Affinity Chromatographic Purification of Angiotensin Converting Enzyme†

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ABSTRACT: The compounds *N*-[1(*S*)-carboxy-5-aminopentyl]-L-phenylalanylglycine and *N*-[1(*S*)-carboxy-5-aminopentyl]-DL-alanyl-L-proline were synthesized and explored as potential ligands for the affinity chromatography of angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE), a membrane-bound zinc metalloprotease. The N-alkylated Ala-Pro derivative has an apparent $K_i < 1$ nM (at pH 7.5, 0.50 M NaCl) while the Phe-Gly derivative is a much less potent competitive inhibitor with an apparent $K_i = 0.20$ μM under the same conditions and thus more suitable for use as an affinity ligand. Immobilization of these compounds via a 28-Å spacer to agarose yields resins with binding capacities of >7 mg of enzyme/mL of resin, while spacers of 22 Å or less result in binding capacities at least 350 times smaller. Immobilized *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly is superior to the Ala-Pro derivative because elution can be affected by raising the pH to 8.9 with 98% yields compared with only 20% from the latter. Thus, a three-step process involving detergent extraction, concentration by ammonium sulfate precipitation, and affinity chromatography on the resin-immobilized Phe-Gly derivative provides 30 mg of homogeneous ACE from 640 g of rabbit lung tissue. An ACE-like metalloprotease has also been isolated from testicular tissue by this same technique.

Angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE),[1] a zinc metalloprotease, catalyzes the hydrolysis of the penultimate peptide bond at the carboxyl terminal of many peptides, but its best known physiological substrates are the vasoactive peptides angiotensin I (Skeggs et al., 1954) and bradykinin (Yang et al., 1970). The recognition of the role of this enzyme in the regulation of blood pressure has stimulated interest in the use of ACE inhibitors as antihypertensive agents (Cushman & Ondetti, 1980; Patchett et al., 1980), many of which have proved to be effective in clinical trials.

A substantial body of mostly kinetic information concerning this enzyme has been reported, largely focusing on drug design. Few detailed physicochemical examinations of the metal, substrate, and anion binding sites of the enzyme by spectroscopic and other incisive techniques have been made. Such studies typically require more material (20–100 mg) than has generally been available from isolation procedures employed thus far or from affinity chromatographic techniques introduced recently (Harris et al., 1981; El-Dorry et al., 1982). While the latter have yielded highly purified enzyme, they have not proved to be satisfactory on a preparative scale. Without exception, the affinity methods reported to date require 1 L or more of resin to purify 20 mg of enzyme. We here describe the design and synthesis of a ligand and spacer combination that constitutes an effective ACE affinity resin with a binding capacity more than 350 times greater than any reported previously and that allows the rapid, convenient isolation of relatively large amounts of homogeneous enzyme.

Materials and Methods

Frozen lungs from young rabbits (8–12 weeks) of mixed sex and breed (95% albino New Zealand and Californian) were obtained from Pel-Freez Biologicals, Inc. (Rogers, AR) and stored at −70 °C. Hepes (Calbiochem-Behring, La Jolla, CA) and NaCl solutions used in assays were made metal free by extraction with 0.01% dithizone in carbon tetrachloride (Thiers, 1957). Pyridine was distilled over BaO, and all other chemicals were of reagent grade and used without further purification.

All assays and absorption measurements were performed on a Cary 219 spectrophotometer equipped with thermostated cuvette holders. All pH determinations were made with a Chemtrix type 60A meter equipped with a Radiometer GK-2321C glass–calomel combination electrode. Amino acid analyses were obtained on a Durrum D-500 instrument with ninhydrin or *o*-phthalaldehyde as detecting reagent. Na-DodSO$_4$–polyacrylamide gel electrophoresis was performed according to Weber & Osborn (1969) and calibrated with molecular weight markers (BRL Inc., Bethesda, MD).

Enzyme Assays. Routine ACE activity measurements for monitoring various purification steps were made spectrophotometrically at 334 nm by using 100 μM Fa-Phe-Gly-Gly buffered at pH 7.5 with 50 mM Hepes at 25 °C and containing 0.3 M NaCl and 1.0 μM zinc acetate, as previously described (Holmquist et al., 1979). One unit of activity produces a ΔA_{334}/min of 1.0. ACE concentrations were estimated by $A_{280}^{0.1\%} = 1.58$ (Bünning et al., 1983).

Organic Syntheses. The synthetic and purification schemes for all inhibitors and spacer groups are given in the supplementary material (see paragraph at end of paper regarding supplementary material).

Preparation of Affinity Resins. Sepharose–14–CA-L-Phe-Gly and Sepharose–14–CA-Ala-Pro were prepared by reacting 4 mL of epoxy-activated Sepharose 6B (Pharmacia, Piscataway, NJ) with 8 mL of a 23 mM solution of either CA-L-Phe-Gly or CA-Ala-Pro in 0.1 M NaOH at room tem-

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[1] Abbreviations: ACE, angiotensin converting enzyme; CA-L-Phe-Gly, *N*-[1(*S*)-carboxy-5-aminopentyl]-L-phenylalanylglycine; CA-Ala-Pro, *N*-[1(*S*)-carboxy-5-aminopentyl]-DL-alanyl-L-proline; Affi-Gel 10–CA-Ala-Pro, affinity resin containing CA-Ala-Pro immobilized on Affi-Gel 10 (Bio-Rad); Sepharose–*X*–CA-Ala-Pro and Sepharose–*X*–CA-L-Phe-Gly, affinity resins containing immobilized CA-Ala-Pro and CA-L-Phe-Gly, respectively, but derived from epoxy-activated Sepharose 6B where *X* is the length of the spacer group in angstroms; EDTA, ethylenediaminetetraacetic acid; Fa, *N*-(2-furanacryloyl); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; NaDodSO$_4$, sodium dodecyl sulfate; Z, benzyloxycarbonyl.

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perature with mixing in a mechanical rotator for 16 h (Sundberg & Porath, 1974). Unreacted epoxide groups were then blocked by making the suspension 1 M in ethanolamine and rotating it for another 4 h. The resulting resin was washed with copious amounts of 0.1 M sodium acetate, pH 3.8, and 0.5 M NaCl and of 0.1 M sodium borate, pH 8.7, and 0.5 M NaCl in alternating cycles.

Similarly, 6-[*N*-(*p*-aminobenzoyl)amino]caproic acid (30 mL of a 90 mM solution) was coupled to 15 mL of epoxy-activated Sepharose 6B. The resulting resin was blocked and washed as described above, activated to the *N*-hydroxy-succinimide ester (Cuatrecasas & Parikh, 1972), reacted with a 10 mM solution of either CA-L-Phe-Gly or CA-Ala-Pro in 0.1 M Na_2CO_3, pH 10.0 at 4 °C for 16 h, and again washed as described above. Resins prepared in this way were labeled Sepharose–28–CA-L-Phe-Gly and Sepharose–28–CA-Ala-Pro. Sepharose–22–CA-Ala-Pro was prepared in the same way except that 6-aminocaproic acid was used as the spacer group instead of 6-[*N*-(*p*-aminobenzoyl)amino]caproic acid. Finally, Affi-Gel 10–CA-Ala-Pro was prepared by direct reaction of a 10 mM solution of CA-Ala-Pro to Affi-Gel 10 (Bio-Rad, Richmond, CA) in 0.1 M Na_2CO_3, pH 10, at 4 °C overnight. The efficiency of the various coupling reactions was determined by amino acid analysis following hydrolysis of 0.5-mL samples of resin in 6 N HCl for 20 h at 110 °C. Glycine, proline, and 6-aminocaproic acid were measured. Resins were stored at 4 °C in 50 mM sodium borate containing 0.03% sodium azide.

Solubilization of ACE from Lung Tissue (Step 1). Ten pairs (103 g) of young rabbit lungs were homogenized in a Waring Blendor with 110 mL of 5 mM Tris-HCl, pH 7.4, for 3–4 min at medium speed and then at high speed for 30 s. The homogenate was kept on ice while the procedure was repeated 6 more times, until 60 pairs of lungs (640 g) were processed.

The combined crude homogenates were centrifuged at 20000*g* for 30 min. The supernatant was discarded, and the pellet was resuspended in an equal volume of cold 5 mM Tris-HCl buffer, pH 7.4, and again centrifuged in the same manner. After the supernatant[2] was discarded, the pellet was suspended in 3.9 L of cold 50 mM Tris-HCl, pH 8.5, containing 1.0% (v/v) Nonidet-P40 (BRL Inc.). This suspension was sonicated, 500 mL at a time, by using six pulses (15 s each) of a 350-W sonifier (Branson Model 350) equipped with a $^3/_4$-in. horn and set at power setting 5 (Lazo & Quinn, 1980). The sonicated membrane fraction was then centrifuged at 50000*g* for 75 min.

Ammonium Sulfate Fractionation of Detergent-Solubilized ACE (Step 2). The solubilized membrane fraction supernatant was cooled in an ice bath, and solid ammonium sulfate was added over 30 min to give a concentration of 18% (w/v). After being stirred for 1 h, the mixture was centrifuged at 20000*g* for 30 min, and the resultant floating "pellet" was removed by filtration through cheesecloth. The solution was adjusted to 34% (w/v) ammonium sulfate as above, stirred for 1 h, and centrifuged as before. The supernatant was discarded, and the pellet was redissolved in 400 mL of 20 mM Mes, pH 6.0, 0.5 M NaCl, and 0.1 mM zinc acetate and dialyzed against the same buffer but containing 0.1% (v/v) Nonidet-P40. The enzyme was concentrated to 110 mL in an ultrafiltration cell (Amicon Model 2000) equipped with an XM-50 membrane (Diaflo) and clarified by passage through a 8.0-μm filter (Millex HA, Millipore). The resulting solution was then ready for affinity chromatography.[3]

Table I: pH Dependence of CA-L-Phe-Gly Inhibition of ACE[a]

pH	buffer	apparent K_i (μM)
6.0	20 mM Mes[b]	0.035
7.5	50 mM Hepes	0.200
8.9	50 mM Tris-HCl	35.0

[a] Inhibition was measured in 50 μM Fa-Phe-Gly-Gly and 0.5 M NaCl. [b] This buffer also contained 0.10 mM zinc acetate.

Solubilization of ACE from Testicular Tissue. Testes from mature rabbits (1–3 years, mixed breed) were obtained from Pel-Freez and stored at −70 °C. This tissue was homogenized, solubilized, and concentrated with ammonium sulfate in the same way as described above for lung tissue, except that centrifugation of the crude homogenate was at 50000*g* for 75 min instead of 20000*g* for 20 min.

Results

Inhibition of Rabbit Pulmonary ACE by Candidate Affinity Ligands. The compounds *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly (CA-Phe-Gly) and *N*-[1(*S*)-carboxy-5-aminopentyl)-DL-Ala-L-Pro (CA-Ala-Pro) were synthesized and tested as potential ligands for the affinity chromatography of ACE. These compounds are analogues of the recently described, pharmacologically active ACE inhibitor *N*-[1-(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Pro (MK 422) (Patchett et al., 1980). Unlike the latter, the former two compounds bear a primary amino group that serves as a point of covalent attachment to insoluble matrices. The synthetic procedures employed for the preparation of CA-Phe-Gly and CA-Ala-Pro, namely, reductive amination of β-(phenylpyruvoyl)glycine and pyruvoylproline, respectively, by N^{ϵ}-benzyloxycarbonyl-L-lysine methyl ester, as outlined by Patchett et al. (1980), result in the formation of two sets of diastereomers (see supplementary material). Ion-exchange chromatography of CA-DL-Phe-Gly provided the pure isomers, CA-L-Phe-Gly and CA-D-Phe-Gly, which exhibit identical amino acid composition and UV absorption spectra but differ markedly in their inhibitory capacity toward ACE. The more effective inhibitor has been assigned to the L,L (*S*,*S*) configuration (CA-L-Phe-Gly) on the basis of the known preference of this enzyme for inhibitors (Cushman et al., 1977; Patchett et al., 1980) and substrates (Rohrbach et al., 1981) containing amino acids of the L(*S*) configuration at the penultimate position. CA-L-Phe-Gly and CA-Ala-Pro were evaluated kinetically by determining their inhibitory effects in solution on enzyme that had been purified from rabbit lungs as previously described (Bünning et al., 1983). Lineweaver–Burk plots at various concentrations of CA-L-Phe-Gly with the substrate Fa-Phe-Gly-Gly at pH 7.5, 50 mM Hepes, and 0.30 M NaCl are linear and indicate competitive inhibition, but Dixon plots of the same data are nonlinear. For this reason, only apparent K_i values at 50 μM substrate (6-fold below K_m) are reported.[4] CA-L-Phe-Gly has an apparent K_i of 0.20 μM under these conditions. This in-

[2] The supernatants from centrifugation of the crude homogenate and subsequent washing of the membrane–organelle fraction routinely contain only about 10% of the total ACE activity released on solubilization of membranes with detergents.

[3] The combination of ammonium sulfate fractionation, dialysis, and Millipore filtering causes a 49% loss of the original ACE activity (see step 2 of Table III). The ammonium sulfate fractionation accounts for half of this loss and is a function of the type, concentration, and volume of detergent used for solubilization. Smaller losses were obtained with 30 mM octyl glucoside, but its high cost made it impractical for large-scale isolations.

[4] An apparent K_i was estimated from Dixon plots obtained under conditions where the substrate was 6-fold below K_m so that K_i and IC_{50} are nearly equal.

Table II: Effect of Affinity Ligand Spacer Length on Resin Binding Capacity

spacer group-ligand	spacer length (Å)[a]	amount of immobilized ligand (μmol/mL of resin)[b]	binding capacity (mg/mL of resin)[c]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2$-CA-L-Phe-Gly	14	3.5	0.02[d]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2NHC_6H_4CONH(CH_2)_5CO$-CA-L-Phe-Gly	28	2.5	>7.0[d]
$-OCH_2CONH(CH_2)_2NHCO(CH_2)_2CO$-CA-Ala-Pro	10	0.3	<0.01[e]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2$-CA-Ala-Pro	14	ND	<0.02[f]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2NH(CH_2)_5CO$-CA-Ala-Pro	22	3.3	<0.02[f]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2NHC_6H_4CONH(CH_2)_5CO$-CA-Ala-Pro	28	2.5	>7.0[g]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2NHC_6H_4CONH(CH_2)_5COO^-$	28	none	<0.001[g]

[a] Lengths were calculated by using the extended conformation of space-filling models. An additional 14 Å must be added to obtain the overall length (spacer + ligand) of the ligand chain. [b] Measured by amino acid analysis after hydrolysis (ND, not determined). [c] Binding capacity is defined as the amount of enzyme removed from solution after it reaches equilibrium with a given volume of affinity resin (normalized per milliliter of resin). For the resins containing immobilized CA-L-Phe-Gly, this process is completely reversible. It is only partially reversible, however, for resins containing immobilized CA-Ala-Pro. [d] pH 6.0 (Mes), 0.5 M NaCl, 0.1 mM $ZnCl_2$. [e] pH 7.0 (Hepes), 0.1 M NaCl. [f] pH 6.5 (Mes), 0.1 M NaCl, 0.1 mM $ZnCl_2$. [g] pH 7.0 (Hepes), 0.5 M NaCl.

Table III: Affinity Purification of Pulmonary ACE on Sepharose-28-CA-L-Phe-Gly

step	vol (mL)	total units	protein (mg)[a]	sp act. (units/mg)	purification (x-fold)	yield (%)	time (days)
(1) detergent solubilization[b]	4050	6480	28700	0.23	1	100	1
(2) ammonium sulfate fractionation	111	3230	5770	0.56	2.4	50	3
(3) affinity chromatography[c]	1.6	2190	26.8	82	356	34	2

[a] Protein was determined by $A_{280}^{0.1\%} = 1.58$. [b] See text for details of individual steps. [c] Affinity chromatography was performed as described for Figure 1. Eluted ACE was pooled and concentrated with a collodion bag apparatus (Schleicher & Schuell, Keene, NH).

hibition is strongly pH dependent with the apparent K_i increasing 1000-fold as the pH is raised from 6.0 to 8.9 (Table I). The moderately strong inhibition near neutral pH and the drastically reduced affinity near pH 9.0 indicate the potential of CA-L-Phe-Gly as a ligand for affinity chromatography.

CA-Ala-Pro is a much more potent inhibitor of ACE than CA-L-Phe-Gly. Its apparent K_i at pH 7.5 was estimated to be less than 1 nM, in agreement with the value reported by Patchett et al. (1980). This is an upper limit in part owing to the marked time dependence of the inhibition of ACE by CA-Ala-Pro.

Immobilization of Affinity Ligands. Affinity resins were prepared by covalently attaching CA-L-Phe-Gly and CA-Ala-Pro to agarose via spacers of varying lengths (Table II). Resins with the shortest spacers (10–14 Å) were prepared by direct coupling of the inhibitors to epoxy-activated Sepharose 6B or Affi-Gel 10 to give Sepharose–14–CA-L-Phe-Gly, Sepharose–14–CA-Ala-Pro, and Affi-Gel 10–CA-Ala-Pro. Resins with longer spacers were prepared by first coupling either 6-aminocaproic acid or 6-[*N*-(*p*-aminobenzoyl)-amino]caproic acid to epoxy-activated Sepharose 6B and then coupling the desired CA dipeptide to give Sepharose–22–CA-Ala-Pro, Sepharose–28–CA-L-Phe-Gly, or Sepharose–28–CA-Ala-Pro. The amount of ligand covalently bound to resin varied from 0.3 to 3.5 μmol/mL (Table II).

Effect of Spacer Length on Binding Capacity. The binding capacities of Sepharose–14–CA-L-Phe-Gly and Sepharose–28–CA-L-Phe-Gly were estimated from the amount of ACE activity eluted from 5 mL of each of these resins at pH 8.9 after it was saturated or nearly saturated with enzyme at pH 6.0. Saturation is reached when the activity (units/mL) of the effluent is equal to that of the sample applied. Doubling the spacer length from 14 to 28 Å increases the binding capacity (mg of enzyme/mL of resin) by more than 350-fold (Table II). The binding capacity of Sepharose–28–CA-L-Phe-Gly is >7.0 mg/mL of resin.

The binding capacities of resins containing immobilized CA-Ala-Pro could not be estimated in the same way due to the conditions required to elute ACE from these resins (see below). Instead, they were estimated from the amount of activity removed from solution when an enzyme preparation was equilibrated with a given volume of resin in either a column or batch experiment. The CA-Ala-Pro resins with the shorter spacer arms, Sepharose–14–CA-Ala-Pro, Affi-Gel 10–Ala-Pro, and Sepharose–22–CA-Ala-Pro, bind negligible amounts of ACE activity. Increasing the spacer length to 28 Å, however, again dramatically increases the binding capacity (Table II), entirely consistent with the results obtained with immobilized CA-L-Phe-Gly. Importantly, the 28-Å spacer arm itself does not exhibit any binding capacity in the absence of an attached ligand (Table II).

Affinity Chromatography of Pulmonary ACE on Immobilized CA-L-Phe-Gly. A crude enzyme preparation from step 2 containing 3330 units of activity in 110 mL was applied at room temperature to a 0.9 × 8.1 cm (5.2-mL) column of Sepharose–28–CA-L-Phe-Gly, previously equilibrated with 20 mM Mes buffer, pH 6.0, containing 0.5 M NaCl, 0.10 mM zinc acetate, and 0.1% Nonidet-P40 at a flow rate of 10 mL/h. More than 99.4% of the applied protein (A_{280}) passed through the column or eluted on subsequent washing with starting buffer, while only 30% of the initial ACE activity could be detected in this breakthrough (Figure 1). After being washed with 12 column volumes (62 mL) of the starting buffer containing 0.1% Nonidet-P40, the column was washed with 24 volumes (125 mL) of the same buffer but without detergent. A subsequent buffer change to 50 mM sodium borate, pH 8.9, caused the elution of bound ACE. Approximately 70% of the starting enzyme activity and virtually all of the bound activity were eluted under these conditions with a 148-fold (this step) increase in specific activity. The purification scheme is summarized in Table III. Similar results are obtained when the sample is applied to the column at pH 7.0 in 20 mM Hepes, 0.5 M NaCl, and 0.1% Nonidet-P40 (v/v).

Affinity Chromatography of Pulmonary ACE on Immobilized CA-Ala-Pro. When crude preparations from (step 2) solubilized lung tissue are applied to a column (0.9 × 10.8 cm) of immobilized CA-Ala-Pro (Sepharose–28–CA-Ala-Pro)

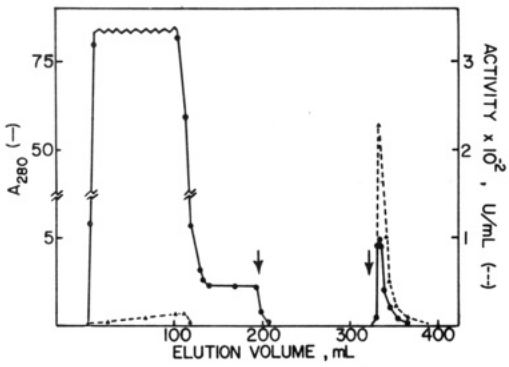

FIGURE 1: Affinity chromatography of solubilized pulmonary ACE on immobilized CA-L-Phe-Gly. A crude extract of ACE, previously precipitated with ammonium sulfate, was equilibrated with the starting buffer (20 mM Mes, pH 6.0, 0.50 M NaCl, 0.10 mM zinc acetate, 0.1% Nonidet-P40), passed through a 0.80-μm filter, and applied to a 0.9 × 8.1 cm column of Sepharose–28–CA-L-Phe-Gly similarly equilibrated. The flow rate was 10 mL/h, and the temperature was 23 °C. The column was washed with 12 column volumes of starting buffer, followed by 24 column volumes of the same buffer but without detergent (first arrow). Elution was initiated with 50 mM sodium borate, pH 8.9 (second arrow). The A_{280} that remains in fractions collected after the column was washed with starting buffer is due to the 0.1% Nonidet.

under conditions similar to those described above (20 mM Mes, pH 6.0, 0.1 M NaCl, 250 μM $ZnCl_2$, 23 °C, flow rate 4.4 mL/h), 23% of the initial ACE activity is detected in the breakthrough peak.[5] A buffer change to 0.1 M sodium borate, pH 9, results in the elution of additional ACE activity, but only 20% of the bound activity can be recovered by this means. The poor yield is not due to denaturation or inhibition of ACE because the eluted enzyme has a specific activity > 70 units/mg. Another 20% of the expected amount of protein, as measured by A_{280}, could be desorbed by elution with a 16 mM solution of the free inhibitor, CA-Ala-Pro, and even more protein (presumably inactive ACE) could be removed from the resin by a further washing with 1 M NH_4OH. At least twice as much protein as that eluted with pH 9 buffer is recovered by this procedure on the basis of the density of stained protein after gel electrophoresis of the various eluted fractions.

Criteria of Enzyme Purity. A 3-μg sample of enzyme affinity purified on Sepharose–28–CA-Phe-Gly was electrophoresed on a 10% polyacrylamide slab gel in the presence of 1% $NaDodSO_4$ and 2-mercaptoethanol and found to migrate as one major band of M_r 130 000 (Figure 2, lane B). Gels loaded with >10 μg of sample contained additional faint bands of M_r 100 000 and >200 000 (not shown).

N-Terminal amino acid analysis of 12 nmol of the affinity purified enzyme by Edman degradation (Edman & Begg, 1967) revealed only threonine, providing additional evidence for virtual homogeneity. The affinity-purified enzyme has a k_{cat}/K_m of 70.1 × 10^6 M^{-1} min^{-1} for hydrolysis of Fa-Phe-Gly-Gly at pH 7.5, 0.3 M NaCl, 1.0 μM $ZnCl_2$, and 25 °C. Its activity toward hippuryl-His-Leu, under the conditions suggested by Cushman & Cheung (1971), is 41.2 units/mg on the basis of an ϵ_{280} = 204 000 M^{-1} cm^{-1}.

Affinity Chromatography of ACE from Rabbit Testes. The same procedure as outlined in Table III has been successfully used for the purification of a dipeptidyl carboxy-

[5] If this experiment is performed at 4 °C, more than 90% of the ACE activity emerges just after the breakthrough. This is believed to be a kinetic problem and is probably related to the slow onset of inhibition observed for the free inhibitor.

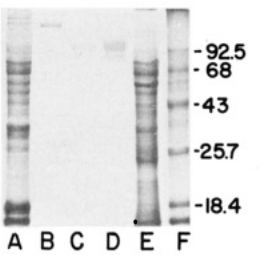

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of pulmonary and testicular ACE on a 10% acrylamide slab gel: (lane A) pulmonary ACE preparation immediately prior to affinity chromatography; (lane B) 3 μg of pulmonary ACE purified by affinity chromatography as shown in Figure 1; (lane C) testicular ACE purified by affinity chromatography (eluted with 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl; (lane D) testicular ACE eluted from the affinity resin at pH 8.9 with 50 mM sodium borate; (lane E) crude testicular ACE prior to affinity chromatography; (lane F) standard molecular weight markers from BRL, Inc.

peptidase from mature rabbit testes. Detergent-solubilized material was concentrated with ammonium sulfate, equilibrated with 20 mM Mes, pH 6.0, 0.5 M NaCl, and 0.1 mM zinc acetate, and applied to a 0.9 × 11.0 cm column of Sepharose–28–CA-L-Phe-Gly, equilibrated with the same buffer. Adsorbed enzyme was eluted by a buffer change to 20 mM Tris-HCl, pH 7.5–0.5 M NaCl. It had a specific activity of 142 units/mg (toward Fa-Phe-Gly-Gly) at a protein concentration based on $A_{280}^{0.1\%}$ = 1.58 and gave one major band at M_r 96 000 and a minor band at M_r = 85 000 on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2). Thus, a single passage through the affinity resin gave a 750-fold purification from the crude membrane extract.

Under these elution conditions, there is no evidence of contamination by the higher molecular weight form of ACE (130 000) that predominates in lung tissue. However, if elution is effected with a buffer change to 50 mM sodium borate, pH 8.9, as was used for the pulmonary ACE preparations, a 130 000-dalton species can be detected coeluting with the 96 000-dalton material as shown in lane D of Figure 2. In addition, a third band at M_r 85 000 is also eluted. The binding capacity of Sepharose–28–CA-L-Phe-Gly for the testicular variant of ACE was found to be comparable to that for the pulmonary enzyme (Table II) when loaded at pH 6.0 in 0.5 M NaCl–0.1 mM zinc acetate.

Lifetime of Affinity Resins. One 5-mL column of Sepharose–28–CA-L-Phe-Gly, the most effective of the affinity resins prepared, has been used nearly 20 times for the large-scale (>10 mg each) purification of rabbit lung ACE over the course of 9 months. In most cases, the specific activity of the eluted enzyme has been >70 units/mg, the ratio of the activity in the eluted peak to that in the breakthrough has been 2:1, and the recovery of activity has been >98%. In addition, the binding capacity does not appear to diminish with use.

Discussion

ACE plays a major physiological role in blood pressure regulation and is subject to control by specific inhibitors such as captopril (SQ 14,225) and enalapril (MK 421). This has stimulated considerable interest in its kinetic, mechanistic, and physiological attributes. However, the low tissue concentration of ACE together with the long and tedious isolation procedures available have limited the types of investigations largely to kinetics, since physical and structural studies can require substantial quantities of material. Several attempts have been made to overcome this problem by employing affinity chromatography for preparative isolation of ACE, but they have met with only limited success. Invariably, past affinity resins

have had extremely low binding capacities. Harris et al. (1981) coupled D-Cys-L-Pro to Sepharose via a six carbon (8-Å) spacer to produce an affinity resin for ACE with a binding capacity of 0.02–0.03 mg/mL resin. More recently, Soffer and co-workers (El-Dorry et al., 1982) described an affinity resin containing the very potent inhibitor N^{α}-1(*S*)-carboxy-3-phenylpropyl]-L-Lys-L-Pro attached to Sepharose through the ϵ-amino group of the lysyl residue via a 14-Å spacer. The binding capacity of this resin for both pulmonary and testicular ACE was only 0.04 mg/mL, and no more than 25% of the bound enzyme could be desorbed even when elution was attempted by competition with the free inhibitor (225 000-fold above IC_{50}). Moreover, subsequent removal of the inhibitor from the eluted enzyme required the formation of apoenzyme by dialysis against EDTA. Immobilized anti-ACE antibodies have been employed for immunoadsorbent chromatography (Das et al., 1977; Lanzillo et al., 1980), but again, the low capacities and denaturing elution conditions needed for this method limit its preparative utility.

We have developed a very effective affinity chromagraphic system for purifying ACE on the basis of an *N*-(carboxyalkyl) dipeptide inhibitor of the enzyme as the affinity ligand. One such compound, CA-Ala-Pro, was among those first prepared by Patchett et al. (1980) and found to be an extremely potent inhibitor with an IC_{50} = 2.2 nM at pH 8.0, in 0.17 M NaCl. We have estimated its K_i to be less than 1 nM at pH 7.5 in 0.50 M NaCl. The other inhibitor used in this study, CA-L-Phe-Gly, is more than 200 times less potent than the Ala-Pro derivative and thus more suitable for use as an affinity ligand. In addition, its apparent K_i of 0.20 μM in solution at pH 7.5 in 0.30 M NaCl increases 175-fold to 35 μM (50 mM Tris-HCl, 0.50 M NaCl) on raising the pH to 8.9. This degree of inhibition and its pH dependence are strikingly similar to that reported for the inhibition of carboxypeptidase A by benzyl-L-succinate (Byers & Wolfenden, 1973), an inhibitor that has been found very useful for the affinity chromatography of a number of carboxypeptidases (Peterson et al., 1976, 1982; Bazzone et al., 1979; Peterson & Holmquist, 1983).

These results suggested that CA dipeptides, and especially CA-L-Phe-Gly, might be effective ligands for affinity chromatography of ACE. Accordingly, CA-L-Phe-Gly and CA-Ala-Pro were covalently linked to agarose via four different spacers of varying lengths (10–28 Å), resulting in six different resins (Table II). Two of these, Sepharose–28–CA-L-Phe-Gly and Sepharose–28–CA-Ala-Pro, which contained the longest spacers (28 Å), were found to bind pulmonary ACE in amounts (>7.0 mg/mL of resin) comparable to the best affinity resins for the carboxypeptidases (Peterson et al., 1976; Bazzone et al., 1979) and other zinc metalloenzymes such as the alcohol dehydrogenases (Lange & Vallee, 1976) and neutral endoproteases (Holmquist, 1977; Nishino & Powers, 1979).

The results summarized in Table II indicate that the binding capacity of affinity resins for ACE is strongly dependent on the distance between the support matrix (agarose) and the immobilized ligand. Lange & Vallee (1976) found that a distance of 6 or 10 Å between matrix and ligand results in very low binding capacities for alcohol dehydrogenases but a further increase of 6 Å raises it 10-fold to 5 mg/mL of resin. Similar results have been observed for other systems (Lowe & Dean, 1974).

Steric hindrance is a commonly held rationale for the need of spacer-arm extensions (Cooper, 1977; Lowe & Dean, 1974). Thus, an elongated spacer would obviously be required if the active site of ACE was recessed deep within the interior of the enzyme molecule rather than at its surface. In the case of carboxypeptidase A, effective affinity resins have been prepared with the ligand only minimally displaced from the matrix (Cuatrecasas et al., 1968; Bazzone et al., 1979), but the active site of this enzyme is situated close to the surface of the protein (Lipscomb et al., 1966). In contrast, the catalytic zinc atom of alcohol dehydrogenase is near the center of the molecule 25 Å from the protein surface (Bränden et al., 1975), consistent with the requirement that the spacer group project the affinity ligand at least 16 Å from the support matrix (Lange & Vallee, 1976). The molecular weight of ACE is more than 3 times that of the subunit of alcohol dehydrogenase. By analogy, this could imply an active site even farther from the surface of the molecule, and in this case, an even longer spacer would be needed. A deeply recessed active site might actually contribute to the specificity of ACE by restricting access and thereby limiting activity to small peptides such as angiotensin I and bradykinin rather than serum proteins. This supposition, rarely considered in studies of substrate specificity, warrants further investigation.

The enzyme isolated with Sepharose–28–CA-L-Phe-Gly is of the highest purity; i.e., it gives a single major band on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2) and a single N-terminal amino acid by Edman degradation. Moreover, its activity toward Fa-Phe-Gly-Gly is at least 10% higher than that observed for ACE purified by procedures hitherto available. Its specific activity toward hippuryl-His-Leu is 41 units/mg, comparable to the 39 units/mg of the enzyme of Harris et al. (1981) that was isolated by their affinity chromatographic procedure and subsequently purified on Procion blue–Sepharose.

The Sepharose–28–CA-L-Phe-Gly affinity resin can be used to isolate ACE-like metalloproteases from other tissues. Chromatography of solubilized membrane concentrates from mature rabbit testes yields a 96 000-dalton variant of ACE with a specific activity of 142 units/mg (Fa-Phe-Gly-Gly) that could be resolved from a 130 000-dalton species (presumably identical with the enzyme in lung tissue) by elution at a lower pH. It is apparent that the 96 000-dalton enzyme predominates in mature rabbit testes and has been reported to be derived from a gene product different from that for the pulmonary enzyme (El-Dorry et al., 1982). The differential elution of these two variants from Sepharose–28–CA-L-Phe-Gly suggests that they differ in more than just physical size. A third band of M_r 85 000 was also observed as a minor component when elution was effected at pH 8.9.

We expect that this affinity procedure will prove useful in the isolation and resolution of ACE-like enzymes from yet other sources. In addition to its utility for the rapid large-scale isolation of enzyme, Sepharose–28–CA-L-Phe-Gly has been found to be a useful tool for resolving the various derivatives that are formed during chemical modification of groups at the active site of ACE critical to function (Shapiro & Riordan, 1983).

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Supplementary Material Available

Experimental details for the synthesis and purification of inhibitors and spacer groups used in this paper (6 pages).

Ordering information is given on any current masthead page.

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