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Cloning and Expression of an Evolutionary Conserved Single-domain Angiotensin Converting Enzyme from *Drosophila melanogaster**

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Mammalian somatic angiotensin converting enzyme (EC 3.4.15.1, ACE) consists of two highly homologous (N- and C-) domains encoded by a duplicated gene. We have identified an apparent single-domain (67 kDa) insect angiotensin converting enzyme (AnCE) in embryos of *Drosophila melanogaster* which converts angiotensin I to angiotensin II (K_m, 365 μM), removes Phe-Arg from the C terminus of bradykinin (K_m, 22 μM), and is inhibited by ACE inhibitors, captopril ($IC_{50} = 1.1 \times 10^{-9}$ M) and trandolaprilat ($IC_{50} = 1.6 \times 10^{-8}$ M). We also report the cloning and expression of a *Drosophila* AnCE cDNA which codes for a single-domain 615-amino acid protein with a predicted 17-amino acid signal peptide and regions with high levels of homology to both the N- and C-domains of mammalian somatic ACE, especially around the active site consensus sequence. Northern analysis identified a single 2.1-kilobase mRNA in *Drosophila* embryos, and Southern analysis of *Drosophila* genomic DNA indicates that the insect gene is not duplicated. When expressed in COS-7 cells, the AnCE protein is a secreted enzyme, which converts angiotensin I to angiotensin II and is inhibited by captopril ($IC_{50} = 5.6 \times 10^{-9}$ M) and trandolaprilat ($IC_{50} = 2 \times 10^{-8}$ M). The evolutionary significance of these results is discussed.

Mammalian angiotensin converting enzyme (EC 3.4.15.1, peptidyl dipeptidase A) (ACE)[1] is a Zn^{2+} metallopeptidase, which plays an important role in blood pressure homeostasis by cleaving the C-terminal His-Leu from angiotensin I (AI) to generate the potent vasopressor, angiotensin II (AII) (1). A number of other small peptides are also hydrolyzed by mammalian ACE, including the vasodilatory peptide bradykinin (2). The bulk of the protein, including the active sites, is extracellular, connected to a short intracellular C-terminal tail by a hydrophobic sequence that traverses the plasma membrane (3, 4). The enzyme exists as two isoforms, a somatic form (M_r 140,000–180,000) which displays a wide tissue distribution and a smaller form (M_r 90,000–110,000) found exclusively in the testes (2, 3). Somatic ACE (sACE) is composed of two highly homologous domains (N- and C-domains), both of which contain the Zn^{2+}-binding motif HExxH found in other Zn^{2+} metallopeptidases (10). It has recently been shown that both the N- and C-domains bind Zn^{2+} and are catalytically active (11–13).

Testicular ACE (tACE) is identical to the C-domain of sACE, apart from a short N-terminal sequence (64–72 amino acid, depending on species), and includes the transmembrane and the intracellular C-terminal domains (14–17). Both sACE and tACE are transcribed from a single gene with the testis-specific transcript under the control of a tissue- and stage-specific intragenic promoter (18–20). The physiological function of tACE with its single catalytic domain is not known.

The close similarity of the N-and C-domains of mammalian sACE and the fact that exons 4–11 and 17–24 of the human ACE gene, which code for the N- and C-domains, are very similar in size and have similar codon phases at exon-intron boundaries (20) strongly indicates that the gene arose from duplication of an ancestral ACE gene coding for a single-domain enzyme.

We have recently found a peptidyl dipeptidase in heads of the housefly, *Musca domestica*, which has very similar enzymatic properties to mammalian ACE (21). The enzyme had an apparent M_r of approximately 80, 000, thus resembling the single-domain enzyme found in mammalian testes. There was no evidence for a larger two-domain protein, implying that the insect enzyme is the product of a non-duplicated gene. In the present study we have purified a soluble 67-kDa ACE (AnCE) from embryos of *Drosophila melanogaster* and have used a cDNA for human sACE as a heterologous probe to clone a cDNA from a *Drosophila* embryo cDNA library, whose predicted translation product has regions of homology to both the C- and N-domains of mammalian sACE. The sequence data predicts that this cDNA encodes a secreted protein with no recognizable C-terminal hydrophobic membrane-anchor sequence. Functional expression of the cDNA in COS-7 cells confirms that we have cloned the cDNA for an insect ACE.

MATERIALS AND METHODS

Insect Culture—Standard culture methods were employed to obtain large numbers of adult *D. melanogaster* (Oregon R strain) which were

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U25344.

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[1] The abbreviations used are: ACE, angiotensin converting enzyme; sACE, somatic ACE; tACE, testicular ACE; AnCE, *D. melanogaster* ACE; AI, angiotensin I; AII, angiotensin II; BK, bradykinin; bp, base pairs; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HPLC, high performance liquid chromatography; kb, kilobase pairs; PAGE, polyacrylamide gel electrophoresis.

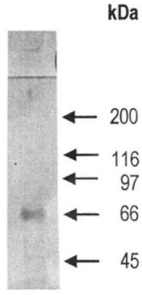


FIG. 1. **SDS-polyacrylamide gel electrophoresis of AnCE purified from *Drosophila* embryos.** Affinity-purified AnCE (~0.1 μg) was loaded on to a Pharmacia PhastGel Homogeneous 7.5 polyacrylamide gel which was subjected to electrophoresis (Pharmacia PhastSystem) with SDS buffer strips. Proteins were visualized by staining with silver nitrate and the molecular mass of AnCE was determined by reference to marker proteins indicated by *arrows*.

allowed to lay eggs on agar plates impregnated with apple juice (22). Eggs were washed from the agar with distilled water and were stored at −70 °C until required.

Enzyme Preparation—Soluble enzyme was extracted from eggs (4–16 h) by homogenization in 10 mM Tris-HCl buffer, pH 8.4 (3 ml of buffer/g of eggs) using a glass homogenizer. The homogenate was centrifuged at 200,000 × *g* for 20 min (Beckman 100.4 rotor for 20 min), and the pellet was resuspended in 10 mM Tris-HCl, pH 7.4, and subjected to centrifugation at 200,000 × *g* for 20 min. The supernatant was kept and the pellet washed in 10 mM Tris-HCl, 0.5 M NaCl, pH 7.4, before being centrifuged for the last time to yield the final supernatant and a pellet which was resuspended in 10 mM Tris-HCl, pH 7.4. All procedures were performed at 4 °C. ACE activity in the combined supernatants and the resuspended pellet were assayed using Hip-His-Leu as the substrate and HPLC to quantify hippurate (Hip) released (21).

Enzyme Purification—*Drosophila* angiotensin converting enzyme (AnCE) was purified from eggs (4–16 h) using a lisinopril-Sepharose affinity column (23) and a procedure developed for the purification of AnCE from the housefly, *M. domestica*.[2] Briefly, eggs were homogenized in 10 mM Tris-HCl, 0.2 M NH_2SO_4, pH 8.4, and the supernatant obtained after centrifugation (20,000 × *g* for 20 min) was loaded onto the lisinopril-sepharose column (20 ml) equilibrated with 10 mM Tris-HCl, 0.2 M NH_2SO_4, pH 8.5. The column was washed with the loading buffer until all the unbound protein had been eluted, as indicated by the UV absorbance (280 nm) of the eluate reaching a base-line level. Bound AnCE was eluted by replacing the loading buffer with 10 mM Tris-HCl, pH 8.5. Fractions were collected and assayed for peptidyl dipeptidase activity as described above using Hip-His-Leu as the substrate. Fractions containing enzyme activity were concentrated using a CentriCell 20 filtration unit (Polysciences Inc., Park Scientific, Northampton, U.K.) with a 30-kDa molecular mass cut-off. The purity of the enzyme was determined by SDS-PAGE using a Pharmacia PhastSystem (Pharmacia Biotech, U.K.) and PhastGel Homogeneous 7.5 polyacrylamide gels with SDS buffer strips and the molecular mass was estimated by comparison to size-marker proteins (Sigma, U.K.) which were analyzed in adjacent lanes on the same gel. Protein bands were visualized by staining with silver nitrate (24), and the protein concentration of the purified sample was estimated using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, U.K.) with bovine serum albumin as the protein standard.

K_m Determinations for the Hydrolysis of AI and BK—The effect of AI concentration on the ACE activity of the native *Drosophila* enzyme was determined by quantifying the amount of AII formed by HPLC using a SuperPac Pep-S reversed phase column (250 mm × 4 mm; Pharmacia Biotech, U.K.), a 10 min gradient of acetonitrile (16–38%) in 0.1% trifluoroacetic acid, and a flow rate of 1 ml/min. The hydrolysis of BK generated BK^{7-9}, BK^{1-7}, and BK^{1-5}, all of which were resolved by HPLC using the SuperPac Pep-S reversed phase column (250 × 4 mm) and a gradient of acetonitrile in 0.1% orthophosphoric acid (flow rate, 1 ml/min). Initially the column was eluted with 5% acetonitrile for 3 min, followed by a linear increase to 28% acetonitrile over 15 min. Authentic peptides (Sigma, U.K.) were used as chromatography markers. The rate of BK hydrolysis was determined by quantifying Phe-Arg. The hydrolysis of AI and BK were performed in the presence of 0.1 M Tris-HCl, 0.3 M NaCl, 10 μM $ZnCl_2$, pH 8.3. Reactions were terminated by heating to 100 °C for 5 min, and samples were diluted with 0.1% trifluoroacetic acid before HPLC analysis. Standard solutions of AII and Phe-Arg were used for calibration, and a curve-fitting computer programme (P.fit, Biosoft, U.K.) was used to obtain K_m values.

Cloning of AnCE cDNA—A *D. melanogaster* 4–8 h embryo cDNA library (25) was screened with a 3.33-kb fragment of the human sACE cDNA (λHEC1922 clone, Ref. 5). [α^{32}P]CTP multiprime-labeled probe (Amersham International) was hybridized to the *D. melanogaster* 4–8 h embryo cDNA library grid in 4 × SSPE, 2% SDS at 65 °C overnight, followed by sequential washes in 0.5 × SSC, 0.1% SDS (65 °C, 30 min) and 0.1 × SSC, 0.1% SDS (65 °C, 30 min). Five hybridizing clones (17C9, 37C5, 48A3, 52F10, and 53B11) were subcloned into *Not*I/*Hin*dIII-digested pBluescript II SK^+ and SK^- (Stratagene) and sequenced using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp.) with T3 and T7 primers and specific synthetic oligonucleotides as internal primers.

Expression of Drosophila AnCE cDNA—A 2.03-kb *Hin*dIII/*Not*I fragment of the *Drosophila* AnCE cDNA was subcloned into the *Hin*dIII-*Not*I sites of pRC/CMV expression vector (Invitrogen) under the transcriptional control of the cytomegalovirus promoter for the immediate early gene. COS-7 cells were cultured and transfected as described previously (26). At 24-h post-transfection, the medium was discarded and the cells were washed five times with phosphate-buffered saline before a further 24-h incubation in fetal calf serum-free Dulbecco's minimum essential medium, since the circulating form of ACE is present in fetal calf serum. The culture medium was collected and assayed for ACE-soluble activity. The cells were washed five times with phosphate-buffered saline and were dislodged from the culture flasks by scraping into 1 ml of 8 mM CHAPS detergent, 5 mM potassium phosphate pH 8.3, 10 μM $ZnSO_4$ and were incubated at 4 °C for 1 h with agitation. Solubilized proteins were recovered in the supernatant after centrifugation at 12, 000 × *g* for 15 min and were assayed for membrane-bound ACE activity.

A control transfection was performed exactly as described above but in the absence of pCMV/AnCE ("mock transfection"). COS-7 cells were also transfected with the cDNA encoding wild-type human ACE (4.02 kb) with the C-terminal membrane anchor (5), in the expression vector pECE (pEACE). In addition, cells were transfected with a mutated human sACE cDNA with a stop codon introduced before the membrane anchoring sequence, also in pECE ($pEACE_{\Delta COOH}$) which produces a secreted enzyme (4). Due to differences in the promoter activities of pECE and pRC/CMV, the culture medium of pEACE and $pEACE_{\Delta COOH}$ transfected cells was changed to fetal calf serum-free Dulbecco's minimum essential medium 48-h post-transfection, and the culture medium and cells were collected after a further 24 h of growth. ACE activity was determined as described previously (4), using either Hip-His-Leu or AI as the substrate.

Western Blotting—Samples of cell culture medium from AnCE and mock transfected cells (800 μl, corresponding to 50 nmol of Hip produced/min from pCMV/AnCE transfected cell culture medium) were concentrated using micron-10 filtration tubes (Amicon) before SDS-PAGE and Western blotting, as described previously (4). Antiserum to AnCE was obtained by three subcutaneous injections of 50 μg of enzyme purified from the housefly *M. domestica*[2] into a New Zealand White rabbit at 3-week intervals.

Genomic Southern Blot Analysis—Genomic DNA isolated using standard procedures (27) was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Xba*I, and *Xho*I and run on a 0.8% agarose gel. The DNA was transferred to a Hybond N membrane (Amersham International, U.K.) and was hybridized with the full-length AnCE cDNA. Membranes were washed in 0.1 × SSC, 0.1% SDS at 65 °C and were exposed to x-ray film.

Northern Blot Analysis—Total RNA (20 μg), extracted from *D. melanogaster* embryos using the hot phenol method (27), was subjected to electrophoresis on a 1% denaturing agarose gel, transferred to a Hybond N membrane (Amersham International, U.K.), oven baked, and hybridized to the full-length AnCE cDNA (5 × Denhardt's solution, 5 × SSC, 1% SDS, 50% formamide at 42 °C overnight). The membrane was washed using stringent conditions with a final wash in 0.1 × SSC, 0.1% SDS at 65 °C. The membrane was exposed to a Fuji imaging plate (BASIIIs) for 4 days, and the plate was then developed using a Fuji BAS 1000 PhosphoImager.

Chromosomal Localization—*In situ* hybridization of the full-length AnCE cDNA to polytene chromosomes of salivary glands of third instar larvae was carried out using a commercial kit (Life Technologies, Inc., U.K.) according to a standard procedure (22).

Sequence Comparisons—The common core region sequences (20) of human sACE (exons 4–11 for the N-domain and exons 17–24 for the C-domain, corresponding to amino acid positions 162–569 and 770-

[2] N. S. Lamango and R. E. Isaac, manuscript in preparation.

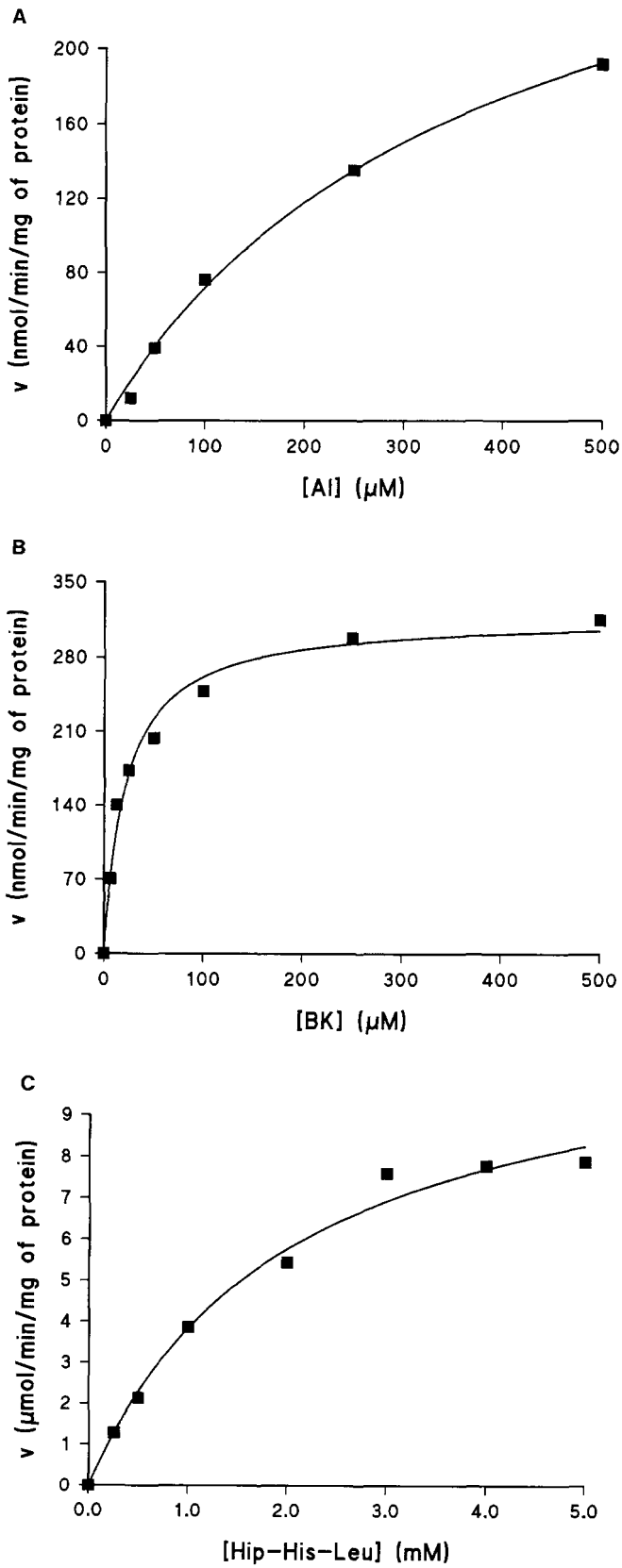


FIG. 2. **Michaelis-Menten plots for the hydrolysis of AI (*panel A*), BK (*panel B*), and Hip-His-Leu (*panel C*) by *Drosophila* AnCE.** The activity of affinity-purified AnCE was measured with a range of substrate concentrations under conditions which resulted in less than 20% degradation of the substrate. The hydrolysis of AI was measured by quantifying the amount of AII formed using HPLC. Peptidyl dipeptidase activity toward BK was determined by quantifying Arg-Phe by HPLC. HPLC methods and the assay of the hydrolysis of Hip-His-Leu are described under "Materials and Methods." Data are the mean ± S.E. of triplicate assays. The Michaelis-Menten plots and the K_m values derived from them were generated by a curve-fitting computer program (P.fit, Biosoft).

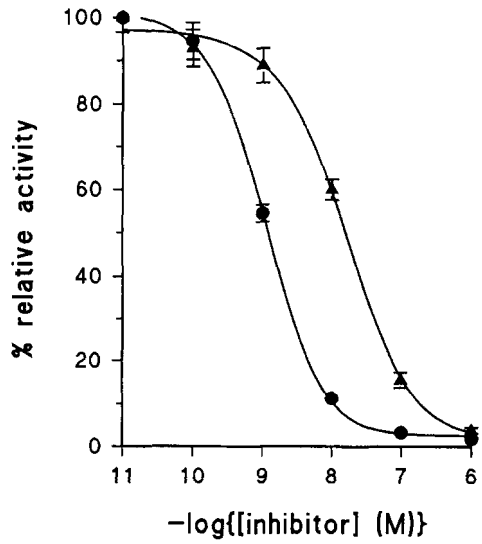


FIG. 3. **Inhibition of *Drosophila* AnCE activity by captopril (●) and trandolaprilat (▲).** The activity of affinity-purified AnCE from *Drosophila* embryos was assayed in the presence of different concentrations of inhibitor with Hip-His-Leu as the substrate, as described under "Materials and Methods." The enzyme was preincubated with inhibitor for 15 min at 37 °C before the addition of substrate. Data are expressed as a percentage of the uninhibited activity and are the mean of triplicate determinations ± S.E. The inhibition plot and the IC_{50} values were generated by a curve-fitting computer program (P.fit, Biosoft).

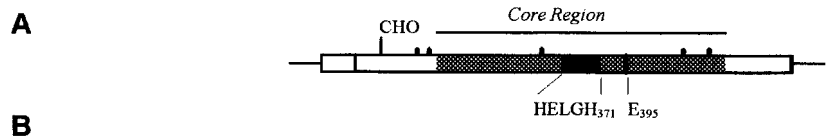
1167) were used to identify the equivalent domains in rat, mouse, rabbit, and bovine (6) sACE and the *D. melanogaster* AnCE. Sequences were compared using the Wisconsin GCG package (28). Alignments produced using ClustalV (29) were analyzed using the PROTDIST (Dayhoff PAM 001 matrix) (30) and KITSCH components of PHYLIP (31) to generate the dendrogram shown in Fig. 4 (optimum tree from 25 randomized cycles of KITSCH).

RESULTS

Identification of a Soluble 67-kDa AnCE in Drosophila Embryos—Peptidyl dipeptidase activity in the homogenate of *D. melanogaster* embryos (4–18 h) measured using Hip-His-Leu as the substrate was found predominantly (77.8 ± 1.9%; $n = 4$, ± S.E.) in the supernatant after centrifugation at 200,000 × *g* for 20 min. Washing the 200,000 × *g* pellet with 10 mM Tris-HCl buffer, pH, 7.4, and subsequently with 10 mM Tris-HCl, 0.5 M NaCl, pH 7.4, left only 11.4 ± 1.1% ($n = 4$, ± S.E.) of the total peptidyl dipeptidase activity associated with the membrane fraction. The hydrolysis of Hip-His-Leu by the soluble enzyme was completely abolished in the presence of either 10 μM captopril or 10 μM trandolaprilat (data not shown).

A 1000-fold purification of the soluble embryo peptidyl dipeptidase was achieved using a lisinopril-Sepharose affinity column. SDS-PAGE analysis of the affinity-purified enzyme revealed a single protein band with an apparent molecular mass of 67 kDa (Fig. 1). The purified enzyme converted AI to AII and removed Phe-Arg from the C terminus of BK with K_m values of 365 and 22 μM for AI and BK, respectively (Fig. 2, *A* and *B*). The K_m for the hydrolysis of Hip-His-Leu was 2.0 mM (Fig. 2*C*), and IC_{50} values of 1.1×10^{-9} M and 1.6×10^{-8} M were obtained for captopril and trandolaprilat, respectively (Fig. 3).

Cloning and Characterization of AnCE cDNA—A cDNA comprising the nucleotide sequence between position 690 and 4024 of the human sACE cDNA sequence (5) was used to screen a 4–8-h *D. melanogaster* embryo cDNA library. Five strongly



B

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v       v       v       v       v       v       v       v       v       v       v       v       v
                                TCAGTTTAGTTCAGACCGCTGGCGAACCGGTCACGTTCGATTGGTTGGGAAATATCGCCGGACAGAACGCGAGAAAGTAGTTGATCAAC      90
ATGAGACTGTTTCTGCTAGCCCTGCTGGCTACTTTGGCGGTAACCCAAGCGCTGGTCAAGGAGGAGATACAGGCCAAGGAGTATCTGGAGAATCTGAACAAGGAGCTGGCCAAGCGAACC     210
M  R  L  F  L  L  A  L  L  A  T  L  A  V  T  Q  A  L  V  K  E  E  I  Q  A  K  E  Y  L  E  N  L  N  K  E  L  A  K  R  T      40
AACGTGGAAAACCGAAGCTGCCTGGGCCTATGGCTCCAACATCACCGACGAGAACGAAAAGAAAAAGAATGAGATATCCGCCGAGTTGGCCAAGTTCATGAAGGAGGTGGCCAGTGATACC     330
N  V  E  T  E  A  A  W  A  Y  G  S  N  I  T  D  E  N  E  K  K  K  N  E  I  S  A  E  L  A  K  F  M  K  E  V  A  S  D  T      80
ACCAAGTTCCAATGGCGCTCGTACCAATCGGAGGATCTCAAGCGCCAGTTCAAGGCTCTAACCAAACTGGGCTATGCTGCTCTACCTGAAGACGACTATGCCGAACTGCTGGACACACTC     450
T  K  F  Q  W  R  S  Y  Q  S  E  D  L  K  R  Q  F  K  A  L  T  K  L  G  Y  A  A  L  P  E  D  D  Y  A  E  L  L  D  T  L     120
TCCGCCATGGAGTCGAATTTCGCCAAGGTCAAGGTGTGCGACTACAAGGATAGCACCAAGTGCGACCTAGCCCTCGACCCGGAGATCGAGGAGGTCATCAGCAAGAGCCGTGACCACGAG     570
S  A  M  E  S  N  F  A  K  V  K  V  C  D  Y  K  D  S  T  K  C  D  L  A  L  D  P  E  I  E  E  V  I  S  K  S  R  D  H  E     160
GAGCTCGCCTACTACTGGCGCGAGTTCTACGACAAGGCCGGAACCGCCGTGCGATCTCAATTCGAACGCTACGTGGAGCTCAACACCAAGGCAGCCAAACTGAATAACTTCACTTCTGGT     690
E  L  A  Y  Y  W  R  E  F  Y  D  K  A  G  T  A  V  R  S  Q  F  E  R  Y  V  E  L  N  T  K  A  A  K  L  N  N  F  T  S  G     200
GCCGAGGCCTGGTTGGATGAGTACGAGGACGATACCTTCGAGCAGCAGCTGGAGGACATCTTCGCGGATATTCGTCCGCTTTTACCAGCAGATCCATGGCTATGTGCGTTCCGCCTGAGG     810
A  E  A  W  L  D  E  Y  E  D  D  T  F  E  Q  Q  L  E  D  I  F  A  D  I  R  P  L  L  P  A  D  P  W  L  C  A  F  R  L  R     240
AAACACTATGGTGACGCGGTGGTCTCCGAGACAGGACCCATTCCCATGCACCTTTTGGGCAACATGTGGGCACAGCAGTGGTCAGAGATTGCGGACATCGTATCCCCCTTCCCGGAGAAG     930
K  H  Y  G  D  A  V  V  S  E  T  G  P  I  P  M  H  L  L  G  N  M  W  A  Q  Q  W  S  E  I  A  D  I  V  S  P  F  P  E  K     280
CCCCTGGTGGATGTGAGCGCTGAGATGGAAAAGCAGGCCTACACGCCACTCAAAATGTTCCAAATGGGCGACGACTTCTTCACGTCTATGAATCTTACCAAGCTGCCACAGGACTTCTGG     1050
P  L  V  D  V  S  A  E  M  E  K  Q  A  Y  T  P  L  K  M  F  Q  M  G  D  D  F  F  T  S  M  N  L  T  K  L  P  Q  D  F  W     320
GACAAGTCGATCATTGAAAAGCCCACCGATGGTCGCGATCTGGTTTGCCATGCCAGCGCCTGGGACTTTTATCTCATCGATGATGTGcGcATCAAGCAGTGCACCCGGGTTACACAGGAT     1170
D  K  S  I  I  E  K  P  T  D  G  R  D  L  V  C  H  A  S  A  W  D  F  Y  L  I  D  D  V  R  I  K  Q  C  T  R  V  T  Q  D     360
CAGCTCTTTACCGTGCATCACGAACTGGGACACATTCAGTATTTCCTTCAGTATCAACACCAACCCTTCGTCTATCGCACTGGTGCCAATCCCGGTTTCCATGAAGCTGTCGGTGATGTG     1290
Q  L  F  T  V  H  H  E  L  G  H  I  Q  Y  F  L  Q  Y  Q  H  Q  P  F  V  Y  R  T  G  A  N  P  G  F  H  E  A  V  G  D  V     400
CTCTCCCTATCCGTATCGACACCCAAACATCTCGAGAAGATTGGCCTGCTGAAGGATTATGTGCGCGATGACGAGGCTCGAATCAACCAGCTCTTCCTCACCGCCCTGGACAAGATCGTT     1410
L  S  L  S  V  S  T  P  K  H  L  E  K  I  G  L  L  K  D  Y  V  R  D  D  E  A  R  I  N  Q  L  F  L  T  A  L  D  K  I  V     440
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F  L  P  F  A  F  T  M  D  K  Y  R  W  S  L  F  R  G  E  V  D  K  A  N  W  N  C  A  F  W  K  L  R  D  E  Y  S  G  I  E     480
CCGCCAGTTGTGCGAAGCGAAAAGGACTTTGATGCCCCGGCCAAGTATCACATTTCCGCCGATGTCGaGTATCTGAGATACTTGGTCTCCTTCATCATTCAGTTCCAGTTCTACAAGTCC     1650
P  P  V  V  R  S  E  K  D  F  D  A  P  A  K  Y  H  I  S  A  D  V  E  Y  L  R  Y  L  V  S  F  I  Q  F  Q  F  Y  K  S     520
GCCTGCATCAAGGCTGGCCAATATGATCCCGACAATGTGGAGTTGCCTCTGGATAATTGCGATATCTACGGAAGTGCTCGAGCTGGTGCTGCTTTCCACAACATGCTGTCCATGGGCGCC     1770
A  C  I  K  A  G  Q  Y  D  P  D  N  V  E  L  P  L  D  N  C  D  I  Y  G  S  A  R  A  G  A  A  F  H  N  M  L  S  M  G  A     560
TCGAAGCCATGGCCCGATGCACTGGAGGCCTTCAACGGCGAGCGCATCATGTCCGGAAAGGCCATCGCCGAATACTTTGAGCCACTGCGCGTTTGGCTGGAGGCGGAGAACATTAAGAAC     1890
S  K  P  W  P  D  A  L  E  A  F  N  G  E  R  I  M  S  G  K  A  I  A  E  Y  F  E  P  L  R  V  W  L  E  A  E  N  I  K  N     600
AATGTCCACATTGGCTGGACTACTTCAAATAAATGCGTCTCATCATAATTCAAATGATTTTAGTGTTCTCACACACAGTTCATTGCTCAATAAAGTTTAACAATATCTTAA     2001
N  V  H  I  G  W  T  T  S  N  K  C  V  S  S  <        615
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C

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          1                                                  50
Human     MGQGWATAGL PSLLFLLLCY GHPLLVPSQE ASQQVTVTHG TSSQATTSSQ
Mouse     MGQGWAT-GL PS-LFLLLC- GH-LLV-SQ- A---VT---G ---QATT-SQ
Rabbit    MGQGWA--GL PSLL-LLLC- GH-LLVPS-- A---VTV--G T-SQATT-S-
AnCE      M-........ ...LFLL... ..-LL--... .....-VT-- ..........
Con.      M--------- ---LfLL--- ---LL----- ----la--qa ----------

          51                                                 100
Human     TTT......H QATAHQTSAQ SPNLVTDEAE ASKFVEEYDR TSQVVWNEYA
Mouse     TTT......H QAT--QT-.Q -PNL-TDEA- A--FVEEYDR T-QV--NEYA
Rabbit    -TT------H Q-TAHQT-.Q SPNLVTDEAE AS-FVEEYDR --Q-VWNEYA
AnCE      ..........  .......... ...LV--E-- A----E---- ------N---
Con.      ---------- ---------- ---L--eE-- A--flEn-nk ------N--t

          101                                                150
Human     EANWNYNTNI TTETSKILLQ KNMQIANHTL KYGTQARKFD VNQLQNTTIK
Mouse     EANW-YNTNI T-E-SKILL- K-----NHTL KYGT-A--FD V---QN--IK
Rabbit    EANWNYNTNI TTE-SKILLQ KNMQIANHTL -YG--AR-FD V---QN-T-K
AnCE      EA-W-Y--NI T-E--K---- -----A---- -------KF- ----Q----K
Con.      EA-W-Y-sNI T-E--K---e -s-elak--- --a--t--Fq --syQs---K

          151                                                200
Human     RIIKKVQDLE RAALPAQELE EYNKILLDME TTYSVATVCH PNGS..C.LQ
Mouse     RIIKK-Q-L- RA-LP--ELE EYN-ILLDME TTYS----C- -NG-..C.--
Rabbit    RIIKKVQDL- RA-LP--ELE EYN-ILLDME T-YSVA-VC- --GS..C.LQ
AnCE      R--K----L- -AALP----- E----L--ME -------VC- ---S--C-L-
Con.      R-fK-l--L- -A-LP--d-- E--d-L--ME s-fa---vC- ---s--C-l-
                                                     ↑          ↑
          201                                                250
Human     LEPDLTNVMA TSRKYEDLLW AWEGWRDKAG RAILQFYPKY VELINQAARL
Mouse     LEPDLTN-MA TSRKYE-LLW AW--WRDK-G RAIL-F-PKY VE--N--A-L
Rabbit    LEPDLTN-MA TSRKY--LLW -W--WRDK-G RAIL---PKY VE--N-AARL
AnCE      L-P----V-- -SR--E-L-- -W----DKAG -A-------Y VEL---AA-L
Con.      LdPei-evis kSR--eeL-w -W--W-DK-G -Av---f-rY VEl--k-AkL

          251                                                300
Human     NGYVDAGDSW RSMYETPSLE QDLERLFQEL QPLYLN...L HAVVRRALHR
Mouse     NGY-DAGDSW RS-YE---LE QDLE-L-QEL QPLYLN...L HAYVRR-LHR
Rabbit    NGYVDAGDSW RSMYETP-LE QDLERLFQEL QPLYLN...L HAYV-RALHR
AnCE      N--------W ---YE----E Q-LE--F--- -PL------L -A-...-L--
Con.      N-f--gaeaW ---YE---fE QqLE-if-di rPL------L -Af----Lrk

          301                                                350
Human     HYGAQHINLE GPIPAHLLGN MWAQTWSNIY DLVVPFPSAP SMDTTEAMLK
Mouse     HYG---INL- GPIPAHLLGN MWAQTWSNIY DLV-PFPSAP --D-TEAM-K
Rabbit    HYGAQHINLE GPIPAHLLGN MWAQTWSNIY DLV-PFPSA- -MD-TEAM-K
AnCE      HYG------- GPIP-HLLGN MWAQ-WS-I- D-V-PFP--P --D----M-K
Con.      HYG---v--- GPIP-HLLGN MWAQ-Ws-I- DiV-PFP--p -vD-s--M-K

          351                                                400
Human     QGWTPRRMFK EADDFFTSLG LLPVPPEFWN KSMLEKPTDG REVVCHASAW
Mouse     QGWTPRR-FK EAD-FFTSLG LLPVPPEFWN KSMLEKPTDG REVVCH-SAW
Rabbit    QGWTPRRMF- EAD-FF-SLG LLPVPPEFWN KSMLEKPTDG REVVCHASAW
AnCE      Q--TP--MF- --DDFFTS-- L---P--FW- KS--EKPTDG R--VCHASAW
Con.      QaWTP-kmF- -gD-FF-Sm- L--lP-dFWd KSiiEKPTDG RdlVCHaSAW
                                                     ↑
          401                                                450
Human     DFYNGKDFRI KQCTTVNLED LVVAHHEMGH IQYFMQYKDL PVALREGANP
Mouse     DFYNGKDFRI KQCT-VN-ED LV-AHHEMGH IQYFMQYKDL PV--REGANP
Rabbit    DFYNGKDFRI KQCTTVN-ED LVV-HHEMGH IQYFMQYKDL PVALREGANP
AnCE      DFY---D-RI KQCT-V---- L---HHE-GH IQYF-QY--- P---R-GANP
Con.      DFY---D-RI KQCT-V--dq L---HHElGH IQYFlQYqh- P--LR-GANP

          451                                                500
Human     GFHEAIGDVL ALSVSTPKHL HSLNLLSSEG GSDEHDINFL MKMALDKIAF
Mouse     GFHEAIGD-- ALSVSTPKHL -SLNLLS-EG ---E-DINFL MKMALDKIAF
Rabbit    GFHEAIGDVL ALSVSTPKHL HS-NLLSSEG G--EHDINFL MKMALDKIAF
AnCE      GFHEA-GDVL -LSVSTPKHL ----LL---- --DE--IN-L ---ALDKI-F
Con.      GFHEAvGDvl sLSVSTPKHL --i-LL---- -d-E--IN-L ---ALDKI-F

          501                                                550
Human     IPFSYLVDQW RWRVFDGSIT KENYNQEWWS LRLKYQGLCP PVPRTQGDFD
Mouse     IPFSYL-DQW RWRVFDGSIT KENYNQEWWS LRLKYQGLCP PVPR-QGDFD
Rabbit    IPFSYLVD-W RWRVFDGSIT KENYNQEWWS LRLKYQGLCP P-PR-QGDFD
AnCE      -PF----D-- RW--F-G--- K-N-N---W- LR--Y-G--P PV-R---DFD
Con.      lPFaf-mD-W RW-lF-G-v- K-NYN--WW- LR--Y-Gi-P P--Rse-DFD

          551                                                600
Human     PGAKFHIPSS VPYIRYFVSF IIQFQFHEAL CQAAGHTGP. .....LHKCD
Mouse     PG-KFH-P-- VPY-RYFVSF IIQFQFHEAL C-AAGHTGP. .....LHKCD
Rabbit    PGAKFHIPSS VPYIRYFVSF IIQFQFHEAL C-AAGHTGP. .....LH-CD
AnCE      --AK-HI--- V-Y-RY-VSF IIQFQF---- C---G---P- --------CD
Con.      a-aKyHisa- V-YlRYlVSF IIQFQF--s- C---Gq-dP- -----Ld-CD
                                                  ↑          ↑
          601                                                650
Human     IYQSKEAGQR LATAMKLGFS RPWPEAMQLI TGQPNMSASA MLSYFKPLLD
Mouse     IYQSKEAG-- LA-AMKLG-S -PWPEAM-LI TGQPNMSASA M--YFKPL--
Rabbit    IYQSKEAG-R LA-AMKLG-S -PWPEAM--I TGQPNMSASA M--YFKPL-D
AnCE      IY-S--AG-- -------G-S -PWP-A---- -G---MS--A ---YF-PL--
Con.      -Y-S--AG-- f---l-mG-S kPWPdAl--f -Ge--MSg-A i-eYF-PL--

          651                                                700
Human     WLRTENELHG EKLGWPQYNW TPNSARSEGP LPDSGRVSFL GLDLDAQQAR
Mouse     WL-TEN--HG E-LGWP-YNW -PN-AR-EG- ---S-RV-FL GL-L--QQAR
Rabbit    WL-TEN--HG EKLGWPQY-W TPNSARSEG- LPDSGRV-FL G--LDAQQAR
AnCE      WL--EN---- ---GW-.... ...S------ .......... ..........
Con.      WL-aEN---- --iGW----- ---s-k--ss ---------- ----------

          701                                                750
Human     VGQWLLLFLG IALLVATLGL SQRLFSIR.H RSLHRHSHGP QFGSEVELRH S
Mouse     VGQW-LLFLG -ALLVAT-GL --RL--IR-H -SL-R---GP QFGSEVELRH S
Rabbit    VGQW-LLFLG -ALL-A-LGL -QRLFSIR.- -SL----HGP QFGSEVELRH S
AnCE      .......... .......... .......... .......... .......... .
Con.      ---------- ---------- ---------- ---------- ---------- -
```

FIG. 4. *A*, cartoon showing the relative positions of the conserved features of AnCE. *CHO* marks the conserved glycosylation site; the *hatched* area represents the conserved core region found in all ACE domains; the *solid bars* mark the Zn^{2+}-binding site, and the *small upright bars* the positions of the conserved cysteines. *B*, nucleic acid sequence of AnCE with the predicted translation product. The putative signal peptide is marked

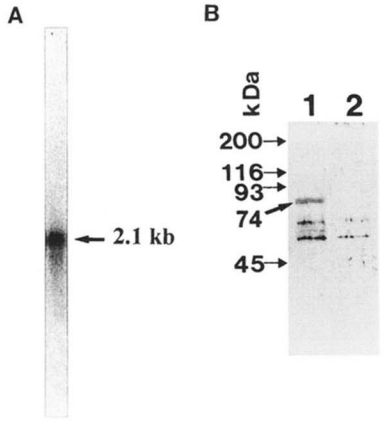


FIG. 5. **Northern (*A*) and Western (*B*) blot analysis of AnCE expression.** *A*, Northern hybridization of 17C9 to 20 μg of total *D. melanogaster* RNA from embryos. A single band can be seen at 2.1 kilobase pairs. *B*, Western blot analysis of recombinant AnCE. Culture medium (800 μl) from AnCE and mock transfected COS-7 cells were concentrated and analyzed by SDS-PAGE followed by Western blotting using antiserum raised against housefly AnCE. The analysis identified a protein band with an M_r of 74,000 which was absent from the mock transfected cell medium. The migration of molecular weight markers is indicated on the *left*.

TABLE I
Expression of recombinant enzymes in COS-7 cells

Data are the mean values ± S.E. of three separate transfections for each construct. Enzyme assays were performed as described under "Materials and Methods" using Bz-Gly-His-Leu (1 mM final concentration) as substrate.

Construct	Total ACE activity expressed		
	Medium	Cells	Secreted
	nmol Bz-Gly/min		%
Mock transfected	0.032 ± 0.036	0.055 ± 0.002	
pCMV/AnCE	170.07 ± 6.40	12.00 ± 0.28	93.4 ± 0.2
$pEACE_{\Delta COOH}$	39.43 ± 5.25	8.60 ± 0.11	81.3 ± 2.5
pEACE	6.19 ± 0.29	8.14 ± 0.18	43.1 ± 0.7

hybridizing clones (17C9, 37C5, 48A3, 52F10, and 53B11) were identified and partially sequenced. Clone 53B11 had a long open reading frame of 1845 bp which was flanked at the 5′ and 3′ ends by 90 and 66 bp of untranslated sequence. Partial sequencing of clones 48A3, 37C5, and 52F10 indicated that they contain partial sequences. The sequence of the full-length cDNA was compiled from the direct sequencing of 17C9 and the partial sequencing of 37C5, 48A3, and 52F10 (Fig. 3*B*). The open reading frame encodes a protein of 615 amino acids with a hydrophobic N terminus, which is probably the signal peptide for the secreted protein (Fig. 4, *A* and *B*). There is a good consensus sequence for the hydrolysis of the peptide bond between residues 17 and 18 by a signal peptidase (32). The calculated M_r for the protein including the predicted signal sequence is 70,746. The deduced primary structure of the *AnCE* gene product has a high degree of homology to both the N- and C-domains of mammalian sACE and to mammalian tACE (see Fig. 4*C* for the comparison of AnCE with tACEs). This strong sequence similarity to mammalian ACE suggests that the clone 17C9 is a cDNA for an insect ACE which, like mammalian tACE, is a single-domain protein. The acronym AnCE, rather than ACE, has been given to this insect gene and its protein product, since ACE has already been accepted as the

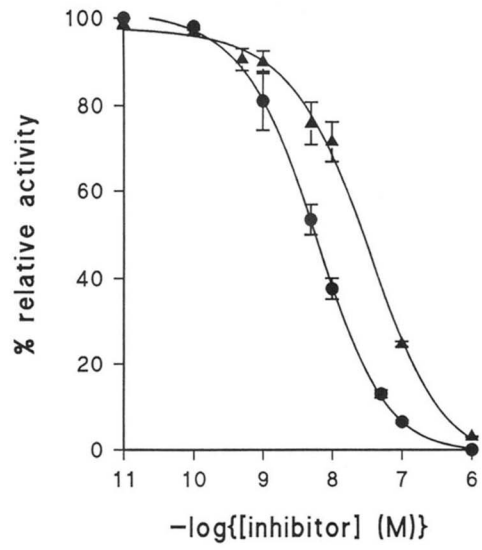


FIG. 6. **Inhibition of recombinant AnCE activity by captopril (●) and trandolaprilat (▲).** The activity of recombinant AnCE from transfected COS-7 cells was assayed in the presence of different concentrations of inhibitor with Hip-His-Leu as the substrate (4). The enzyme was preincubated with inhibitor for 2 h at 37 °C before the addition of substrate. Data are expressed as a percentage of the uninhibited activity and are the mean of triplicate determinations ± S.E. The inhibition plot and the IC_{50} values were generated by a curve-fitting computer program (P.fit, Biosoft).

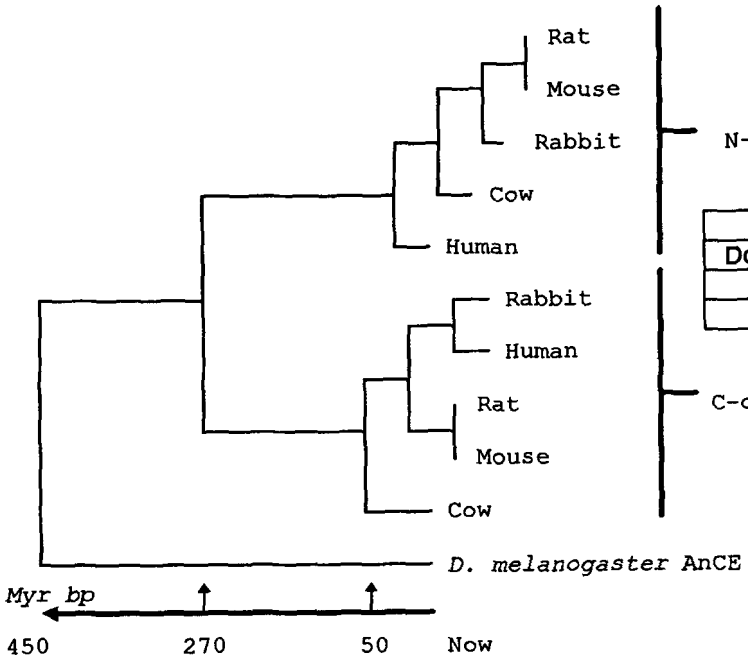
abbreviation for the *D. melanogaster* acetylcholinesterase.

A comparison of the deduced amino acid sequence of AnCE with the sequences of the two domains of mammalian sACEs reveals a number of regions that have common identity. For example, the peptide sequence between residues 311 and 395 of AnCE displays 72 and 76% identity with the same region in the N- and C-domains of human sACE, respectively. This region includes the consensus sequence HExxH in which the 2 histidines serve as zinc ligands and the glutamate is the base donor in catalysis in human ACE (11). In addition, Glu^{395} is conserved, which provides the third amino acid zinc ligand in mammalian ACE (33). The AnCE sequence consistently shows a slightly more favorable alignment with tACEs and with the C-domains of sACEs as opposed to the N-domains. The alignment of the AnCE amino acid sequence with human (14, 16), rabbit (15), and mouse (17) tACEs (Fig. 4*C*) identifies 5 conserved cysteines. There are three consensus sequences for *N*-glycosylation sites in AnCE, the first of which has been conserved and corresponds to the first potential *N*-glycosylation site of both the N- and C-domains of sACE.

Analysis of hydrophobicity confirms the presence of a potential hydrophobic signal sequence at the N terminus of AnCE, but there is no hydrophobic region toward the C terminus. This is in contrast to the situation in mammals, where ACE is anchored to the plasma membrane by a hydrophobic transmembrane domain close to the C terminus (3). This suggested the possibility that the insect cDNA codes for a soluble form of AnCE.

Southern blot analysis of genomic *D. melanogaster* DNA digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Xba*I gave single hybridizing bands of 10.8, 19.1, 6.8, 3.7, and 7.7 kb when probed with the complete AnCE cDNA (clone 53B11). Using the same cDNA probe, the *AnCE* gene was localized to a single site

in *italics*, the three glycosylation sites are *underlined*, and the conserved HExxH motif and the conserved E^{395} are *double underlined*. *C*, alignments of the human, mouse, and rabbit tACE and AnCE. *Con.* is the consensus sequence; *capitalized residues* are conserved in all sequences; *lower case* are conservative substitutions. A *dash* represents a site where there is no consensus, and a *dot* represents gaps inserted for optimal alignment. *Arrows* mark the conserved cysteines; *underlining* is as in *B*.



Average % similarity			
Domains	N	C	AnCE
N	93	73	62
C		91	64

FIG. 7. **Dendrogram showing the relationships between the core sequences of the mammalian N- and C-domains, and *D. melanogaster* AnCE.** The line below gives the estimated time points for the original divergence (450 million years before present), the duplication event (270 million yearsr before present), and the radiation of the mammals (50 million years before present) as calculated by the KITSCH algorithm based on these sequences. The table at right summarizes the average relative percent similarities within and between domains and between the N- and C-domains of the mammalian enzyme (as defined under "Materials and Methods") and AnCE, as calculated using the GCG Distances programme.

at band 34A on chromosome 2. Northern blot hybridization of total RNA extracted from *D. melanogaster* embryos revealed a single transcript of around 2.1 kb (Fig. 5*A*).

Expression of AnCE in COS-7 Cells—Expression of AnCE in COS-7 cells resulted in 93% of the total ACE activity in the serum-free medium, which was inhibited by trandolaprilat (10 μM), with little enzymatic activity in the cells (Table I), whereas medium and cells from mock transfected cell cultures contained negligible levels of peptidase activity. COS-7 cells transfected with $pEACE_{\Delta COOH}$, which codes for human sACE minus the membrane spanning and cytoplasmic domains, also produced a soluble form of ACE. In contrast, cells transfected with pEACE, which codes for the entire sequence of human sACE including the C-terminal membrane anchor, synthesized membrane-bound ACE (Table I). However, 43% of this activity was found in the medium, which we assume resulted from post-translational cleavage of the C-terminal anchor peptide of the membrane-bound enzyme, as observed previously when pEACE was expressed in Chinese hamster ovary cells (4). Immunoblot analysis of serum-free medium taken from pCMV/AnCE transfected cells with a polyclonal antibody raised to housefly AnCE revealed a band with an M_r of 74,000 on SDS-PAGE, which was not present in medium from mock transfected COS-7 cells (Fig. 5*B*).

Recombinant AnCE from the medium of transfected cells, gave a K_m of 1.64 mM for the hydrolysis of Hip-His-Leu, and this activity was inhibited by captopril (IC_{50} value, 5.6×10^{-9} M) and trandolaprilat (IC_{50} value, 2×10^{-8} M), both of which are potent and selective inhibitors of mammalian ACE (Fig. 6). Recombinant AnCE also catalyzed the conversion of AI to AII, and this activity was inhibited by both captopril and trandolaprilat.

DISCUSSION

We have purified a peptidyl dipeptidase with an apparent M_r of 67,000 from embryos of *D. melanogaster* using a lisinopril-Sepharose column which is routinely used to affinity purify ACE from various mammalian sources (5, 23, 34, 35). The enzyme is very similar to mammalian ACE in that it converts AI to AII (K_m, 365 μM), removes the C-terminal dipeptide from BK (K_m, 22 μM), and is inhibited by the ACE inhibitors, captopril and trandolaprilat. Some differences between the insect and mammalian proteins can be inferred from the lower affinity of the *Drosophila* enzyme for both AI and BK (K_m for hydrolysis of AI and BK by mammalian ACEs, 16–70 and 0.18–1.00 μM, respectively) (36–38). The insect enzyme is much smaller than mammalian sACE (M_r 140,000–180,000) and tACE (M_r 90,000–110,000), both of which are heavily glycosylated, but is similar in size to the non-glycosylated form of the single-domain tACE (76–84 kDa, depending on species) (3, 39) and the N-domain ACE (68 kDa), recently found in human ileal fluid (35).

The purification of the *Drosophila* AnCE did not yield sufficient protein for sequence analysis which would have been necessary for the design of oligonucleotide probes for use in screening insect DNA libraries. An alternative strategy of using the cDNA for human endothelial ACE as a heterologous probe led to the isolation of cDNA clones with regions of homology to human ACE cDNA from a 4–8-h *Drosophila* embryo cDNA library. The deduced amino acid sequence of the AnCE protein predicted a number of structural features of an insect protein with an M_r of 68,921 after cleavage of the putative signal peptide. The strong sequence homology around the active site region and the conservation of the amino acid residues known to contribute to catalysis and coordination with the active site Zn^{2+} of human ACE provides strong evidence for the identification of a cDNA coding for a *Drosophila* AnCE. The functional expression of the *AnCE* cDNA in COS-7 cells confirmed that the translated protein product is an ACE which is inhibited by the selective ACE inhibitors, captopril and trandolaprilat. The potencies of these inhibitors against the recombinant (captopril, $IC_{50} = 5.6 \times 10^{-9}$ M; trandolaprilat, $IC_{50} = 2 \times 10^{-8}$ M) and native enzyme (captopril, $IC_{50} = 1.1 \times 10^{-9}$ M; trandolaprilat, $IC_{50} = 1.6 \times 10^{-8}$ M) are very similar. The

molecular mass of the recombinant AnCE expressed in COS-7 cells appears to be ~5 kDa larger than the affinity-purified enzyme and is probably the consequence of differences between insect and mammalian cells in post-translational modification of the protein. The absence of a hydrophobic region toward the C-terminal part of the protein identifies an important difference between insect and mammalian ACEs. Most of the mammalian ACE occurs as a membrane-bound form, although a soluble two-domain form of enzyme is found in plasma, cerebrospinal fluid, seminal fluid, and lymph (3, 40). It has been clearly shown in mammalian cells that the soluble two-domain ACE can be derived from membrane sACE by a proteolytic cleavage, which releases the extracellular domain of this enzyme (4, 41, 42). Recently, a soluble N-domain of human sACE has been found in human ileal fluid, and it is believed that this enzyme is derived from proteolysis of the intact two-domain sACE of the intestine (35). There is no evidence for the direct biosynthesis in mammalian tissues of a soluble ACE from an alternatively spliced mRNA. In contrast, the *AnCE* cDNA from *Drosophila* embryos codes for a soluble enzyme which is secreted from transfected COS-7 cells. A hydrophobic signal sequence is predicted, which in some ectopeptidases is not cleaved and serves as a membrane anchor (43). We cannot at present be certain that this does not occur to some extent with the product of the *AnCE* gene *in vivo*, but we have shown that only around 11% of the AnCE activity of *Drosophila* embryos is associated with cellular membranes.

The fact that mammalian sACE consists of two highly homologous domains indicates that the mammalian genes arose from duplication of an ancestral ACE gene. The evidence obtained in this study indicates that the insect *AnCE* gene is much smaller than the corresponding ACE genes of mammalian species and, therefore, might resemble the ancestral form of the ACE gene prior to the duplication event. Comparison of the core regions of the two mammalian sACE domains with the single AnCE core domain shows three significant events in evolutionary terms: the divergence of the higher insect and mammal lineages (usually put at 550 million years ago), the radiation of the modern mammals (65 million years ago), and the gene duplication event that led from a single-domain ACE-like protein to the mammalian two domain sACE. As noted previously (6, 20), the duplication event predates the radiation of the mammals because the percent similarity between the core domains is less than the percent similarity within the C- and N-domain groupings (Fig. 7, 73% as against 91–93%). The KITSCH algorithm used to produce the tree in Fig. 4 attempts to calculate time scales (31). Clearly the time spans it comes up with are underestimates, presumably because the sequences involved are under strong selection. It is possible to say that the duplication probably occurred in the Deuterostome lineage, as seen by the greater similarity between the N- and C-domains than between either N- or C-domains and AnCE. Secondly, an estimate can be made of the latest time at which the duplication must have occurred. Correcting for the time compression introduced by selection gives a minimum estimate of 330–350 million years ago before present, at about the time of the appearance and radiation of the amphibians, during the mid-Paleozoic. Already there is biochemical evidence that *Torpedo marmorata* has a double-domain ACE, based on size estimates of the protein (34). This puts the date for the duplication much earlier in history, at the time of the development of the Chordata, or represents a different event, either another duplication or some other alteration leading to a larger ACE protein.

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