Kidney International, Vol. 33 (1988), pp. 652–655

Partial protein sequence of mouse and bovine kidney angiotensin converting enzyme

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Partial protein sequence of mouse and bovine kidney angiotensin converting enzyme. Angiotensin converting enzyme (ACE) plays an important role in the regulation of renal blood pressure by the hydrolysis of the inactive precursor peptide angiotensin I to the potent vasopressor angiotensin II. Renal ACE is a surface membrane protein of both endothelium and tubular epithelium. Enzymatically active ACE was isolated from renal homogenates by chromatography using an affinity column constructed by linking an ACE inhibitor, lisinopril, to Affi-Gel 15. Analysis of eluates from this column showed that ACE activity was increased greater than 500-fold. SDS-polyacrylamide gel electrophoresis demonstrated a single band of molecular weight 144 kD (mouse) and 149 kD (bovine). N-terminal amino acid sequence analysis revealed:

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          1          10          20
          |          |           |
Mouse     LDPGLQPGNFSPDEAGAQLF
Bovine  ELDPALQPGNFPADEAGAQIFA
          |          |           |
          1          10          20
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Though bovine ACE has one additional N-terminal amino acid, these two partial sequences are highly homologous (16 of 20 positions are identical). Mouse ACE was digested with trypsin and the peptides were isolated by reverse phase HPLC. Analysis of the amino acid sequences showed that these tryptic peptides were unique to ACE. Thus, we were able to isolate ACE from bovine and mouse kidneys and show that they had substantial structural homology. They were also quite similar to that from rabbit lung.

Angiotensin converting enzyme (ACE) (EC 3.4.15.1) is a protease that catalyzes the hydrolysis of dipeptides from the carboxyl terminus of polypeptides. In this way the enzyme degrades bradykinin, a potent vasodilator, and converts angiotensin I (Ang I) to the potent vasoconstrictor, angiotensin II (Ang II) [1–6]. Ang II markedly influences kidney hemodynamics and thus ACE, by converting the non-vasoactive Ang I to Ang II plays an important role in the control of hemodynamics within the kidney [7].

We are interested in studying the control of ACE expression on kidney endothelial and epithelial cell surfaces, and as an initial step towards that goal, report the isolation and partial sequence of bovine kidney cortex and mouse kidney ACE. Building on the work of Bull et al, Pantoliano et al, and Harris et al in the use of ACE inhibitors and other ACE substrate analogues in constructing affinity columns [8–10], we used the ACE inhibitor lisinopril linked to Affi-Gel 15 to isolate bovine kidney cortex and mouse kidney ACE. These proteins were found to have substantial N-terminal amino acid sequence homology.

Received for publication May 20, 1987
and in revised form October 15, 1987

Methods

Lisinopril (N^{α}-[(5)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline) used in this study was from Merck Sharp and Dohme Research Laboratories (Rahway, New Jersey, USA). Affi-Gel 15 and acrylamide were purchased from BioRad Lab (Richmond, California, USA). Proteins were analyzed using 8% SDS-polyacrylamide gels as described by Laemmli [11]. Silver staining of SDS-protein gels was performed by the method of Wray et al [12]. Protein concentration was measured using albumin as standard by the method of Bradford with Commassie Brilliant Blue G-250 dye concentrate purchased from BioRad [13]. Diethylaminoethyl DE 52 cellulose was purchased from Whatman. Other chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). All sequencer reagents were from Applied Biosystems, Foster City, California, USA.

ACE activity was measured by the hydrolysis of hippuryl-glycine-glycine (Boehringer-Mannheim #789011) as described by Neels, van Sande and Scharpe [14]. Here one unit is the amount of enzyme required to release 1 micromole of hippuric acid per minute per liter. ACE was also occasionally measured by the hydrolysis of ^{3}H-benzoyl-phenylalanine-alanine-proline (Ventrex) as described by Ryan et al [15].

One hundred milligrams (227 μM) of lisinopril was dissolved in 15 ml of reagent grade methanol. Twenty-two milliliter of Affi-Gel 15 was warmed to 4°C and washed rapidly with cold methanol. After draining, the gel was added to the lisinopril solution and mixed by constant inversion at room temperature overnight. Thirty-two microliters (229 μM) of ethanolamine was then added and mixed at room temperature for an additional 24 hours. This organic base was added to maintain the rate of lisinopril-Affi-Gel 15 coupling by keeping the primary amine of lisinopril in the non-protonated form. The reaction was stopped by adding an excess of glycine to bind to unoccupied sites on the agarose. No attempt was made to quantitate the coupling efficiency of lisinopril to Affi-Gel 15. The affinity resin was extensively washed with methanol, then water, then a solution

Table 1. Purification of mouse kidney ACE[a]

	Protein *mg/ml*	Enzyme *units/ml*	Specific activity *units/mg protein*	Yield[b] %	Purification[c] *fold*
Detergent extract of kidney	8.13	1,754	216	100	1
DEAE cellulose	7.65	4,412	576	32	2.7
Affinity chromatography	0.51	149,114	292,380	15	1354.7

[a] ACE activity was measured as described by Neels, van Sande and Sharpe [14]. Units are expressed as micromoles of hippuric acid generated per min per liter. Protein concentration was measured by the method of Bradford [13].

[b] Minimum yield values as portions of the detergent extract and the DEAE cellulose eluate were not further purified.

[c] Comparison of ACE activity to that of the starting detergent extract.

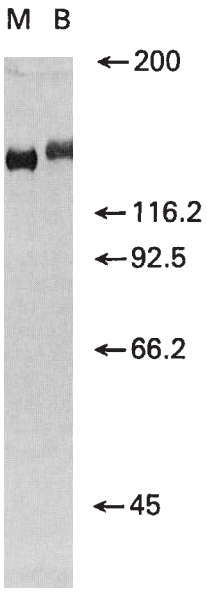


Fig. 1. *Purified mouse (M) and bovine (B) ACE.* Aliquots (1 μg) of purified mouse and bovine kidney ACE were separated on an 8% SDS-polyacrylamide gel as described by Laemmli [11]. The gel was fixed in 50% methanol containing 0.038% formaldehyde and silver stained as described by Wray et al [12]. The size in kilodaltons as determined by protein standards (Pharmacia) is indicated on the right.

of 20 mM MES (2-N-Morpholinoethanesulfonic Acid), pH 6.0, 0.5 M NaCl, 100 μM zinc acetate and 0.1% Triton X-100 and was loaded onto a column 1 × 20 cm.

Seventy grams of kidney from adult male NIH Swiss mice were crushed in liquid nitrogen. Subsequent manipulations until affinity chromatography were carried out at 4°C. The powdered kidneys were washed in 400 ml of 5 mM Tris, pH 7.75 and then 400 ml of 5 mM Tris, pH 7.75 with 0.5% of the detergent Triton X-100. This was stirred for one hour and then sonicated for three minutes with a Sonifier sonicator set at 70% burst and 100 watts. After centrifugation for 15 minutes at 20,000 x g, the liquid phase was dialyzed against 10 mM potassium phosphate buffer, pH 7.5 containing 0.025% Triton X-100 (Buffer A) overnight. Aliquots of the kidney extract were then loaded onto 2.5 × 40 cm DE-52 DEAE cellulose columns as described by Gonhagen-Roska and Fyhrquist [16]. The columns were washed with several column volumes of Buffer A and eluted with 0.2 M KCl in Buffer A. Those fractions containing ACE activity were pooled and dialyzed against 20 mM MES, pH 6.0, 0.5 M NaCl, 100 μM zinc acetate, and 0.1% Triton X-100 (Buffer B). The kidney extract was warmed to room temperature and passed over the lisinopril affinity column at a rate of approximately 40 ml/hour. The affinity column was washed with Buffer B and then Buffer B without Triton X-100 until the optical density at 280 nanometers was less than 0.01. The column was eluted with 1 ml aliquots of 50 mM sodium borate, pH 8.9 as described by Pantoliano, Holmquist and Riodan [9]. Those aliquots with ACE activity were pooled and concentrated with a Centricon 30 microconcentrator (Amicon, Lexington, Massachusetts, USA). Bovine kidney was purchased frozen from Pel-Freeze, thawed at 4°C, and the cortex removed. This tissue served as the basis for developing the above protocol. We have not investigated if the affinity column will isolate ACE without a preliminary DEAE ion exchange step.

Purified mouse kidney ACE (265 μg) was denatured by boiling for three minutes and digested for four hours at 37°C with 3.0 μg of trypsin (Sigma T-8642) in 45 mM Tris, pH 8.0. Half of this was loaded onto a Vydac C-4 HPLC column mounted in a Beckman 344 HPLC. Elution of the column was as follows: Solution A consisted of 0.12% trifluoroacetic acid in water, Solution B consisted of 0.1% trifluoroacetic acid in acetonitrile. The column was eluted with a linear gradient of 0 to 60% Solution B over a one hour period, with O.D. measurements at 225 nm. Peaks were collected manually, brought to dryness, and resuspended in 30% acetic acid prior to amino acid sequence analysis.

Amino terminal sequence determinations of the whole ACE protein and tryptic peptides were performed using an Applied Biosystems Model 470A gas-phase sequencer equipped with a Model 120 A on-line PTH analyzer. To determine N-terminal sequence of both bovine and mouse ACE, approximately 160 picomoles of protein were applied to the glass fiber filter (previously treated with bioprene plus) and the sequence determined using the standard PTH program (Applied Biosystems).

Results

Table 1 presents the result of the isolation of mouse kidney ACE. In this protocol the lisinopril affinity column resulted in a single pass purification exceeding 500-fold. To assess the purity and molecular weight of the recovered protein, 1 μg aliquots of bovine and mouse ACE were denatured and analyzed on an SDS-polyacrylamide gel (Fig. 1). A single protein of molecular weight 149 kDa from bovine kidney and 144 kDa from mouse kidney was identified. These molecular weights are very similar to the 139 to 140 kDa reported for rabbit lung ACE [4]. The isolated proteins enzymatically degraded two ACE synthetic substrates hippuryl-glycine-glycine and benzoyl-phenylalanine-alanine-proline and were inhibited by 1 μM lisinopril and 1 mM EDTA as has previously been described for ACE [17].

The amino acids in 16 of the 20 positions available for comparison between mouse and bovine ACE were identical (Fig. 2). In three of the four positions that differ, the hydrophobic nature of the side chains was conserved (glycine/alanine,

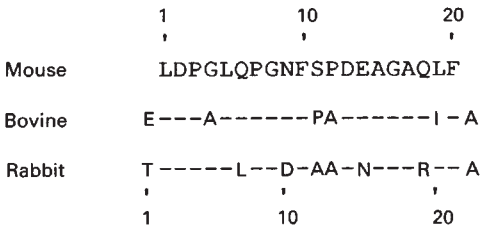
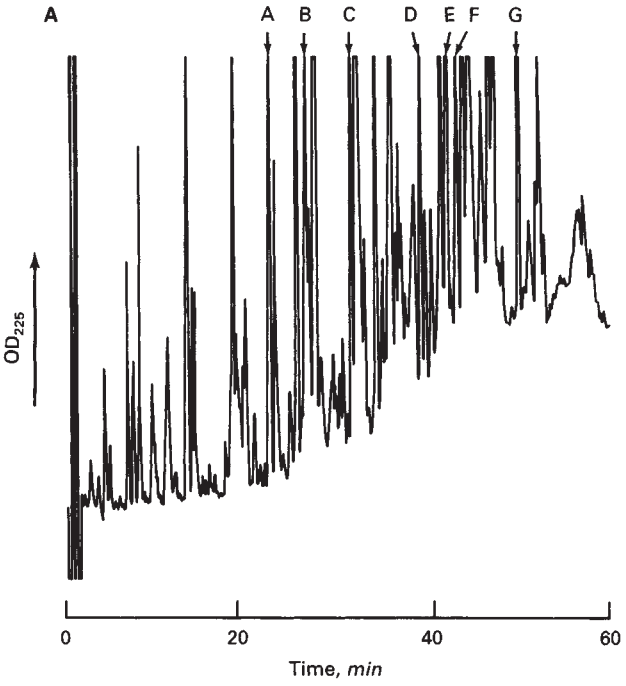


Fig. 2. *N-terminal amino acid sequence of mouse and bovine ACE.* The N-terminal amino acid sequence of mouse and bovine kidney ACE is compared to the published sequence of rabbit lung ACE [17]. The mouse sequence contains one less N-terminal amino acid than that of bovine or rabbit. Non-identical sequences are indicated. The one letter amino acid code is: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

proline/alanine, and leucine/isoleucine at mouse positions 4, 12, and 19) [18]. At position 11, the polar residue serine in the mouse was replaced with the nonpolar proline in bovine ACE. A comparison of the sequence of rabbit ACE showed them to be homologous though less conserved than a mouse-bovine comparison (rabbit ACE is identical to bovine ACE at 13 of 21 positions and to mouse ACE at 14 of 20 positions). A computer search of the Protein Identification Resource, National Biomedical Research Foundation's protein sequence bank showed that the sequences determined had no significant homologies with other reported protein sequences.

The pattern of elution of the tryptic fragments of mouse ACE are shown in Figure 3A. Figure 3B shows the partial amino acid sequence determined from these peptides. Computer analysis of the amino acid sequences of these fragments demonstrated no consistent homologies with other protein sequences including rabbit kidney neutral endopeptidase [19].



B

Peak	Time, *min*	Amino acid sequence
A	24.08	T L G P A
B	28	E L Y E S
C	32.63	K Y E E L
D	40.1	V S E E F
E	42.83	W Y E S P
F	43.9	F H V P A
G	50.53	G L Y E S

Fig. 3. *Tryptic fragments of mouse kidney ACE.* Mouse ACE (265 μg) was digested with trypsin and loaded on a Vydac C4 reverse phase HPLC column. The column was eluted over 60 min with a linear 0 to 60% solution of 0.1% trifluoroacetic acid in acetonitrile. **A.** The eluted pattern of tryptic fragments with letters A-G indicating fragments found suitable for amino acid sequence analysis. **B.** The time of elution and partial N-terminal amino acid sequence of these ACE fragments. The one letter amino acid code is as described in Figure 2.

Discussion

We report the isolation of ACE from mouse and bovine kidney. Eluates from an affinity column revealed single protein bands with a molecular weight similar to that previously described for ACE [4]. These proteins enzymatically degraded artificial ACE substrates and were inhibited by small amounts of the ACE inhibitor lisinopril. EDTA was found to inhibit the enzymatic action of these proteins as expected for ACE which requires Zn++ for activity [1, 4]. Finally the N-terminal amino acid sequences reported here were highly homologous to that previously reported for rabbit lung ACE and identical to that of bovine kidney ACE published by St. Clair et al [20]. These data establish that it is angiotensin converting enzyme that has been isolated from bovine and mouse kidney.

The majority of kidney cortical tissue is composed of proximal tubules which have a large amount of ACE in their brush border, and thus the majority of the ACE isolated and sequenced in this study must be of tubular epithelial origin. Whether ACE produced by renal or pulmonary endothelium is identical to that produced by tubular epithelium is not known. However, studies of the immunogenicity, molecular weight and kinetics suggest the ACE from the lung and kidney are indistinguishable [21–24]. This contrasts to the ACE-like enzyme produced by the testis which differs in size, N- and C-terminal amino acids and pattern of expression from ACE produced in the kidney or lung [25–29]. The exact number of genes encoding ACE and the complete sequence of this large protein awaits the cloning and analysis of the ACE gene.

Though very little amino acid sequence data has been published, many reports have previously detailed the isolation of ACE from a variety of organs and animals. Recent studies have used affinity chromatography to simplify ACE isolation protocols [5]. Thus Pantoliano used the ACE inhibitor N-[1(S)-carboxy-5-aminopentyl]-L-phenylalanyglycine immobilized to Sepharose with a long spacer arm to isolate rabbit lung ACE [9]. Bull, Thornberry and Cordes used the ACE inhibitor lisinopril bound to Sepharose to purify rabbit lung and human sera ACE [8]. Lanzillo used the same protocol to isolate ACE from other human tissues [24].

The protocol used in this study took advantage of the previous studies but differed in several significant ways. A commercially available matrix, Affi-Gel 15, was used to bind lisinopril in constructing an affinity resin. Affi-Gel 15 contains a

large number of ligand binding sites and a long spacer arm. The spacer arm is supplied derivatized with an active ester group so that a ligand such as lisinopril, containing a primary amine and soluble in methanol, can bind efficiently under nonaqueous conditions. The lisinopril-Affi-Gel 15 affinity matrix bears a positive charge at physiologic pH. This may aid in binding ACE which as been reported to be an acidic protein with a pI of 4.3 to 5.2 [5]. Finally some ligands have a marked change in affinity for ACE over a small change in pH [9]. The lisinopril affinity matrix in this study behaves similarly to those described by Pantoliano et al in that a small change of pH (from 6.0 to 8.9) resulted in elution of the enzyme from the affinity matrix [9]. As the eluted enzyme was catalytically active, this method proved to be much simpler than those requiring elution of the enzyme by excess ligand followed by extensive dialysis as described by others [8].

Angiotensin converting enzyme has been intensively studied since it was first described by Skeggs et al [1, 2]. It has become clear that this protease is produced in a regulated fashion by a number of tissues including endothelium, renal tubular epithelium, activated macrophages, testis, and brain [4, 5]. Here we report the isolation of mouse ACE, an animal model that will allow future study of the control of ACE gene expression to use the many strains, the large amount of genetic data, and the cultured glomerular endothelial cells available in this species [30]. The information obtained from the tryptic digestion of mouse kidney ACE provides the basis for future studies to identify ACE cDNA.

Acknowledgments

Lisinopril used in this study was a gift from Dr. Herbert Bull, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, USA. The authors thank William Eliason and Kara Maysak for assistance in amino acid sequencing and Dr. John Inman, NIDDK, for helping in chemical protocols. We also thank Linda Adams and Alpharetta Scales for their typing.

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