-Gly-Gly (Stevens et al., 1972) as substrates.

The binding specificity of amino acids in angiotensin II for smooth muscle receptor sites in the peripheral circulation of higher mammals has been extensively investigated and recently reviewed by Regoli et al. (1974). However, the binding specificity of amino acids in angiotensin I for the active site of CE has not yet been clearly elucidated. A method for determining substrate specificity, as suggested by Bakhle (1974), is to examine substrates "derived from angiotensin I by the loss of successive amino acids from the N-terminal". Angus et al. (1973) attempted to determine the kinetic constants of angiotensin I and several peptides related to angiotensin I, but were forced to allow the reaction to proceed to 40% hydrolysis of substrate in order to assay the hydrolysis products with an amino acid analyzer.

It was felt that a more direct and accurate method for measurement of these important kinetic constants was needed. With this in mind, a series of *p*-nitrophenylalanyl peptides related to the C-terminal of angiotensin I was synthesized to be used in a continuous spectrophotometric assay procedure. These substrates allowed determination of the initial reaction velocities before significant amounts of product had been formed, a condition which is necessary in order to estimate the Michaelis constants and velocity maxima reliably (Dixon and Webb, 1960; Webb, 1963).

This report describes a kinetic study of eight [(NO_2)Phe^8]-angiotensin I like substrates, their kinetic behavior with purified rabbit lung CE, and an analysis of those amino acid residues in the angiotensin I molecule that contribute to binding at the active site of the enzyme.

Experimental Procedures

Synthesis of Substrates. The peptide substrates were prepared by solid-phase synthesis.

Homogeneity of the peptides was demonstrated by TLC on Merck Silplate F22 plates in three solvent systems: (A) BuOH–AcOH–H_2O (6:2:3), (B) BuOH–AcOH–H_2O–pyridine (9:2:6:7), and (C) propanol–NH_4OH (7:3). Amino acid analyses of acid hydrolysates prepared in 6 N HCl at 140 °C for 6 h under N_2 were performed on a Jeol JLC-5AH amino acid analyzer. Solvents employed in solid phase synthesis were AR grade or Spectroquality (DMF) and were used without further purification. Boc-amino acids were prepared as described by Schnabel (1967).

Solid-Phase Synthesis. The resin esters were prepared from Bio-Beads SX-2 by standard procedures. The synthesis was started with approximately 2 meqiv of Boc-amino acid resin ester. The Boc-protecting group was removed at each stage with 0.8 N HCl in HOAc for 40 min. The resin hydrochloride was neutralized with 10% Et_3N in CH_2Cl_2 for 10 min. The Boc-amino acids were coupled to the peptide resin with DCI in CH_2Cl_2 employing a 3 molar excess of both reagents for 18 h. Because of poor solubility, Boc-histidine was coupled in DMF, and 1-hydroxybenzotriazole was added to the Boc-histidine coupling step to reduce racemization.

Tyrosine was introduced as the Boc-*O*-benzyl derivative and arginine as the Boc N^6-tosyl derivative. Asparagine was coupled using the Boc-*p*-nitrophenyl ester derivative in DMF for 48 h. After the synthesis was complete, the peptide resin was washed with HOAc and EtOH and dried in vacuo. The peptides were removed from the resin by treating a mixture of the resin and trifluoroacetic acid with dry HBr (2 × 30 min). The filtrates from the cleavage reaction were evaporated to an oil which solidified upon trituration with ether.

[Asn^1,(NO_2)Phe^8]angiotensin I (substrate 8) was deprotected with liquid HF after cleavage from the resin as described.

The peptides were purified by ion-exchange chromatography on Sephadex SP-C-25 (2.5 × 60 cm) by gradient elution with 0.1–0.5 M NH_4OAc in 1% acetic acid. The buffer salts were removed by lyophilization, yielding the desired peptides as hydrated acetate salts.

Z-(NO$_2$)Phe-His-Leu (*Substrate 1*). The synthesis of the N-terminal carbobenzoxy-blocked derivative of (NO_2)Phe-His-Leu has been described (Stevens et al., 1972).

Pro-(NO$_2$)Phe-His-Leu (*Substrate 2*). $[\alpha]^{20}D = 10.4°$ (c = 0.51, 1 N HOAc). R_f A, 0.33; R_f B, 0.55; R_f C, 0.65. Amino acid analysis: Leu, 1.00; Pro, 1.08; His, 0.96; (NO_2)Phe, 1.02.

Gly-Pro-(NO$_2$)Phe-His-Leu (*Substrate 3*). $[\alpha]^{20}D = 50.5°$ (c = 0.51, 1 N HOAc). R_f A, 0.45; R_f B, 0.58; R_f C, 0.70. Amino acid analysis: Leu, 1.00; Pro, 1.05; His, 0.91; (NO_2)Phe, 0.87; Gly, 0.93.

His-Pro-(NO$_2$)Phe-His-Leu (*Substrate 4*). $[\alpha]^{20}D = 43.0°$ (c = 0.51, N HOAc). R_f A, 0.38; R_f B, 0.48; R_f C, 0.90. Amino acid analysis: Leu, 1.00; Pro, 1.07; His, 1.76; (NO_2)Phe, 0.98.

Acetyl-His-Pro-(NO$_2$)Phe-His-Leu (*Substrate 5*). The pentapeptide resin was treated with acetic anhydride in DMF prior to cleavage of the peptide. $[\alpha]^{20}D = 53.0°$ (c = 0.50, 1 N HOAc). R_f A, 0.48; R_f B, 0.58; R_f C, 0.90. Amino acid analysis: Leu, 1.00; Pro, 0.94; His, 2.14; (NO_2)Phe, 0.97.

Tyr-Ile-His-Pro-(NO$_2$)Phe-His-Leu (*Substrate 6*). $[\alpha]^{20}D = -54.7°$ (c = 0.52, 1 N HOAc). R_f A, 0.45; R_f B, 0.65; R_f C, 0.65. Amino acid analysis: Leu, 1.00; Pro, 1.03; His, 1.97; (NO_2)Phe, 1.06; Ile, 0.87; Tyr, 92.

Gly-Ile-His-Pro-(NO$_2$)Phe-His-Leu (*Substrate 7*). $[\alpha]^{20}D = 69.3°$ (c = 0.50, 1 N HOAc). R_f A, 0.41; R_f B, 0.55; R_f C, 0.60. Amino acid analysis: Leu, 1.00; Pro, 1.04; His, 1.78; (NO_2)Phe, 0.94; Ile, 0.86; Gly, 0.93.

[Asn^1,(NO$_2$)Phe^8]Angiotensin I (*Substrate 8*) was purified by elution with a 0.4–0.8 M NH_4OAc in 1% acetic acid. $[\alpha]^{20}D = -64.4°$ (c = 0.51, 1 N HOAc). R_f A, 0.40; R_f B, 0.53. Amino acid analysis: Leu, 1.00; His, 2.01; (NO_2)Phe, 1.06; Pro, 1.07; Ile, 0.97; Tyr, 0.96; Val, 0.97; Arg, 1.06; Asp, 1.03.

Preparation of Rabbit Lung Converting Enzyme. Rabbit lung CE was prepared as previously described (Massey and Micalizzi, 1974), except for an additional purification step. Enzyme obtained from the 45% saturated–2% (w/v) Na DOC (sodium deoxycholate) subphase was concentrated on a Diaflow PM-30 membrane and made 10% Triton X-100 at pH 6.0. The 10-ml sample was loaded on a DEAE-cellulose (Whatman DE-32) column equilibrated with 5 mM sodium

maleate, pH 6.0 (2.5 × 20 cm), and washed with 800 ml of 5 mM sodium maleate, pH 6.0, 1 μM $ZnSO_4$, and 1% Triton X-100. The column was eluted overnight with a total of 1 l. of 5 mM sodium maleate, pH 6.0, 1 μM $ZnSO_4$, and 0.1% Triton X-100 with a linear NaCl gradient of 0–0.2 M. The leading peak, determined by 280-nm absorption, was concentrated and applied to a Sepharose 4B column (3.2 × 230 cm) packed with 6-mm glass beads, according to the method of Sachs and Painter (1972), and eluted with 5 mM Tris-Cl, pH 8.0, and 0.1 M NaCl. The leading peak, determined by 280-nm absorption, contained CE and was concentrated by Diaflow filtration. The enzyme had a specific activity of 25 μmol min^{-1} mg of $protein^{-1}$ using Z-(NO_2)-Phe-His-Leu as substrate at 0.2 mM, pH 7.2, and 23 °C, and was comparable to the purity obtained by Cushman and Cheung (1972). The enzyme was stored without loss of activity at 4 °C for 3 months at 0.5 mg/ml in 50 mM Tris-Cl and 0.1 M NaCl at pH 8.0.

Converting Enzyme Assay. One unit of enzyme activity is defined as that amount of enzyme which hydrolyzes 1 μmol of substrate/min. Specific activity is defined as units/mg of protein.

CE activity was measured by a method similar to that described by Stevens et al. (1972) with the use of a Cary 16 double-beam spectrophotometer coupled to a 10-in. linear recorder with the aid of a Cary recorder interface accessory. The reaction mixture contained 50 mM Tris–acetate and 0.1 M NaCl at pH 7.2 and the appropriate substrate at a concentration no greater than 0.2 mM. One milliliter of the assay solution was pipetted into a quartz semimicrocuvette with a 1-cm light path after which no more than 10 μl (containing from 0.2 to 2 μg or 0.005 to 0.05 unit) of enzyme was added. All substrates were soluble at or below 0.2 mM and did not require methanol, as used by Stevens et al. (1972) for the Z-(NO_2)-Phe-His-Leu substrate. The assay mixture was gently mixed by capping with Parafilm and inverting the cuvette. All assays were performed at 23 °C. The hydrolysis of the substrate was continuously recorded with time at the wavelength for which a maximum absorbance difference was observed (see below). Enzyme units were then calculated with the aid of the difference extinction coefficient obtained for each substrate.

The rate of hydrolysis for any concentration was determined from the slope of the curve starting at the beginning of the recorder trace. During the time required for mixing and placing the cuvette in the spectrophotometer, roughly 1–5% of the substrate was hydrolyzed. Since the recorder trace showed a linear increase in optical density with time for up to 10% hydrolysis, it was assumed that steady-state kinetics existed during the early part of the assay; this assumption made it possible to interpret the kinetic constants meaningfully (Dixon and Webb, 1960).

Difference Spectra of the Substrates. The substrates were dissolved in the reaction mixture at a concentration of 0.2 mM. About 5 μg of enzyme in 10 μl was added to 1 ml and the substrate was incubated at 23 °C for 2 h. Hydrolysis of the substrates was essentially complete within 1 h, as determined in the next section. To another 1 ml of the same substrate solution, 5 μg of enzyme in 10 μl, which had been completely inactivated by exposure to 80 °C for 15 min, was added. The cuvette containing the substrate solution treated with active enzyme was placed into the sample position of the spectrophotometer, while the cuvette containing the substrate solution treated with identical amounts and volumes of heat-inactivated enzyme was placed into the reference position. The cuvettes were matched for the wavelength range of interest. The difference spectrum was then obtained with the aid of a Cary wavelength drive accessory and a Cary multipot accessory design to insure a flat baseline.

Determinations of Difference Extinction Coefficients. Because the free-energy change for hydrolysis of a peptide bond is relatively large, it was assumed that essentially complete hydrolysis of the (NO_2)Phe–His bond in the substrates was obtained in the presence of CE. Therefore, the absorbance difference between the substrate and its hydrolysis products was used for estimating the difference extinction coefficient for the substrates. One milliliter of a solution of 0.2 mM substrate in 50 mM Tris–acetate and 0.1 M NaCl, pH 7.2, was pipetted into a semimicrocuvette with a 1-cm light path. Ten microliters of enzyme solution was added to initiate hydrolysis and the reaction was allowed to go to completion (zero slope of the reaction rate). The time required to mix the enzyme in the reaction mixture and place the cuvette in the spectrophotometer was measured, so that the optical density at zero time for the reaction mixture could be obtained by extrapolation on the recording trace. The change in optical density was measured three times and corrections for dilution with the enzyme were made. The difference extinctions were calculated for a 1 molar solution and expressed as the mean difference molar extinction coefficients ($\Delta\bar{\epsilon}$) with the standard deviation.

Thin-Layer Chromatography. Analysis of the substrates and the hydrolytic products after incubation with CE was performed by TLC using a modified method of Stevens et al. (1972). The substrates were dissolved in 50 mM Tris–acetate, pH 7.2, at a concentration of 1 mM and spotted on a 20 × 20 cm silica gel plate. To 0.2 ml of the substrate solutions were added 20 μl of 1.0 M NaCl and 25 μl of enzyme, and the solutions were allowed to incubate for 2 h to ensure complete hydrolysis, after which they were spotted on another silica gel plate. The plates were developed overnight in a solvent system containing 1-butanol–acetic acid–water (6:2:3). The spots were detected by spraying with a ninhydrin solution and incubation in a 60 °C oven for 1 h.

Standards of His-Leu and leucine were spotted on the TLC plates and developed simultaneously with spots of substrates not incubated with CE (Figure 2A) and spots of substrates previously incubated with CE (Figure 2B).

Kinetic Analysis of the Substrates. Kinetic behavior of the angiotensin I like substrates was determined at six different concentrations with duplicate assays. Substrate concentrations were chosen both higher and lower than the K_M, allowing maximum accuracy in the determination of the kinetic constants. Michaelis constants and velocity maxima were determined by the graphic method of Lineweaver and Burk (1934) in which a linear regression analysis was performed to obtain the best fit of the data. The kinetic constants were determined three times and the mean with its standard deviation was obtained.

Chloride Dependency of Converting Enzyme. The effect of chloride on the activity of CE with the various substrates was tested by substituting sodium acetate for sodium chloride in the assay mixture, to reduce chloride ion without varying the ionic strength. The substrate concentration, different for each substrate,izas set at twice the K_M. This was done to ensure that variations in chloride dependency could not be attributed to variation in degrees of saturation of the enzyme by the substrates.

Protein Determinations. Protein concentrations of CE were analyzed by the method of Lowry et al. (1951) with crystallized bovine serum albumin as a standard.

EDTA Inhibition of Converting Enzyme. A concentration

TABLE I: Angiotensin I Like Substrates Containing a Nitrate Group on Phenylalanine.

Substrate	Compound
1	Z-(NO_2)Phe-His-Leu
2	Pro-(NO_2)Phe-His-Leu
3	Gly-Pro-(NO_2)Phe-His-Leu
4	His-Pro-(NO_2)Phe-His-Leu
5	Ac-His-Pro-(NO_2)Phe-His-Leu
6	Tyr-Ile-His-Pro-(NO_2)Phe-His-Leu
7	Gly-Ile-His-Pro-(NO_2)Phe-His-Leu
8	Asn-Arg-Val-Tyr-Ile-His-Pro-(NO_2)Phe-His-Leu

TABLE II: Wavelength Maximum and Extinction Coefficients for the Difference in Absorbance between the Nitrated Angiotensin I Analogues and the Hydrolysis Products Obtained with Purified CE.

Substrate	λ_{max}[a] (nm)	$\Delta\tilde{\epsilon}\left(\frac{\Delta OD}{M}\right)$	$\pm\sigma$	No. of Determinations
1	313	845	24	4
2	308	766	33	4
3	309	678	38	3
4	310	604	34	3
5	310	691	17	3
6	311	735	41	3
7	313	742	43	3
8	310	684	10	3

[a] All values are given for pH 7.2 in 50 mM Tris-Cl–0.1 M NaCl.

of 0.1 mM EDTA was added to the assay mixture which contained substrate 1, Z(NO_2)Phe-His-Leu at 0.2 mM. The reaction was initiated by addition of purified CE.

Results

The structures of the [(NO_2)Phe^8]angiotensin I like substrates evaluated are numbered 1 through 8 in Table I.

Ultraviolet Difference Spectra. The uv difference spectra for the substrates and the converting enzyme hydrolysis products appeared similar for all the compounds. Only slight differences were seen in the wavelength maxima and δ molar extinction coefficients. A typical difference spectrum is shown for substrates 1 and 8 in Figure 1. The tripeptide substrate (substrate 1) had the highest difference spectrum of any of the substrates. All the difference spectra had a wavelength maximum near 310 nm, which agrees with the result of Yang et al. (1970) in which Boc-(NO_2)Phe-Phe-Gly gave a difference absorbance maximum at the same wavelength after treatment with CE. The wavelength maxima and mean difference molar extinction coefficients with standard deviations are in Table II.

Determination of the Hydrolysis Products with Converting Enzyme by Thin-Layer Chromatography. The specificity for hydrolysis of the angiotensin I like substrates by CE was examined by means of TLC (Figure 2). Six substrates, not incubated with enzyme (Figure 2A), gave a single ninhydrin-positive spot. Four of these spots (substrates 2, 3, 4, and 6) migrated faster than the His-Leu spot on the same TLC plate. Substrates 2, 3, and 7 gave yellow spots after treatment with ninhydrin and all migrated faster than the His-Leu spot. There was no difference in migration between the spot of substrate 8, [Asn^1,(NO_2)Phe^8]angiotensin I, and the spot of His-Leu

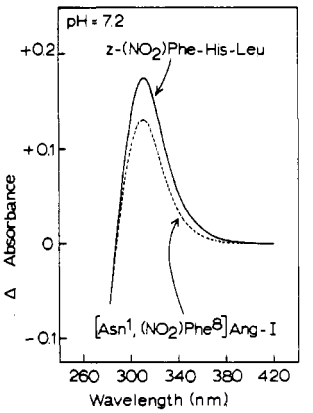

FIGURE 1: Difference spectra for substrate 1 (Z-(NO_2)Phe-His-Leu) and substrate 8 ([Asn^1,(NO_2)Phe^8]angiotensin I) incubated with CE.

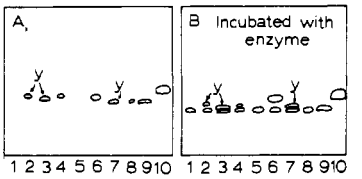

FIGURE 2: Thin-layer chromatograms of the nitrated angiotensin I like substrates with His-Leu and leucine as standards. Substrates 1 through 8 are numbered 1–8, His-Leu is 9, and leucine is 10. (A) Substrates and standards; (B) substrates and standards incubated with CE. Those spots that showed a yellow color with ninhydrin are marked Y.

(spot no. 9, Figure 2A). Substrates 1 and 5, which have the α-amino group blocked, did not give a positive ninhydrin reaction.

Upon incubation of the substrates with purified CE followed by TLC (Figure 2B), a slower moving ninhydrin-positive spot appeared which migrated a distance identical to that found for His-Leu, spotted on the same plate (spot no. 9, Figure 2B). Only one spot corresponding to His-Leu appeared for substrates 1 and 5. The yellow spots for substrates 2, 3, and 7 were easily distinguished from the purple spot corresponding to His-Leu. Substrate 8 (Figure 2B) appeared to migrate on the plate at a rate identical to the His-Leu spot; however, using the amino acid analyzer, analysis of the hydrolysis products of [Asn^1]angiotensin I resulting from exhaustive incubation with a similar CE preparation indicated that only the dipeptide, His-Leu, had been released. It is concluded from these data that purified CE hydrolyzes substrates 1 through 8 and releases only the dipeptide His-Leu.

Determination of the Kinetic Constants for CE with the [(NO_2)Phe^8]Angiotensin I Like Substrates. CE showed linear Lineweaver–Burk plots of reciprocal velocity vs. reciprocal substrate concentration for all eight substrates. A plot of velocity, expressed as units/mg of protein, vs. substrate concentration, is shown in Figure 3 for substrate 8. The insert shows the double-reciprocal plot allowing a measure of Michaelis constant (K_M) and velocity maximum (V_m) by taking the reciprocal of the abscissa and ordinate intercepts, respectively. Mean values for K_M and V_m with the standard deviations for all eight substrates are given in Table III.

Chloride Dependency. For all substrates the activity dropped by 50 to 75% when the chloride concentration was lowered from 0.1 to 0.01 M. There appeared to be no marked differences in the chloride requirement for hydrolysis of [(NO_2)-Phe^8]angiotensin I like substrates.

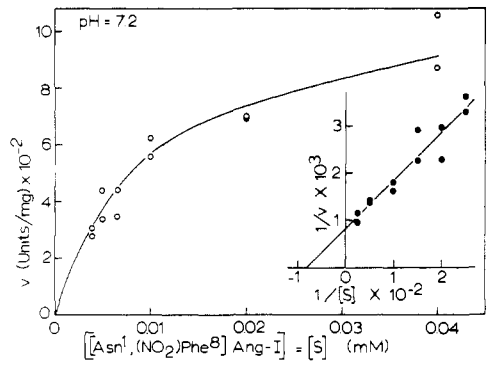

FIGURE 3: Kinetic analysis of substrate 8 ([Asn^1,(NO_2)Phe^8]angiotensin I). Plot of velocity vs. substrate concentration. Insert shows Lineweaver-Burk plot with regression analysis used to draw the line.

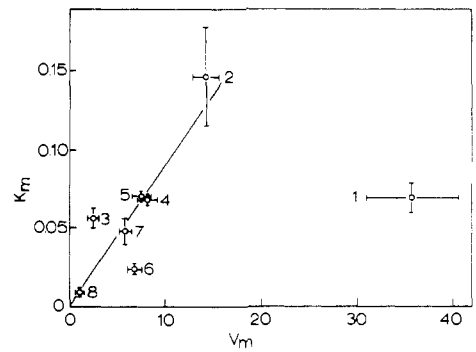

FIGURE 4: Test of proportionality for variation of the kinetic constants with the nitrated angiotensin I like substrates. Plot of Michaelis constants, K_M, vs. velocity maxima, V_m. The curve was plotted according to a linear regression analysis of all data points except for substrate 1. Vertical and horizontal bars next to each point represent the standard deviation of the K_M and V_m, respectively.

TABLE III: Michaelis Constants and Velocity Maxima of Nitrated Angiotensin I Like Substrates Obtained With Purified CE at pH 7.2.[a]

Substrate	k_M (mM)	$\pm\sigma$	$\bar{V}_m$ (units/mg)	$\pm\sigma$
1	0.067	0.010	35.7	4.9
2	0.146	0.036	14.4	1.2
3	0.057	0.006	2.5	0.29
4	0.067	0.025	8.2	1.1
5	0.069	0.003	7.7	1.0
6	0.023	0.003	6.9	0.7
7	0.047	0.008	5.8	0.5
8	0.0097	0.002	1.2	0.03

[a] Values are obtained by averaging three results obtained from linear regression analysis of Lineweaver-Burk plots in which duplicate assays were performed at each substrate concentration.

EDTA Inhibition. Upon initiation of the reaction containing 0.1 mM EDTA and 0.2 mM substrate 1, by addition of purified CE (0.01 unit of enzyme), an increase in optical density occurred, indicating active enzyme was present in the assay mixture. However, as time progressed, the activity of the enzyme decreased until 100% inhibition of CE was obtained after 1.5 min. Only about 50% of the substrate had been hydrolyzed. This result demonstrated that purified CE was inhibited by EDTA but that complicated kinetics for the onset of inhibition exists which is beyond the scope of this paper.

Discussion

All eight [(NO_2)Phe^8]angiotensin I like peptides are substrates for purified CE and are cleaved only at the nitrophenylalanyl-histidyl bond, as determined by TLC of the hydrolysis products. The enzyme also showed a requirement for chloride ion with all substrates and is inhibited by EDTA for the case of substrate 1. These results conform to the requirements for angiotensin I CE as defined according to Bakhle (1974) and Erdös (1975), which state that: (1) CE should hydrolyze the substrate angiotensin I, only at the phenylalanyl-histidyl bond, thereby releasing the two products, angiotensin II and the dipeptide, His-Leu, (2) that the hydrolysis of angiotensin I by CE should be chloride ion dependent, and (3) that the enzyme should be inhibited by EDTA. It has been previously reported that short-chain angiotensin I fragments, beginning from the carboxyl end, have shown a chloride dependency for activity as determined by Piquilloud et al. (1970), Cushman and Cheung (1972), and Angus et al. (1973). Inhibition of the hydrolysis of Hip-His-Leu by EDTA was also demonstrated by Cushman and Cheung (1971) for the rabbit lung enzyme.

The affinity of a substrate for the active site of an enzyme cannot be determined by Michaelis constant, K_M, data alone (Dixon and Webb, 1960). Knowledge of V_m and K_M data for a series of related substrates, such as presented in this paper, can be used to suggest a ranking order for relative affinity to the enzyme. There is no data to indicate the effect of peptide length on the rate of hydrolysis of the (NO_2)Phe-His bond in the angiotensin I molecule. Until such information becomes available it is assumed that as additions to the N-terminal end of the substrate are made, several bond lengths removed from the (NO_2)Phe-His bond, the possibility of perturbing the catalytic properties of the active site of the enzyme are minimal.

Dissociation of one or both of the two products, His-Leu and the nitrated angiotensin II like peptides, could determine the V_m if the dissociation constant is small. The product, His-Leu, is known to bind poorly for CE (Angus et al., 1973; Lanzillo and Fanburg, 1974; Yang et al., 1970; Lee et al., 1971b; Sander et al., 1971) and, therefore, probably does not affect V_m. However, a high affinity of angiotensin I for the active site of CE is suggested by the results of Cushman and Cheung (1971) who reported I_{50} values of 0.01 mM for angiotensin I and 0.02 mM for angiotensin II with the rabbit lung enzyme when Hip-His-Leu was used as the substrate. This result suggests that the dissociation of angiotensin II from CE could be the rate-limiting step in V_m.

As a preliminary assessment of the relative affinities of the angiotensin I like substrates presented in Table I, it is postulated that a proportional decrease in both K_M and V_m be observed as the N-terminal end of the substrate is modified or lengthened. A test of this hypothesis is seen in Figure 4 in which the K_M is plotted against V_m for all eight substrates. A straight line, obtained by linear regression analysis of all points, except that for substrate 1, is drawn and indicates that the overall trend is for the K_M and V_m to decrease in a proportional fashion as the N-terminal end of the substrates is lengthened. Substrate 1 shows abnormal behavior and is far removed from the curve. This result is expected because of possible perturbations of the catalytic process with the substitution of a carbobenzoxy group instead of a proline group close to the bond that is cleaved by the enzyme.

Substrate 1 is probably the best substrate for purposes of

assaying CE, because of the relatively low K_M and very high V_m. The V_m for substrate 1 is about 30 times that of substrate 8, which does not agree well with the result of Piquilloud et al. (1970) in which Z-Phe-His-Leu has a hydrolysis rate ten times that of angiotensin I. The value of the K_M for substrate 1, Z-(NO_2)Phe-His-Leu, does agree roughly with that obtained by Piquilloud et al. (1970) for the nonnitrated species in which a pH of 7.5 was used.

It appears that the pentapeptides (substrates 3 and 4) may bind significantly more than the tetrapeptide (substrate 2) in which a significantly greater K_M and V_m are observed for the tetrapeptide. The N-terminal amino acids in the pentapeptides (substrates 3 and 4) seem to enhance binding of the substrate only by blocking the imino nitrogen of proline and thereby moving the positive charge three bond lengths away from the region of catalysis on the enzyme surface. Angus et al. (1973) showed that with the hog plasma CE addition of histidine to the imino nitrogen of proline in the tetrapeptide to give the pentapeptide gave a decrease in K_M *and* a drop in V_m. It was tentatively concluded that the histidine residue played a role in increasing the affinity of the substrate for the enzyme. However, the effect of "blocking the nitrogen function of proline and thereby eliminating a positive electrical charge at this locus" was not ruled out.

Blocking the N-terminal positive charge in the histidine-containing pentapeptide (substrate 4) by acetylation (substrate 5) gives no significant change in K_M or V_m, which suggests that simply moving the positive charge out by three bond lengths (substrates 3 and 4 compared to substrate 2) may not add to binding by possibly picking up a binding site for a positive charge. These results imply that a positive charge on the substrate *near* the site of hydrolysis by the enzyme actually repels the substrate and decreases its affinity for the enzyme. The fact that tripeptides with a free positive charge on the N-terminal nitrogen are not substrates for converting enzyme (Yang et al., 1970) further substantiates that a positive charge located close to the point of hydrolysis of the peptide substrate is not tolerated by the enzyme.

Binding of angiotensin I to converting enzyme does not seem to require the imidazole moiety of the His^6 position of angiotensin I. The glycine-substituted pentapeptide (substrate 3) gave a somewhat smaller K_M and a significantly smaller V_m than the histidine-substituted pentapeptide (substrate 4), suggesting that histidine may not play an important role in binding to the enzyme but may, in fact, slightly decrease the binding.

The involvement of Tyr^4 in the binding of angiotensin I to CE cannot be determined, because a comparison of the two heptapeptides, one containing tyrosine (substrate 6) and the other glycine (substrate 7), shows that K_M and V_m do not change in a proportional fashion.

[Asn^1,(NO_2)Phe^8]angiotensin I (substrate 8) appears to bind with, by far, the greatest affinity, as seen by its very low K_M (0.0097 mM) and the low V_m (1.2 units/mg). Because the K_M and V_m of the heptapeptides (substrates 6 and 7) are much larger compared to substrate 8, it is suggested that the N-terminal sequence Asn-Arg-Val contributes significantly to binding to the lung enzyme. It is not known whether this is due to a specific binding site on the enzyme surface for one or more of the amino acids in the tripeptide sequence, or the overall decapeptide is forced into a preferred conformation that has a greater affinity for the active site on the enzyme. This result differs from that obtained by Angus et al. (1973) in which they claimed that "the hog plasma converting enzyme does not have an important recognition site for these N-terminal amino acid residues". It is noted that, besides studying a different enzyme preparation, Angus et al. (1973) used the naturally occurring substrate, angiotensin I, which contains an aspartic acid residue in the N-terminal position of the decapeptide.

The possibility that the nitro group of the (NO_2)Phe^8 position of angiotensin I affects the binding of the substrate to the enzyme was studied. Incubation of [Asn^1,(NO_2)Phe^8]-angiotensin I (substrate 8) and [Asn^1]angiotensin I (synthesized in a fashion similar to substrate 8) with the purified lung enzyme, and analysis of the amount of His-Leu formed by the fluorometric method of Piquilloud et al. (1970), gave a K_M of 0.027 mM for the nitrated species and 0.029 mM for the nonnitrated species. This result indicates that the nitro group on the Phe^8 position does not alter the affinity of the substrate for the enzyme. The fact that a higher K_m was obtained with the purified enzyme when the hydrolytic products were measured by the method of Piquilloud et al. (1970) indicates the difficulty of obtaining kinetic constants for the enzyme using different methods. The slope of the optical density trace obtained for the assay of CE with [Asn^1(NO_2)Phe^8]angiotensin I was determined at the very beginning of the assay. Reaction velocities obtained with the end-point assay of Piquilloud et al. (1970), after the reaction has progressed, could give slower rates and an increased K_M due to product inhibition (Webb, 1963).

A very low K_M (0.0056 mM) for lung CE was reported by Huggins et al. (1970) with [Asp^1]angiotensin I. This result was obtained by a bioassay and a radiometric method, either of which would be sensitive for product formation and could give reaction rates early in the assay before significant product formation had occurred.

Other values reported for the K_M of [Asp^1]angiotensin I have varied from 0.020 mM for guinea pig lung and 0.026 mM for porcine lung (Lee et al., 1971c) to 0.050 mM for the rabbit lung enzyme (Cushman and Cheung, 1972). It has not been determined if the Asn^1 and Asp^1 forms of angiotensin I differ in binding affinity. Enzyme preparations, assay conditions, and methods for analysis of the products vary greatly and all could contribute to the variation in the reported Michaelis constants using [Asp^1]angiotensin I as substrate. Further studies with the Asp^1 and Asn^1 derivatives of angiotensin I assayed with the same CE preparation under identical conditions would be necessary to determine this.

References

Angus, C. W., Hyun-J. L., and Wilson, I. B. (1973), *Biochim. Biophys. Acta 309,* 169.

Bakhle, Y. S. (1974), *Handb. Exp. Pharm. 37,* 41.

Boucher, R., Kurihara, H., Crise, C., and Genest, J. (1970), *Circ. Res. Suppl. 26,* 83.

Cushman, D. W., and Cheung, H. S. (1971), *Biochem. Pharmacol. 20,* 1637.

Cushman, D. W., and Cheung, H. S. (1972), *Hypertens., Proc. Symp., 1971* 532.

DePierre, D., and Roth, M. (1972), *Experientia 28,* 154.

Dixon, M., Webb, E. C. (1960), *Enzymes,* 2nd Ed. 10.

Dorer, F. E., Kahn, J. R., Lentz, E. K., Levine, M., and Skeggs, L. T. (1972), *Circ. Res. 31,* 356.

Erdös, E. G. (1975), *Circ. Res. 36,* 247.

Huggins, C. G., Corcoran, R. J., Gordon, J. S., Henry, H. W., and John, J. P. (1970), *Circ. Res. Suppl. 26, 27,* 93-108.

Igic, R., Erdös, E. G., Yeh, H. S. J., Sorrells, K., and Nakajima, T. (1972), *Circ. Res. Suppl. 30, 31,* 51-61.

Lanzillo, J. J., and Fanburg, B. L. (1974), *J. Biol. Chem. 249,* 2312.

Lee, H. J., Larue, J. N., and Wilson, I. B. (1971a), *Arch. Biochem. Biophys. 142,* 548.
Lee, H. J., Larue, J. N., and Wilson, I. B. (1971b), *Biochim. Biophys. Acta 235,* 521.
Lee, H. J., Larue, J. N., and Wilson, I. B. (1971c), *Biochim. Biophys. Acta 250,* 549.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56,* 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Massey, T. H., and Micalizzi, E. R. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1308 (Abstract).
Nakajima, T., Oshima, G., Yeh, H. S. J., Igic, R., and Erdös, E. G. (1973), *Biochim. Biophys. Acta 315,* 430.
Page, I. H., and Bumpus, F. M. (1961), *Physiol. Rev. 41,* 331.
Page, L. B., Haber, E., Kimura, A. Y., and Purnode, A. (1969), *J. Clin. Endocrinol. 29,* 205.
Piquilloud, Y., Reinharz, A., and Roth, M. (1970), *Biochem. Biophys. Acta 206,* 136.
Regoli, D., Park, W. K., and Rioux, F. (1974), *Pharmacol. Rev. 26,* 69.
Sachs, D. H., and Painter, E. (1972), *Science 175,* 782.
Sander, G. E., West, D. W., and Huggins, C. G. (1971), *Biochim. Biophys. Acta 242,* 662.
Schnabel, E. (1967), *Justus Liebigs. Ann. Chem. 702,* 188.
Skeggs, L. T. Jr., Kahn, J. R., and Shumway, N. S. (1956), *J. Exp. Med. 103,* 295.
Stevens, R. L., Micalizzi, E. R., Fessler, D. C., and Pals, D. T. (1972), *Biochemistry 11,* 2999.
Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, New York, N.Y., Academic Press, p 140.
Yang, H.Y. T., and Erdös, E. G. (1967), *Nature (London) 215,* 1402.
Yang, H. Y. T., Erdös, E. G., and Levin, Y. (1970), *Biochim. Biophys. Acta 214,* 374.

Isolation, Purification, and Cross-linking Profiles of Elastin from Lung and Aorta†

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ABSTRACT: Elastin from anatomically defined regions of young calf lung and dog aorta was isolated and purified by a procedure which sequentially removed lipids, collagen, structural glycoproteins, and the microfibrillar proteins without apparent damage to the cross-linking residues, which have been shown to be sensitive to autoclaving and hot alkali treatment. One of the methods described was effective in obtaining pure elastin from lung parenchyma. Visceral pleura was found to be the richest source (25% dry weight) of elastin in the lung tissues examined. The amino acid compositions of the elastins purified by different methods were compared for purity and for the detection of possible damage to cross-linking compounds. Cross-linking profiles were obtained by column chromatography either after reduction with ^{3}[H]$NaBH_4$ or after reaction with ^{14}[C]NaCN and NH_3. The ^{3}[H]$NaBH_4$ method, under carefully controlled conditions, proved not to be quantitatively reproducible. The reaction of elastin with ^{14}[C]NaCN and NH_3 appeared preferable due to its reproducibility; this procedure required one type of hydrolysis for the analysis of all the cross-linking compounds. Examination of the cross-linking profiles of the elastins from various tissue regions revealed differences in the type, distribution, and quality of cross-links.

Elastin is an insoluble, extensively cross-linked protein found in lung, ligamentum nuchae, aorta, skin, and elastic cartilage. It may be isolated from tissues after other more soluble connective tissue elements have been removed, most often by the use of harsh methods involving autoclaving and exposure to hot alkali (Lansing et al., 1952; Partridge et al., 1955). Such procedures can remove collagen and other components, leaving behind "intact" but undoubtedly "degraded" elastin. In these elastin preparations, a number of N-terminal amino acid residues have been found (Gotte et al., 1963; Serafini-Fracassini and Smith, 1974), demonstrating the probable hydrolysis of peptide bonds in elastin. Richmond (1974) also noted that elastin preparations obtained after hot alkali treatment are altered, as evidenced by a decrease in the level of the desmosine types of cross-links, when compared with milder, albeit not yet entirely satisfactory methods of extraction.

Elastin is a vital constituent of lung and aorta. In lung it participates with surfactant in the maintenance of elastic recoil and in the stabilization of the alveolar volume (Johanson and Pierce, 1973; Wright, 1961; Snider et al., 1974). In addition, the continuity of elastin fibers, as demonstrated histologically (Oderr, 1964; Pierce and Ebert, 1965; Pump, 1974), helps to maintain the patency of small airways and blood vessels during all phases of the respiratory cycle. A loss of elastin integrity, as may occur in emphysema, will result in an increase in the size of the air spaces distal to the terminal bronchioles due to dilatation and destruction of the alveolar walls. As examples, individuals with homozygous α_1-antitrypsin deficiency show a high incidence of emphysema, a probable result of the diminution of plasma antielastase activity (Erikson, 1964; Pierce et al., 1969). Johanson and Pierce (1972) induced emphysema in laboratory animals by the introduction of papain or elastase

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