Drosophila melanogaster angiotensin I-converting enzyme expressed in *Pichia pastoris* resembles the C domain of the mammalian homologue and does not require glycosylation for secretion and enzymic activity

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Drosophila melanogaster angiotensin I-converting enzyme (AnCE) is a secreted single-domain homologue of mammalian angiotensin I-converting enzyme (ACE) which comprises two domains (N and C domains). In order to characterize in detail the enzymic properties of AnCE and to study the influence of glycosylation on the secretion and enzymic activity of this enzyme, we overexpressed AnCE (expression level, 160 mg/l) and an unglycosylated mutant (expression level, 43 mg/l) in the yeast *Pichia pastoris*. The recombinant enzyme was apparently homogeneous on SDS/PAGE without purification and partial deglycosylation demonstrated that all three potential sites for N-linked glycosylation were occupied by oligosaccharide chains. Each N-glycosylation sequence (Asn-Xaa-Ser/Thr) was disrupted by substituting a glutamine for the asparagine residue at amino acid positions 53, 196 and 311 by site-directed mutagenesis to produce a single mutant. Expression of the unglycosylated mutant in *Pichia* produced a secreted catalytically active enzyme ($AnCE_{\Delta CHO}$). This mutant displayed unaltered kinetics for the hydrolyses of hippuryl-His-Leu, angiotensin I and *N*-acetyl-Ser-Asp-Lys-Pro (AcSDKP) and was equally sensitive to ACE inhibitors compared with wild-type AnCE. However, $AnCE_{\Delta CHO}$ was less stable, displaying a half-life of 4.94 h at 37 °C, compared with AnCE which retained full activity under the same conditions. Two catalytic criteria demonstrate the functional resemblance of AnCE with the human ACE C domain: first, the k_{cat}/K_m of AcSDKP hydrolysis and secondly, the k_{cat}/K_m and optimal chloride concentration for hippuryl-His-Leu hydrolysis. A range of ACE inhibitors were far less potent towards AnCE compared with the human ACE domains, except for captopril which suggests an alternative structure in AnCE corresponding to the region of the S_1 subsite in the human ACE active sites.

INTRODUCTION

Angiotensin I-converting enzyme (ACE, peptidyl dipeptidase A, EC 3.4.15.1) is a zinc metallopeptidase which plays an important role in blood pressure homoeostasis by cleaving the C-terminal dipeptide from angiotensin I to produce the vasopressor peptide angiotensin II [1] and by inactivating the vasodilatory peptide bradykinin [2,3]. ACE is primarily expressed as an ectoenzyme and two distinct ACE isoenzymes have been identified in mammalian tissues, the primary structures of which have been determined by molecular cloning (for reviews see [4–6]). The larger isoenzyme (somatic ACE, 170 kDa in humans) displays a wide tissue distribution and is composed of two highly similar domains (referred to here as the N and C domains) each of which bears a functional catalytic site [7]. However, the two active sites display some contrasted enzymic properties; for example, the C domain hydrolyses angiotensin I and substance P at a faster rate compared with the N domain and the N-terminal cleavage of luteinizing hormone-releasing hormone is performed faster by the N-domain active site [8]. In addition, an N-domain-specific natural substrate has been identified recently as the haemoregulatory peptide, *N*-acetyl-Ser-Asp-Lys-Pro (AcSDKP) [9]. The high level of internal homology of the somatic ACE molecule led to the hypothesis that somatic ACE is encoded by a duplicated gene [10,11]. The other isoenzyme (germinal ACE, 100 kDa in humans) is localized exclusively to the testes and is associated with mature germ cells [12,13]. Germinal ACE is comprised of a single domain with a single catalytic site and is derived from the same duplicated gene as somatic ACE but is under the transcriptional control of a germinal ACE-specific intragenic promoter [14–16].

ACE-like enzymes have been identified in a number of non-mammalian species including the *Torpedo marmorata* electric organ [17], the goldfish *Carassius auratus* [18], the chicken [19], and an insect angiotensin-converting enzyme (referred to as 'AnCE') has been identified in the housefly *Musca domestica* [20] and in *Drosophila melanogaster* [21,22]. Interestingly, the molecular sizes of AnCE from *M. domestica* and *D. melanogaster* are consistent with single-domain enzymes (87 and 74 kDa respectively). The molecular cloning of *Drosophila* AnCE confirmed that it comprises a single domain bearing a single catalytic site and notably, unlike both mammalian somatic and germinal ACE, AnCE has no hydrophobic sequence in the C-terminal region for membrane anchorage [21,22]. Furthermore, Southern blot analysis of *Drosophila* genomic DNA indicated that, in contrast to germinal ACE, AnCE is encoded by a non-duplicated gene [21]. The AnCE amino acid sequence is highly homologous to both the N and the C domains of human somatic ACE (residues 311–395 of AnCE bear 72 and 76 % sequence similarity to the corresponding sequences in the N and C domains of human ACE, respectively). Therefore, from primary structure analysis it is not possible to deduce whether AnCE is more closely related to either the N or C domains of the mammalian enzyme.

The primary structure of AnCE contains the Asn-Xaa-Ser/Thr motif three times for the potential addition of N-linked oligo-

Abbreviations used: ACE, angiotensin I-converting enzyme; AnCE, *Drosophila* angiotensin I-converting enzyme; $AnCE_{\Delta CHO}$, unglycosylated AnCE mutant; Hip, hippuryl (benzoyl-glycine); AcSDKP, *N*-acetyl-Ser-Asp-Lys-Pro; *AOX1*, alcohol oxidase gene.

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saccharide chains. This is in contrast to 17 such sites in human somatic ACE [10] and eight in human germinal ACE [23], which is also extensively O-glycosylated in the N-terminal region [24,25]. The absence of the O-glycosylation from human and rabbit germinal ACE does not affect the enzyme's catalytic activity or stability. In contrast, rabbit germinal ACE devoid of both O- and N-linked carbohydrate chains is rapidly degraded in cells in culture, indicating a possible role for the oligosaccharide chains in the protection of the enzyme from intracellular proteolytic degradation [25].

We report the high level expression of AnCE and a catalytically active unglycosylated AnCE mutant ($AnCE_{\Delta CHO}$) in the yeast *Pichia pastoris* by replacement of the *Pichia AOX1* (alcohol oxidase) gene by homologous recombination [26]. This enabled the comparison of the catalytic properties, inhibitor sensitivities and relative stabilities of recombinant AnCE and $AnCE_{\Delta CHO}$. Finally, we compared the enzymic properties of AnCE with both domains of human ACE, which highlights some structural differences existing between the AnCE and human ACE N- and C-domain active sites.

EXPERIMENTAL

Construction of transfer vector for *Pichia* spheroplast transformation

A unique *Eco*RI site was introduced by PCR into the AnCE cDNA (Figure 1) contained in the expression vector pRC/CMV (pCMVAnCE [21]) such that cleavage by *Eco*RI occurs after nucleotide 138, which removes the nucleotide sequence encoding the signal peptide [21]. A 167 bp *Eco*RI/*Bsp*HI fragment of the PCR product was excised and ligated with the 1.7 kb *Bsp*HI/*Not*I fragment of the AnCE cDNA into the *P. pastoris* transfer vector pPIC9 (Invitrogen) digested with *Eco*RI/*Not*I. The resulting construct was termed pPIC9AnCE and was characterized by restriction mapping and sequencing of the region 5′ to the AnCE cDNA to ensure an in-frame fusion between the secretion signal peptide sequence of prepro-α-factor (present in the vector pPIC9)

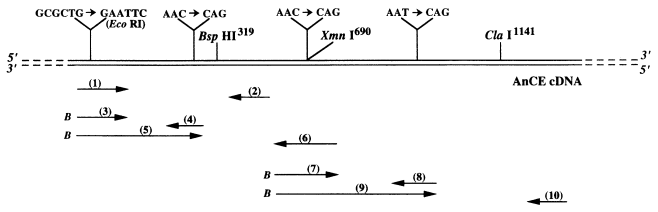

Figure 1 Introduction of mutations into the AnCE cDNA by PCR-mediated mutagenesis

The unique restriction sites used for the digestion of PCR products are shown and the numbers in superscript indicate the nucleotides after which the enzymes cleave. Primers 3, 5, 7 and 9 are biotinylated (indicated by *B*) to allow the purification of the sense strand. The first mutation introduces an *Eco*RI site into the AnCE cDNA to allow the removal of the sequence encoding the signal peptide as described in the Experimental section. The other three mutations were introduced to replace the asparagine residues at amino acid positions 53, 196 and 311 for glutamine residues, thus destroying the consensus sequences for N-linked oligosaccharide addition. Primer numbers are shown in parentheses and primer sequences are as follows (mismatched bases are underlined): primer 1, 5′-^{123}TTGGCGGTAACCCAAGAATTCCTGGTCA-AGGAG^{154}-3′; primer 2, 5′-^{352}AACTTGGTGGTATCA^{338}-3′; primer 3, 5′-biotin-^{123}TTGGCGGT-AACCCAAGAATTCGTCAAGGAGGAGATA^{154}-3′; primer 4, 5′-^{275}CTCGTCGGTGATCTGGGAGCC-ATAGGC^{249}-3′; primer 5, 5′-biotin-152 base megaprimer (nucleotides 123–275); primer 6, 5′-^{703}CCAGAAGTGAACTGATTCAGTTTGG^{679}-3′; primer 7, 5′-biotin-^{679}CCAAACTGAATCAGTTC-ACTTCTGG^{703}-3′; primer 8, 5′-^{1048}AGCTTGGTAAGCTGCATAGACGTGA^{1024}-3′; primer 9, 5′-biotin-369 base megaprimer (nucleotides 679–1048); primer 10, 5′-^{1235}CTGAAGGAAA-TACTG^{1221}-3′.

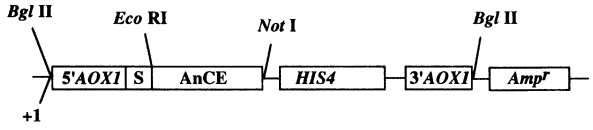

Figure 2 Transfer vector construction (pPIC9AnCE) for spheroplast transformation

A unique *Eco*RI site, introduced into the AnCE cDNA by PCR-mediated mutagenesis, allowed the fusion of the AnCE cDNA, minus the sequence encoding the signal peptide, in the same open reading frame as the *Saccharomyces cerevisiae* α-factor secretion signal peptide and prepro sequence (denoted by S) thus producing *S–AnCE*. The *HIS4* gene product is histidinol dehydrogenase which acts as a selectable marker by growth of transformants on histidine-deficient plates. *S–AnCE* and *HIS4* are flanked by sequences specific to the alcohol oxidase gene (*AOX1*) which allow replacement of the *AOX1* gene in the *Pichia* genome. The *AOX1* gene product (alcohol oxidase) enables the utilization of methanol as a source of carbon; thus recombinants in which the *AOX1* gene has been replaced by the desired *S–AnCE* sequence exhibit slow or no growth on plates containing methanol as the sole carbon source. The *Bgl*II sites allow linearization of the plasmid construct for maximum transformation efficiency [44] and *Amp^r* is the ampicillin resistance gene.

and the AnCE cDNA. A schematic diagram of the construct pPIC9AnCE is shown in Figure 2.

PCR-mediated mutagenesis of N-linked oligosaccharide consensus sequences

The AnCE cDNA contains three consensus sequences (Asn-Xaa-Ser/Thr) for the potential addition of N-linked oligosaccharides. For each of the three consensus sequences, the asparagine was mutated to a glutamine (Asn-53 → Gln, Asn-196 → Gln and Asn-311 → Gln) to produce a single mutant with all three N-glycosylation sites mutated. All mutations were introduced by PCR-mediated mutagenesis using the megaprimer method described previously [27,28] except that the 5′-flanking primers were biotinylated to enable purification of the sense strand on magnetic streptavidin beads (Dynabeads, Dynal) after purification of the PCR product on an agarose gel to eliminate unused biotinylated primer. PCR products were ligated to form the final construction, termed $pPIC9AnCE_{\Delta CHO}$, according to Figure 1.

Expression in *P. pastoris*

pPIC9AnCE or $pPIC9AnCE_{\Delta CHO}$ (10 μg) was linearized by digestion with *Bgl*II (Figure 2) before transformation of the yeast *P. pastoris* strain GS115 spheroplasts prepared by zymolase partial digestion according to the manufacturer's instructions (Invitrogen *Pichia* expression kit). All methods for the screening of recombinant colonies and for protein expression were carried out as described in the users' manual for the *P. pastoris* expression system (Invitrogen). After expression of recombinant AnCE and $AnCE_{\Delta CHO}$, yeast cells were removed by centrifugation (4000 ***g*** for 10 min at room temperature), the supernatant was dialysed against water, centrifuged (10000 rev./min for 60 min at 4 °C) and concentrated using a pressurized cell fitted with a membrane with a 50 kDa cut-off limit (Amicon). Protein concentrations were determined according to the method described by Lowry et al. [29] and were verified by quantitative amino acid analysis.

Characterization of AnCE expressed in *P. pastoris*

Hip-His-Leu

Reactions were performed in duplicate in 100 mM potassium phosphate, pH 8.3, 10 μM $ZnSO_4$, 300 mM NaCl, 1 mg/ml

lysozyme and the liberated hippuric acid was resolved and quantified by reverse-phase HPLC [30]. The specificity of the reaction was confirmed by a control incubation in the presence of the specific ACE inhibitor captopril (10 μM). K_m and k_{cat} values for Hip-His-Leu hydrolysis were determined as described previously [30a] using 0.15×10^{-9} M enzyme.

Apparent K_i values for the specific ACE inhibitors trandolaprilat, captopril, enalaprilat, lisinopril and fosinoprilat were determined as described previously [31] using a final enzyme concentration of 0.5×10^{-10} M. Trandolaprilat was a gift from Roussel-Uclaf, captopril and fosinoprilat were gifts from the Bristol-Myers Squibb Institute, and enalaprilat and lisinopril were gifts from Merck, Sharp and Dohme Research Laboratories.

For determination of enzyme stability, equal concentrations of recombinant AnCE and $AnCE_{\Delta CHO}$ (50 μg of enzyme/ml of culture supernatant) were preincubated at 37 °C for various time intervals (0–300 min) before quantification of residual activity as described above using a final concentration of 5 mM Hip-His-Leu. The half-life of the $AnCE_{\Delta CHO}$ activity was determined by plotting log (% of original activity) against the preincubation time and was calculated from the following: slope $= -k/2.3$ and $k = \ln 2/t_{\frac{1}{2}}$ where k is the inactivation rate and $t_{\frac{1}{2}}$ is the half-life of enzyme activity.

Angiotensin I

Reactions were performed in duplicate using 0.5×10^{-9} M enzyme in 50 mM Hepes, pH 7.5, 50 mM NaCl, 10 μM $ZnSO_4$ and 1 mg/ml BSA. Reactions were initiated by the addition of angiotensin I (3–300 μM, final concentration) in a total volume of 250 μl and incubated at 37 °C for 10 min to produce 5% angiotensin I hydrolysis. Reactions were terminated by the addition of 12% H_3PO_4 (50 μl). Angiotensin II was resolved and quantified by isocratic reverse-phase HPLC in 18% (v/v) acetonitrile/86 mM triethylammonium phosphate, pH 3, at a flow rate of 1.0 ml/min on a 3 μm Hypersil C_{18} column at 45 °C. K_m and k_{cat} values were determined from Lineweaver–Burk plots.

AcSDKP

Reactions were performed in duplicate using 1×10^{-8} M enzyme in 100 mM Hepes, pH 7.0, 50 mM NaCl, 10 μM $ZnSO_4$ and 1 mg/ml BSA. Reactions were initiated by the addition of AcSDKP (0.004–1 mM, final concentration) in a total volume of 250 μl and incubated for 10 min at 37 °C to produce 5% AcSDKP hydrolysis. Reactions were terminated by the addition of 5% trifluoroacetic acid (50 μl). Lysyl-proline was resolved and quantified by isocratic reverse-phase HPLC in 0.1% trifluoroacetic acid in water on a 3 μm Nucleosil C_{18} column at 37 °C. K_m and k_{cat} values were determined from Lineweaver–Burk plots.

Deglycosylation of AnCE expressed in *P. pastoris*

AnCE and $AnCE_{\Delta CHO}$ (200 ng) were denatured by heating at 95 °C for 5 min in the presence of 0.1% SDS and 1% 2-mercaptoethanol in a total volume of 10 μl. After denaturation, samples were incubated in the presence of 250 mM sodium phosphate, pH 7.5, 0.5% Triton X-100 and 0.8 unit of endoglycosidase F (PGNase F) (Boehringer Mannheim) in a total volume of 20 μl at 37 °C overnight. For partial deglycosylations, non-denatured AnCE (200 ng) was incubated in the presence of 250 mM sodium phosphate, pH 7.5, and endoglycosidase F (0.6 unit) in a total volume of 10 μl at 37 °C overnight. For each deglycosylation a mock-treated AnCE or $AnCE_{\Delta CHO}$ sample was included where the denatured enzyme was incubated under the same conditions except in the absence of endoglycosidase F.

N-terminal amino acid sequencing

Recombinant AnCE (90 pmol) was resolved on SDS/10%-PAGE and transferred to an Immobilon P (Millipore) membrane. The AnCE protein was visualized by staining for 3–5 s with 0.1% Amido Black in 40% (v/v) methanol and 1% (v/v) acetic acid and the membrane was washed thoroughly with distilled water. The stained band corresponding to AnCE was excised and subjected to automated Edman degradation on an Applied Biosystems model 473A sequencer.

RESULTS

Expression of AnCE in *P. pastoris*

After the induction of expression from 20 putative *Pichia* recombinants and yeast cell removal, supernatants were analysed by SDS/PAGE, revealing the high-level expression of a secreted protein which migrated with a molecular mass of 74 kDa, which is consistent with the molecular mass of AnCE expressed by COS-7 cells [21]. This protein was expressed from 17 out of 20 putative recombinants and was correlated with the presence of high levels of ACE activity using the synthetic ACE substrate Hip-His-Leu (10.2–17.1 μmol of Hip produced/min per ml of supernatant) which was abolished by the specific ACE inhibitor captopril (10 μM). In addition, a polyclonal antibody raised against purified *Musca domestica* AnCE cross-reacted with the 74 kDa protein on Western blotting (result not shown). The recombinant yeast clone producing the highest level of Hip-His-Leu hydrolysing activity produced an expression of AnCE at a level of 160 mg/l of culture medium supernatant and SDS/PAGE analysis revealed the apparent absence of contaminating secreted proteins in the culture supernatant after yeast cell removal (Figure 3, lane 1).

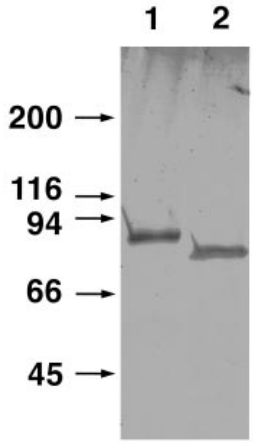

Figure 3 Expression of AnCE and $AnCE_{\Delta CHO}$ in *P. pastoris*

After induction of the *AOX1* promoter by methanol, yeast cells were removed by centrifugation and an aliquot of the supernatant (10 μl) was analysed by 4–15% SDS/PAGE and proteins were visualized by silver staining. Numbers on the left of the Figure indicate the molecular masses of molecular standards in kDa. AnCE (lane 1) migrated with an apparent molecular mass of 74 kDa and the unglycosylated mutant, $AnCE_{\Delta CHO}$ (lane 2), migrated with an apparent molecular mass of 69 kDa.

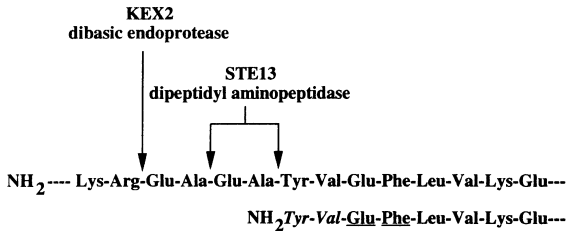

Figure 4 N-terminal sequence of AnCE expressed in *P. pastoris*

The sequence at the top of the Figure shows the cDNA predicted amino acid sequence of part of the prepro-α-factor fused to the AnCE sequence (Glu-Phe-Leu-Val-Lys-Glu-). The sequence at the bottom of the Figure shows the N-terminal sequence of AnCE secreted from *P. pastoris*. The residues in *italics* are those which are derived from prepro-α-factor and the underlined residues are derived from the introduction of the *Eco*RI site to enable the in-frame fusion of the prepro-α-factor and the AnCE sequences. The sites of prepro-α-factor processing identified in *Saccharomyces cerevisiae* are also shown, comprising a dibasic endoprotease cleavage by the *KEX2* gene product [40] and, subsequently, two consecutive dipeptidyl aminopeptidase cleavages by the *STE13* gene product [39].

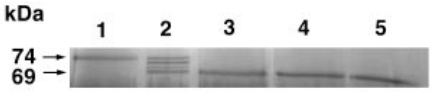

Figure 5 Deglycosylation of AnCE under denaturing and non-denaturing conditions

Deglycosylation of denatured or non-denatured AnCE by endoglycosidase F as described in the Experimental section. Proteins were resolved on an SDS/10%-PAGE and visualized by silver-staining. Lane 1, mock-treated denatured AnCE (200 ng); lane 2, non-denatured AnCE (200 ng), partially deglycosylated; lane 3, denatured AnCE (200 ng), deglycosylated; lane 4, mock-treated denatured $AnCE_{\Delta CHO}$ (200 ng); lane 5, denatured $AnCE_{\Delta CHO}$ (200 ng) treated with endoglycosidase F. Numbers on the left of the Figure indicate the molecular masses of glycosylated and unglycosylated forms.

Post-translational modifications of the prepro-α-factor–AnCE fusion protein

Signal peptide processing

N-terminal sequencing of recombinant AnCE identified unambiguously a single amino acid sequence which corresponded to the predicted sequence of the secretion signal peptide of prepro-α-factor–AnCE fusion protein after signal peptide processing by the dibasic endoprotease *KEX2* gene product and the sequential removal of the two Glu-Ala N-terminal dipeptides by the *STE13* gene product (Figure 4).

N-linked glycosylation

Complete removal of N-linked oligosaccharides by endoglycosidase F produced a protein migrating on SDS/PAGE with an apparent molecular mass of 69 kDa (Figure 5, lane 3), consistent with the size of the cDNA-predicted AnCE polypeptide chain (68.9 kDa [21]). Partial digestion of recombinant AnCE without prior denaturation of the protein produced a ladder of four discrete bands with apparent molecular masses between 69 and 74 kDa (Figure 5, lane 2) corresponding to the presence of 0, 1, 2 and 3 oligosaccharide chains, thus demonstrating the occupation of all potential sites for N-linked glycosylation by glycan chains.

Expression of $AnCE_{\Delta CHO}$ in *P. pastoris*

We disrupted the three consensus sequences for the addition of N-linked oligosaccharides by mutating the asparagine residue in the Asn-Xaa-Ser/Thr motif to a glutamine. The mutant enzyme ($AnCE_{\Delta CHO}$) was expressed in *P. pastoris* as for the wild-type enzyme and SDS/PAGE of yeast culture supernatants revealed the expression of a single secreted protein which migrated with an apparent molecular mass of 69 kDa (Figure 3, lane 2). $AnCE_{\Delta CHO}$ was expressed at a level of 43 mg/l of culture medium supernatant, and the mutant enzyme co-migrated on SDS/PAGE with deglycosylated wild-type AnCE (Figure 5, lanes 4 and 3, respectively) and incubation of $AnCE_{\Delta CHO}$ with endoglycosidase F did not alter the migration of the protein on SDS/PAGE compared with the mock-treated sample (Figure 5, lanes 5 and 4, respectively).

Enzymic characterization of AnCE and $AnCE_{\Delta CHO}$

The kinetic constants of recombinant AnCE and $AnCE_{\Delta CHO}$ for the ACE substrates Hip-His-Leu, angiotensin I and the specific substrate of the N-domain active site, AcSDKP [9], are shown in Table 1. AnCE and $AnCE_{\Delta CHO}$ displayed similar enzymic characteristics towards these three substrates with both recombinant enzymes displaying K_m, k_{cat} and k_{cat}/K_m values in the same order of magnitude for each substrate and both enzymes exhibiting an

Table 1 Kinetic constants for the hydrolysis of Hip-His-Leu, angiotensin I and Ac-SDKP

Data are the means of three independent determinations and assay conditions were as described under the Experimental section. $AnCE_{wt}$ is wild-type recombinant AnCE and $AnCE_{\Delta CHO}$ is the unglycosylated recombinant AnCE mutant.

	Hip-His-Leu			Angiotensin I			Ac-SDKP		
Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
Drosophila ACE (AnCE)									
$AnCE_{wt}$	1740	155	0.09	33.5	17.9	0.53	53.4	1.2	0.02
$AnCE_{\Delta CHO}$	1091	141	0.13	69.1	16.2	0.19	44.6	0.7	0.02
Human ACE									
N domain	2000	40	0.02*	15.0	11.0	0.73‡	31.0	15.5	0.50‡
C domain	1620	99	0.07†	18.0	34.0	1.89‡	39.0	0.4	0.01‡

* Data from Wei et al. [7].
† Data from Williams et al. [30a].
‡ Data from Rousseau et al. [9].

Table 2 Inhibition constants using some specific ACE inhibitors

Data are the mean of three to four independent determinations and assay conditions were as described in the Experimental section. $AnCE_{wt}$ is wild-type recombinant AnCE and $AnCE_{\Delta CHO}$ is the unglycosylated recombinant AnCE mutant. ND, not determined.

	$10^{10} \times$ Apparent K_i (M)				
Inhibitor	Captopril	Trandolaprilat	Lisinopril	Enalaprilat	Fosinoprilat
Drosophila ACE (AnCE)					
$AnCE_{wt}$	11.0	140	180	250	470
$AnCE_{\Delta CHO}$	8.6	106	144	231	250
Human ACE*					
N domain	8.9	3.10	44.0	26.0	ND
C domain	14.0	0.29	2.4	6.3	ND

* Data from Wei et al. [31].

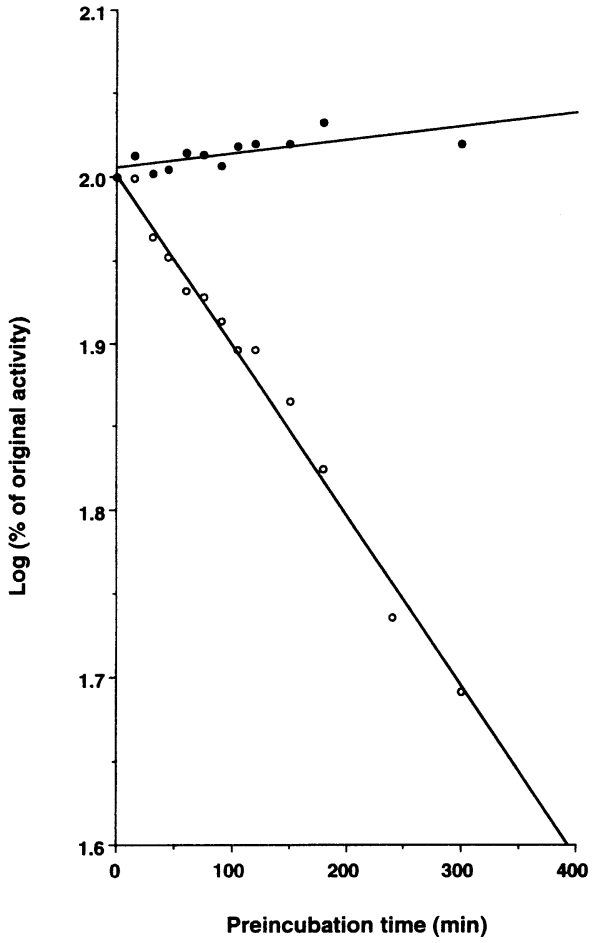

Figure 6 Relative stability of AnCE and $AnCE_{\Delta CHO}$ at 37 °C

Recombinant wild-type AnCE (●) and $AnCE_{\Delta CHO}$ (○) were preincubated at 37 °C for various time intervals (0–300 min) before assaying residual enzyme activity at 37 °C for 30 min using 5 mM Hip-His-Leu as substrate as described in the Experimental section. The activity of the enzyme determined without preincubation at 37 °C was taken as 100% activity. Each point is the mean of three independent determinations performed in duplicate.

optimal chloride concentration for the hydrolysis of Hip-His-Leu in the range of 800–1000 mM (results not shown). In addition, the apparent K_i' values of the specific ACE inhibitors

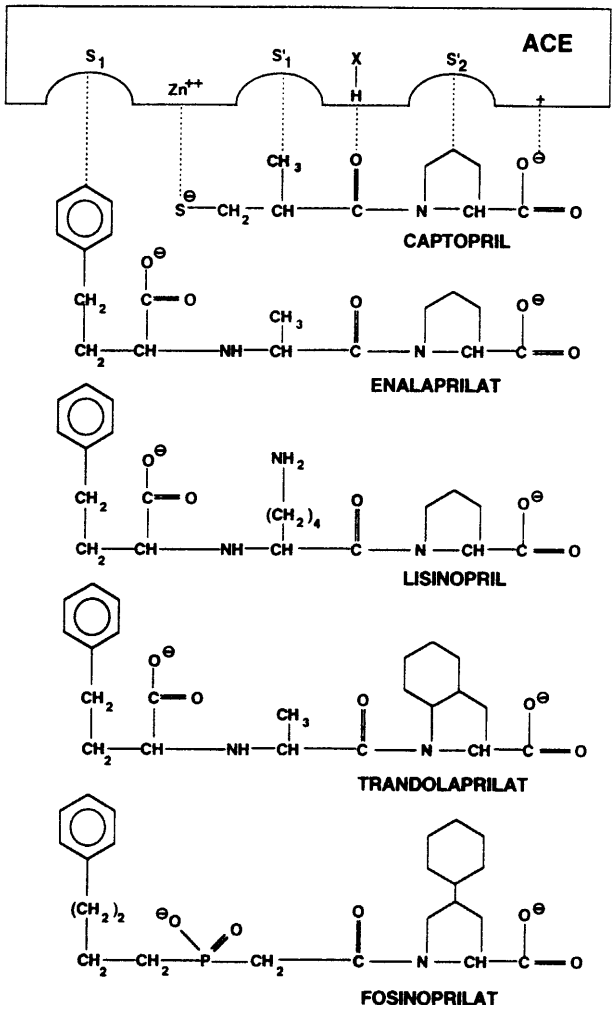

Figure 7 Structures of the specific ACE inhibitors used in the present study

Enalaprilat, lisinopril and trandolaprilat co-ordinate the active-site zinc ion of ACE via their carboxyl groups, whereas captopril and fosinoprilat co-ordinate the active-site zinc via their sulphydryl and phosphoryl groups, respectively. Captopril does not interact with the theoretical S_1 subsite of ACE. This Figure is derived from that of Cushman et al. [45].

captopril, trandolaprilat, lisinopril, enalaprilat and fosinoprilat were similar for both wild-type AnCE and the unglycosylated AnCE mutant (Table 2). However, $AnCE_{\Delta CHO}$ was less thermostable compared with the wild-type enzyme, displaying a half-life for Hip-His-Leu-hydrolysing activity of 4.94 h at 37 °C (Figure 6) in contrast to the wild-type enzyme which displayed full activity after a 5 h preincubation at 37 °C.

Comparison of the enzymic properties of AnCE with the N and C domains of human somatic ACE

The recombinant AnCE enzymic activity displayed a k_{cat}/K_m for the hydrolysis of Hip-His-Leu (0.09 $s^{-1} \cdot \mu M^{-1}$) similar to that of the C domain of human ACE (0.07 $s^{-1} \cdot \mu M^{-1}$), whereas the k_{cat}/K_m of AnCE for angiotensin I (0.53 $s^{-1} \cdot \mu M^{-1}$) was closer to that for the human ACE N domain (0.73 $s^{-1} \cdot \mu M^{-1}$) compared with the C domain (1.89 $s^{-1} \cdot \mu M^{-1}$). Interestingly, recombinant AnCE displayed a k_{cat} for the hydrolysis of the N-domain-specific substrate AcSDKP close to that of the C domain (k_{cat} = 1.2 s^{-1}, 0.4 s^{-1} and 15.5 s^{-1} for AnCE and the C and N domains

of human ACE, respectively) and the k_{cat}/K_m values of AnCE and the human ACE C domain were highly similar (0.02 and 0.01 $s^{-1} \cdot \mu M^{-1}$, respectively). In contrast, the k_{cat}/K_m for the ACE N domain was 25-fold higher compared with that for AnCE (Table 1). In addition, AnCE displayed a high optimal chloride concentration for the hydrolysis of Hip-His-Leu (800–1000 mM, results not shown) as described previously for the C domain of human ACE [7] and for the ACE-like activity purified from the housefly *Musca domestica* [20].

To compare further the enzymic characteristics of AnCE and the N and C domains of human ACE we tested the potency of a number of specific ACE inhibitors whose structures are shown in Figure 7. With the exception of captopril, all inhibitors displayed markedly reduced potencies for the inhibition of AnCE, with apparent K_i values 1–3 orders of magnitude higher compared with the corresponding values for the N and C domains of human ACE (Table 2).

DISCUSSION

Mammalian somatic ACE consists of two highly similar domains, which led to the proposal that the ACE gene arose from the duplication of an ancestral gene [10,11]. The human ACE gene comprises 26 exons and its structure provides further support for the hypothesis of the duplication of an ancestral gene: exons 4–11 and exons 17–24, which encode the two homologous domains of somatic ACE, are highly similar in size and in sequence and the intron–exon boundaries have been conserved [14]. The recent molecular cloning of *Drosophila* ACE (AnCE) revealed a single-domain homologue of mammalian ACE, thus implying the resemblance of AnCE to an ancestral form of ACE prior to the duplication event (estimated as occurring 270 million years ago [21]).

We have previously expressed a secreted form of the C domain of human ACE using the baculovirus-infected insect cell system; however, the purified protein was heterogeneous, appearing as a diffuse band on SDS/PAGE, and the enzyme was expressed at a maximum level of 4 mg/l of culture medium [32]. Recently, the high-level expression of some foreign proteins in the yeast *P. pastoris* has been achieved with considerable success [33–37]. *P. pastoris* is able to perform many of the protein-processing characteristics observed in higher eukaryotes, whereas, unlike *Saccharomyces cerevisiae*, it does not hypermannosylate N-linked oligosaccharide chains [38]. In addition, the absence of protein components in the culture medium and the low secretion level of native *Pichia* proteins is a considerable advantage for the subsequent purification of secreted proteins. Therefore, this system was chosen to express AnCE and to study in detail some of its biochemical and enzymic characteristics.

We expressed AnCE in *Pichia* by replacement of the alcohol oxidase gene (*AOX1*) in the *Pichia* genome with the AnCE cDNA to produce stable *Pichia* transformants. Secretion of the protein was directed by the secretion signal peptide for the *Saccharomyces* α-factor mating pheromone. After the induction of expression from putative recombinants and yeast cell removal, supernatants were analysed by SDS/PAGE, revealing the high-level expression of wild-type AnCE which was apparently homogeneous on SDS/PAGE. The recombinant AnCE was expressed at a level of 160 mg/l of culture medium and migrated on SDS/PAGE with an apparent molecular mass of 74 kDa, which is consistent with the molecular size of AnCE expressed in COS-7 cells [21]. The N-terminal sequence of recombinant AnCE indicated that the yeast prepro-α-factor secretion signal sequence had been correctly processed and was consistent with a cleavage on the C-terminal side of the dibasic Lys-Arg by the endoprotease *KEX2* gene product and, subsequently, the sequential removal of two N-terminal dipeptides (Glu-Ala) by the dipeptidyl aminopeptidase *STE13* gene product as determined previously for the processing of α-prepro factor in *Saccharomyces cerevisiae* [39,40].

The cDNA deduced primary sequence for *Drosophila* AnCE reveals the presence of three consensus sequences for the potential addition of N-linked oligosaccharides [21,22]. Endoglycosidase digestion of recombinant AnCE demonstrated that the N-linked oligosaccharides contribute a total of 5 kDa to the molecular mass of AnCE expressed in *P. pastoris*. A partial digestion of recombinant AnCE produced a ladder of polypeptides on SDS/PAGE corresponding to the removal of zero, one, two and three N-linked carbohydrate chains. Therefore, all three consensus sequences for N-linked glycan addition in the AnCE primary sequence are in fact occupied by carbohydrate. We also expressed an unglycosylated AnCE mutant ($AnCE_{\Delta CHO}$) in *P. pastoris*. As for the wild-type enzyme, $AnCE_{\Delta CHO}$ was expressed and secreted into the culture medium (43 mg/l) with the apparent absence of contaminating secreted proteins. Therefore, the N-linked oligosaccharide chains do not play a key role in the intracellular transport and secretion of AnCE, unlike for many other secreted proteins, for example, human renin [41] and human lipoprotein lipase [42]. Furthermore, the unglycosylated mutant was catalytically active and the kinetic parameters of $AnCE_{\Delta CHO}$ demonstrated that the enzymic properties of this mutant were unaltered compared with those of the wild-type enzyme. In particular, the synthetic ACE substrate Hip-His-Leu, the physiological ACE substrate angiotensin I and the specific substrate for the N domain of human somatic ACE, the haemoregulatory peptide AcSDKP, were hydrolysed in a similar fashion by both AnCE and $AnCE_{\Delta CHO}$. This is the first time that an unglycosylated form of ACE has been produced which remains catalytically active. These observations suggest that the tertiary structure of the active-site region in the unglycosylated AnCE mutant is unaltered compared with the wild-type enzyme, thus indicating that the added carbohydrate chains do not assist in protein folding. In contrast, the presence of the N-linked glycan chains stabilizes the enzymic activity of AnCE consistent with a function in the stabilization of protein conformation. Previous studies on the function of the glycosylation of testicular (germinal) ACE have shown that O-glycosylation is not essential for enzyme activity and stability [24,25]. However, Kasturi et al. [25] demonstrated that unglycosylated rabbit testicular ACE, that is without both O- and N-linked glycosylation, is rapidly degraded in the cell. In contrast, partially N-glycosylated ACE, but without O-glycosylation, is stable and not susceptible to proteolytic degradation [25]. Thus, it would appear that the presence of some but not all N-linked glycans is required to protect germinal ACE from intracellular degradation.

We investigated whether the AnCE activity resembles more closely that of either the N or the C domains of human somatic ACE. The following observations favour a closer similarity of the AnCE activity with the C domain of somatic ACE: (a) the hydrolysis of the N-domain-specific substrate AcSDKP at a rate comparable with that of the C domain of human ACE, and (b) the high optimal chloride concentration for the hydrolysis of Hip-His-Leu similar to that described previously for the human ACE C domain [7] and for an ACE-like activity identified in the housefly *Musca domestica* [20]. Recently, two previously identified lethal mutations in *D. melanogaster* have been shown to map to the AnCE locus. Interestingly, heteroallelic combinations of these two mutations produced occasional adult male survivors which exhibit sterility [22], thus raising the possibility that AnCE plays a role in male fertility. Furthermore, the inactivation of both somatic and germinal/testicular forms of ACE in mice,

although not lethal, produces fertile female homozygotes for the mutation whereas male homozygotes display reduced fertility [43]. Therefore, besides the catalytic similarity demonstrated in the present study between AnCE and the C domain of human ACE, which is essentially germinal/testicular ACE, it would appear that AnCE and mammalian germinal ACE share some common functional features.

Finally, the sensitivity of AnCE and the N and C domains of human ACE to a number of specific ACE inhibitors was investigated. Trandolaprilat, lisinopril, enalaprilat and fosinoprilat display markedly reduced efficacy towards AnCE compared with both domains of human ACE. In contrast, captopril, which does not interact with the S_1 subsite in the theoretical model of ACE–inhibitor interaction (Figure 7), displayed a similar potency towards AnCE and the N and C domains. Thus, these observations highlight structural differences existing between *Drosophila* AnCE and human ACE and indicate an alternative structure in AnCE corresponding to the region of the theoretical S_1 subsite in the human ACE N and C domains. Therefore, although many functional similarities exist between AnCE and mammalian ACE, some structural differences are apparent which probably reflect subtle changes which have accumulated in the ACE active site over the course of evolution towards the optimization of the structure of the ACE active site/s to perform their present functions.

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