ACE for all – a molecular perspective

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Abstract Angiotensin-I converting enzyme (ACE, EC 3.4.15.1) is a zinc dependent dipeptidyl carboxypeptidase with an essential role in mammalian blood pressure regulation as part of the renin-angiotensin aldosterone system (RAAS). As such, it has long been targeted in the treatment of hypertension through the use of ACE inhibitors. Although ACE has been studied since the 1950s, only recently have the full range of functions of this enzyme begun to truly be appreciated. ACE homologues have been found in a host of other organisms, and are now known to be conserved in insects. Insect ACE homologues typically share over 30 % amino acid sequence identity with human ACE. Given that insects lack a mammalian type circulatory system, they must have crucial roles in other physiological processes. The first ACE crystal structures were reported during the last decade and have enabled these enzymes to be studied from an entirely different perspective. Here we review many of these key developments and the implications that they have had on our understanding of the diverse functions of these enzymes. Specifically, we consider how structural information is being used in the design of a new generation of ACE inhibitors with increased specificity, and how the structures of ACE homologues are related to their functions. The Anopheles gambiae genome is predicted to code for ten ACE homologues, more than any genome studied so far. We have modelled the active sites of some of these as yet uncharacterised enzymes to try and infer more about their potential roles at the molecular level.

Keywords Angiotensin-I converting enzyme (ACE) · Molecular structure · Drosophila melanogaster · Anopheles gambiae · Inhibitor design

Introduction

Angiotensin-I converting enzyme (EC number 3.4.15.1) or ACE as it is more commonly known, is an extensively glycosylated integral membrane protein (Hooper et al. 1987; Hooper and Turner 1987; Wei et al. 1991; Ripka et al. 1993) which functions as a zinc dependent dipeptidyl carboxypeptidase to remove the C-terminal dipeptide from its peptide substrates in a zinc dependent manner (Cushman and Cheung 1971; Das and Soffer 1975).

ACE is a key component of the renin angiotensin aldosterone system (RAAS), an essential hormone system responsible for the homeostasis of blood pressure in mammals. ACE processes the precursor peptide angiotensin I to give angiotensin II which is a potent vasoconstrictor. It also destroys the vasodilating properties of a second peptide, bradykinin (Skeggs et al. 1954; Zaman et al. 2002; Acharya et al. 2003; Gonzalez-Villalobos et al. 2013). ACE therefore functions to increase blood pressure, and, as such, has long been targeted in the treatment of hypertension and other cardiovascular ailments through the use of ACE inhibitors (Turner and Hooper 2002).

Mammalian ACE

There are two isoforms of mammalian ACE; somatic (sACE) and testicular (tACE) ACE (Soubrier et al. 1988). Both are integral membrane proteins transcribed from the same twenty-six exon gene. sACE consists of two homologous domains (the N-domain and the C-domain) arranged in tandem, each with its own functional active site. tACE expression is driven from an intragenic promoter, giving rise to a single domain protein identical to the C-domain of sACE, as illustrated in Fig. 1 (Ehlers et al. 1989; Hubert et al. 1991; Wei and Clauser 1992; Corvol et al. 1995). Whilst sACE is widely expressed,
brane domain (TM) and the C-terminus (CT) is positioned inside the cell (LR) in sACE. Like sACE, most of tACE is extracellular with a transmembrane (TM) domain towards the C-terminus (CT) which is intracellular. In contrast, tACE is a single domain protein with only one active, shown here by a single HEExH zinc binding motif. The 36 residues at the N-terminus of sACE followed by the C-terminus (CT) which is intracellular.

Important differences have also been observed in the catalytic properties of the two domains. The C-domain is able to process angiotensin I to angiotensin II much more efficiently than the N-domain, whilst both domains are equally able to hydrolyse bradykinin. The C-domain alone appears sufficient for in vivo regulation of blood pressure (Georgiadis et al. 2003).

A number of substrates have also been identified which are hydrolysed more efficiently by the N-domain compared to the C-domain. These include GnRH (gonadotropin-releasing hormone), A-beta42 (amyloid beta peptide), angiotensin 1–9 and AcSDKP (N-acetyl-seryl-aspartyl-lysyl-proline) (Rousseau et al. 1995; Deddish et al. 1998; Anthony et al. 2012; Masuyer et al. 2012). This is further evidence that the N-domain has, by divergent evolution, become involved in other physiological processes.

The activity of the N-domain towards the peptide AcSDKP is particularly interesting as AcSDKP is involved in regulating the proliferation of fibroblasts in response to injury. A recent detailed study by Bernstein et al. showed that in mice treated with the chemotherapy drug bleomycin to induce lung injury, the level of lung damage was much greater in mice with a mutated sACE N-domain compared to in wild-type mice or those with sACE C-domain mutations. There is strong evidence therefore that the N-domain plays an important role in preventing lung injury, likely due to its role in regulating the proliferation of AcSDKP (Bernstein et al. 2011).

The involvement of ACE in processes outside of blood pressure regulation, and in particular the differences in substrate specificity of the two domains of sACE highlight the growing need for a new generation of ACE inhibitors. The current ACE inhibitors were designed with no structural information and are instead based on peptides isolated from the venom of the Brazilian pit viper Bothrops jararaca (Ferreira et al. 1965; Ferreira et al. 1970; Ondetti et al. 1971; Cushman et al. 1977; Patchett et al. 1980). Most inhibit both domains of sACE equally. Structural studies will be crucial in understanding the reasons for these observed differences and in achieving the goal of a new generation of ACE inhibitors.

Mammalian ACE structures

The initial discovery and partial purification of ACE occurred in the 1950s yet it was not until 2003 that the first human ACE structure, of tACE, was reported (Natesh et al. 2003). One of the main reasons for the 50 years that elapsed between the discovery of ACE and the report of the first structure is the extensive glycosylation, estimated at 30% of the weight of the enzyme (Acharya et al. 2003) but shown to be essential for expression and function (Gordon et al. 2003; Corradi et al. 2007). Ultimately, the structure of tACE was determined using a minimally glycosylated, truncated construct, lacking the transmembrane domain (Natesh et al. 2003).

The structure of tACE is predominantly helical, with twenty-seven α-helices and only six short β-strands, which combine to form an ellipsoid shaped molecule (Fig. 2a and b). The structure shows six glycosylation sites, all located on the surface of the protein. Key features of the molecule include a large central channel and an N-terminal lid. The channel stretches approximately 30 Å across almost the entirety of the molecule, effectively dividing it into two subdomains. The channel narrows at the centre, where the catalytic zinc ion is found. As shown in Fig. 2c, the N-terminal lid is formed by the three N-terminal
helices; \(\alpha_1\); \(\alpha_2\) and \(\alpha_3\) and appears to prevent the enzyme from hydrolysing large, folded substrates (Natesh et al. 2003).

In 2006, the structure of the N-domain of sACE was determined, providing the first opportunity for the two domains of sACE to be compared. The overall structure of the N-domain is essentially identical to that of the C-domain; a predominantly helical, ellipsoid shaped molecule with a long channel that narrows at the centre where the catalytic zinc ion is found. In total it comprises 27 helices, of which 18 are \(\alpha\)-helices and only 6, relatively short, \(\beta\)-strands (Fig. 3a and b).

Comparing the structures of the N and C-domains provides some insight into the observed differences in substrate specificity. The structures of both domains were solved with lisinopril, an inhibitor that is slightly selective for the C-domain, bound. Although lisinopril is bound in broadly the same conformation in both structures, minor differences, mainly concentrated in the \(S_1'\) binding pocket help to explain the selectivity. For example, Asp140 in the N-domain takes the place of Glu162 in the C-domain. Glu162 forms electrostatic interactions with the lysyl side chain of lisinopril, which Asp140 is unable to replicate (Fig. 3c) (Corradi et al. 2006).

By studying the structures of the N- and C-domains of sACE significant progress has already been made towards the design of domain specific inhibitors. RXPA380 was developed from a phosphinic peptide library and is highly selective for the C-domain, with a \(K_i\) three orders of magnitude lower than for the N-domain (Georgiadis et al. 2004). The structure of the C-domain bound to RXPA380 was reported in 2007 (Corradi et al. 2007) and provides an insight into the reasons for the specificity of this inhibitor (Fig. 4).

In the C-domain Phe391 in the \(S_2\) subsite makes an aromatic interaction with Phe at the \(P_2\) position of RXPA380. In the N-domain Phe391 is replaced by Tyr369, which would not be able to replicate this aromatic interaction. Furthermore, the structure suggests that the hydroxyl group of Tyr369 would be within 2 Å of the \(P_2\) Phe, preventing the inhibitor from binding in the same conformation (Fig. 4d).

Further relevant differences are observed in the \(S_2'\) subsite. During the development of RXPA380 it was suggested that Pro and Trp at the \(P_1'\) and \(P_2'\) positions respectively were important determinants of C-domain selectivity. The \(S_2'\) subsite of the C-domain is lined by two valine residues (Val379 and Val380) which create a hydrophobic environment for the Pro and Trp. In the N-domain, these valines are replaced by Ser357 and Thr358, destroying the hydrophobic environment (Fig. 4d).

A second phosphinic peptide, RXP407, has been developed which selectively inhibits the N-domain (Fig. 5a and b). The structure of the N-domain in complex with

![Fig. 2](image-url)  
Fig. 2 The structure of human tACE. (a) and (b) cartoon representation of the tACE structure with helices shown in cyan, \(\beta\)-strands in magenta and loops in pink. The catalytic zinc ion is shown as a grey sphere at the centre of the long substrate binding channel which effectively divides the molecule into two subdomains. Also visible in the active site is the inhibitor lisinopril and the two chloride ions shown as orange spheres. Three N-terminal helices form a “lid” over the substrate binding channel and this is shown more clearly by viewing the molecule from the angle used in (b). The role of the lid in capping the substrate binding channel is highlighted in (c), where the three N-terminal helices which form the lid are shown in purple with the rest of the molecule shown as a cyan surface representation. The lisinopril and zinc are shown bound in the active site. In (c) the molecule is viewed from the same angle as in (b).
RXP407 was reported in 2010 (Anthony et al. 2010). It shows twelve hydrogen bonds from nine residues forming between the enzyme and inhibitor (Fig. 5c). All but two of these would be conserved in the C-domain. Interestingly, the two residues that differ; Arg381/Glu403 and Tyr369/Phe391 are located in the S2 subsite (Fig. 5d), further illustrating that interactions between the enzyme and inhibitors at this site are an important determinant of domain selectivity.

Structural studies of inhibitor binding to ACE have been able to provide essential information relating to the subtle differences in the substrate binding pockets of the two domains. This will be able to be exploited in the design of new, domain specific ACE inhibitors. These may include not only C-domain specific inhibitors for use in the treatment of hypertension, but also N-domain specific inhibitors targeting the roles of ACE in other physiological processes.

ACE2

In addition to the increasing understanding that mammalian ACE is involved in a range of processes outside of blood pressure regulation, in recent years a number of other homologues of ACE have been identified which further emphasise the diverse range of functions of these enzymes.
In the year 2000, a study aiming to find novel genes involved in heart failure identified a cDNA coding for a human homologue of ACE (Tipnis et al. 2000). This was the first evidence of an ACE homologue in humans, and the protein was appropriately named ACE2 (Angiotensin Converting Enzyme 2, EC number 3.4.17.23). Like ACE, ACE2 is a type I integral membrane protein. It has a single catalytic domain that includes a zinc binding site. ACE2 and each domain of sACE share 33% sequence identity, so it appears that the two enzymes have arisen following a gene duplication event which then allowed considerable divergence in ACE2 sequence (Krege et al. 1995).

In contrast to the widespread expression of sACE, ACE2 expression appears to be confined to the heart, kidneys and testis. Furthermore, ACE and ACE2 have quite distinct biochemical properties. There is no evidence of ACE2 having dipeptidyl carboxypeptidase activity as is routinely found in ACE. Instead it removes a single residue from the C-terminus of its peptide substrates, which include neuropeptide and kinetensin. Bradykinin is not a substrate for ACE2, although it is able to cleave angiotensin I, but removes only a single C-terminal residue to give the nonapeptide Ang1-9. ACE2 is not inhibited by the ACE inhibitors captopril, lisinopril and enalaprilat (Tipnis et al. 2000; Donoghue et al. 2000; Crackower et al. 2002).
The structure of ACE2, reported in 2004, provides explanations both for the function of ACE2 as a zinc dependent carboxypeptidase and for many of the observed differences between ACE and ACE2 (Towler et al. 2004). The extracellular portion of ACE2 was shown to consist of two distinct domains. The first (residues 19–611) is a zinc metallopeptidase sharing 42% sequence identity with the catalytic domains of ACE. The second (residues 612–740) is 48% identical to human collectrin. Poor electron density was observed for the “collectrin-like” domain (Towler et al. 2004). Poor electron density was observed for the “collectrin-like” domain (Towler et al. 2004).

The first, “ACE-like” domain is, like ACE, predominantly helical. It consists of two subdomains (I and II) forming the sides of a cleft stretching 40 Å along and 15 Å across the molecule, with a depth of 25 Å. At the base of this cleft is the catalytic site. It is located approximately halfway along the length of the molecule towards one side, as illustrated in Fig. 6a. The location of the active site is marked by the presence of the catalytic zinc ion. As in ACE, and characteristic of zinc dependent proteases, the catalytic zinc ion is coordinated by two histidines and a glutamic acid: His374, His378 and Glu402. In the absence of the inhibitor a water molecule completes the coordination of the zinc ion.

The location of the catalytic site means that it is shielded in the cleft that separates the two subdomains (Fig. 6a). A similar effect is achieved in ACE with the catalytic site being located at the centre of the long substrate binding channel capped by the N-terminal lid. Like ACE, ACE2 is extensively glycosylated with electron density observed at six N-linked glycosylation sites. There are three disulphide bonds in the ACE2 structure, all of which are conserved in ACE.
An interesting feature of ACE2, which has not been seen in ACE, is a large conformational change on inhibitor binding. The two subdomains move relative to each other to close in around the inhibitor in the active site, illustrated in Fig. 6. Differences in the subsites of ACE2 compared to ACE help to explain the observed differences in substrate specificity and catalytic properties. In ACE2 the S₁ subsite is defined by four residues with large, bulky side chains, (Tyr510, Arg514, Phe504 and Thr347) likely to restrict the size of residues that can be accommodated at the P₁ position of substrates. Bradykinin and angiotensin, both substrates of ACE but not ACE2, have phenylalanine and tyrosine respectively at the P₁ position of substrates which are unlikely to be accommodated by the restricted S₁ subsite, Fig. 6e.

Another significant difference between the two enzymes is observed in the S₂’ subsite, where a glutamine residue in ACE is replaced in ACE2 by Arg273. The large arginine side chain effectively blocks the S₂’ subsite, prohibiting ACE2 from acting as a dipeptidyl peptidase as ACE does (Fig. 6e). This also explains why ACE2 is not inhibited by classical ACE inhibitors (captopril, lisinopril and enalaprilat) which make contacts with the S₂’ subsite (Crackower et al. 2002).
ACE2 is an exceedingly important enzyme to study. It has been shown to have an important role in heart failure (Crackower et al. 2002; Luft 2012; Kuba et al. 2013; Santos et al. 2013) and it has also been identified as a receptor for the coronavirus which was linked to the outbreak of Severe Acute Respiratory Syndrome (SARS) (Li et al. 2003). The structural details of this recognition have been characterised and were reported in 2009 (Wu et al. 2009). It is also important to study ACE2 to improve our understanding of ACE homologues. The diverged properties of ACE2 highlight the range of processes that ACE homologues may be involved in and the potential benefits that may come from studying them further.

**Insect ACE homologues**

For many years it was thought that ACE was confined to mammals, where it has a critical role in blood pressure homeostasis. More recently, it has become apparent that ACE has many other substrates in addition to angiotensin I and bradykinin, and that it is involved in a large number of physiological processes outside of the RAAS. Furthermore, the discovery of ACE2 illustrates how homologues of ACE could have arisen from gene duplication events and then evolved to have different, but equally important functions.

In 1994, the first ACE homologue outside of mammals was identified in the housefly *Musca domestica* (Lamango and Isaac 1994). Since then, ACE homologues have been found in every insect genome sequence to date (Isaac et al. 2007). This conservation among insects is indicative of an essential role for ACE homologues. Yet, given that insects lack any form of circulatory system resembling that found in mammals, significant questions remain as to the exact nature of this role or roles.

**Drosophila melanogaster AnCE**

One of the first insect ACE homologues to be studied in detail was AnCE from *Drosophila melanogaster*. First identified in 1995, AnCE shares 45% sequence identity with human tACE and, as such, has been used successfully as a model for studying the structural basis of inhibitor binding to human ACE (Cornell et al. 1995; Tatei et al. 1995).

Although AnCE is able to hydrolyse angiotensin I and bradykinin, two of the major substrates of mammalian ACE, no insect homologues of these peptides have been identified. A lot of research has been performed to try and elucidate the physiological function of AnCE and thus insect ACE homologues in the wider sense.

Ance mutant embryos develop normally but die during the early larval stages. This indicates that AnCE has an essential role in a physiological process critical for survival (Isaac et al. 2007). High levels of Ance expression are observed in the gut epithelium and amniosera, suggesting that AnCE may be involved in processing peptides required for contraction of the heart and gut muscle (Cornell et al. 1995; Tatei et al. 1995).

Ance expression is also concentrated around the reproductive organs of both male and female flies, suggesting a role in reproduction. Further evidence for this was obtained in a 1999 study using insects with hypomorphic Ance alleles. Male flies homozygous for the hypomorphic Ance alleles were infertile as a result of sperm failing to develop properly, suggesting that AnCE may be required for processing a peptide involved in spermatogenesis (Isaac et al. 1998, 1999; Schoofs et al. 1998).

Further investigation in 2007 showed that AnCE expression is concentrated in the secondary cells of the accessory glands of the testis. The accessory glands produce a number of peptides that mix with the sperm and the seminal fluid and induce physiological and behavioural changes in the female insect after mating. It is reasonable to suggest then that AnCE is not only involved in spermatogenesis in the male, but may also have a role in the female after mating (Isaac et al. 2007).

With this in mind, Dup99B was suggested as a potential AnCE substrate. Dup99B can induce egg laying in females, but in order to be functional the basic C-terminal dipeptide must be removed from the propeptide. In theory, this would make Dup99B a good substrate for AnCE. As yet it has not been possible to investigate this hypothesis further, as AnCE is already expressed by the female reproductive organs (Rylett et al. 2007). In order to understand any effect of AnCE on the female after mating tissue specific AnCE knockdowns in female flies would need to be performed.

AnCE is not the only insect ACE homologue which has been implicated in reproduction and it seems increasingly likely that this is a conserved function of these enzymes (Wijffels et al. 1996; Hurst et al. 2003; Ekbote et al. 2003a,b; Macours and Hens 2004; Vercruysse et al. 2004). For example, ACE has been shown to be necessary for egg laying by female *Anopheles stephensi* mosquitoes, where it has been suggested that it may be required to process a myotropic peptide needed for contractions of the oviduct (Ekbote et al. 1999).

**Drosophila melanogaster ACER**

Drosophila also produces a second ACE homologue; ACER. ACER cDNA was first identified in 1996, and from the outset it has been clear that Ance and Acer code for distinct proteins with unique physiological roles. Unlike Ance, Acer is found neither in the amniosera nor the midgut, but it is found in developing heart cells where it has been shown to be necessary for heart development (Taylor et al. 1996; Houard et al. 1998). Additionally, ACER is involved in heart function in adult flies. RNAi was used to specifically knockdown Acer in adult Drosophila and resulted in age-associated changes in heart contraction, leading to a decreased life span. As yet, the
mechanisms behind this remain to be determined (Liao et al. 2014).

Acer has also been implicated in the regulation of circadian rhythms, with the suggestion that its expression is temporally regulated by the expression of clock. Flies lacking active ACER display decreased night-time sleep and increased fragmentation of the sleep that they do get. The mechanisms behind this are unclear, but it has been suggested that ACER processes peptides involved in metabolism, with the resulting metabolic changes upon losing ACER causing behavioural changes leading to the observed effects on sleep, rather than ACER being involved in circadian rhythms per se (Carhan et al. 2011; Ishimoto et al. 2012).

**ACER and AnCE: structural comparison**

Based on the evidence obtained so far, it is clear that AnCE and ACER have distinct physiological roles; it is therefore not surprising that they have different substrate and inhibitor specificities. For example, unlike AnCE and mammalian ACE, ACER is unable to cleave angiotensin I and also has a much lower affinity for bradykinin. On the basis of these differences, one would predict that there must be some variations between the active sites of these two enzymes (Taylor et al. 1996; Coates et al. 2000; Bingham et al. 2006).

The structure of AnCE was first published in 2003 at 2.4 Å resolution (Kim et al. 2003). This was followed in 2010 by higher resolution (~2.0 Å) structures, in a different crystal form, of the native enzyme and a number of enzyme inhibitor complexes (Akif et al. 2010, 2012). The overall architecture of AnCE is conserved from human ACE and the molecule is also phobicity of this pocket which accommodates the tryptophan moiety of RXPA380 hence decreasing the affinity of the inhibitor for AnCE compared to ACER and the C-domain (Kim et al. 2003; Bingham et al. 2006).

These are just some of the potentially interesting features of the ACER active site proposed based on the modelled structure (Bingham et al. 2006). Such small differences can have significant implications on substrate and inhibitor specificity. Whilst there remains a great deal more to be learnt about the in vivo functions of ACER, and indeed AnCE, it is clear that like mammalian ACE, both of these enzymes are involved in a number of important physiological processes. It follows then, that studying these enzymes, particularly from a structural perspective, can only increase our understanding of the diverse range of functions of ACE homologues.

**Anopheles gambiae ACE homologues**

*Anopheles gambiae* is one of the mosquito vectors responsible for the transmission of *Plasmodium falciparum*, a causative agent of malaria. This makes *A. gambiae* an exceedingly important organism to study.

It transpires that *A. gambiae* is also a particularly interesting organism to study in terms of ACE. There are ten *A. gambiae* ACE homologues (AnoACEs); the largest number to be found in any insect genome so far (Holt et al. 2002). The reasons for there being so many AnoACE homologues are not yet clear, but understanding the different functions of these enzymes will surely help us to understand more about the biological roles of other insect ACE homologues, and indeed of mammalian ACE outside of blood pressure regulation. Furthermore, one of these, AnoACE9, appears to consist of two catalytic domains arranged in tandem, as seen in sACE. This is the first instance of a two domain ACE homologue to be reported in insects (Burnham et al. 2005) and indicates that this two domain arrangement has been selected for on more than one occasion and so must surely be in some way advantageous. Studying AnoACE9 may help us to understand the benefits of such an arrangement.

In 2005 Burnham and co-workers investigated the gene expression patterns of the different AnoACE homologues to try and infer more about their potential physiological roles. One of their key observations was that the expression of *AnoACE3*, 7 and 9 was upregulated for 48 h following a blood meal (Burnham et al. 2005). A similar observation was previously reported for the *A. stephensi* mosquito (Ekbote et al. 1999), suggesting that insect ACE homologues may have a conserved role in processing peptides involved in metabolism.

The *AnoACE* expression levels were also observed to alter in response to challenging the immune system of the
mosquitoes. AnoACE7 was upregulated on infection with *Salmonella typhimurium* and AnoACE1 on infection with *Staphylococcus aureus*. Conversely, AnoACE9 expression was reduced when the mosquitoes were challenged with *Beauveria bassiana* (Burnham et al. 2005). These observations alone are of course not enough to conclude that AnoACE homologues have an important role in the immune response, but they do indicate that this area should be investigated further.

Given the evidence for mammalian tACE and other insect ACE homologues being involved in reproduction, the likelihood of some or all of the AnoACE homologues having a similar role cannot be ignored. At this early stage there is no indication as to which, if any of the AnoACE proteins may fulfil this role and further studies, likely knock down studies, will be required to investigate this further. Identifying an enzyme with such a role could be highly significant, with the possibility of designing *A. gambiae* specific ACE inhibitors for use in insecticides.

**AnoACE models**

Of the nine *AnoACE* genes, six (AnoACE2, 3a, 3b, 4, 5, 6 and 7) are clustered together on the same chromosome and are likely to have arisen from relatively recent gene duplication events. Indeed AnoACE5 and 6 are predicted to share 97 % sequence identity whilst AnoACE3a and 3b are coded for by the same gene and are identical save for the first exon (Burnham et al. 2005).

AnoACE2-6 share many common features with each other and *D. melanogaster* AnCE and ACER. All six of these AnoACE proteins were successfully modelled based on the structure of AnCE (PDB code 2X8Y) (Akif et al. 2010) using the automated mode of SWISS-MODEL (Arnold et al. 2006; Kiefer et al. 2009). As would be predicted given the high sequence identity, the crucial active site residues from AnCE were conserved in all of the models. There were though differences in the subsites, indicating probable differences in substrate preference. These differences are summarised in Table 1.

The S2 subsite has been identified as a crucial region conferring inhibitor and substrate binding specificity on the N- and C-domains of human sACE (Hubert et al. 1991; Gordon et al. 2003; Dive et al. 2004; Corradi et al. 2006, 2007; Kröger et al. 2009; Anthony et al. 2010). For example, the substitution of Tyr369 in the N-domain for Phe391 in the C-domain is crucial for the C-domain selectivity of RXPA380, with Phe391 forming an aromatic interaction with the phenyl moiety at the P2 position of RXPA380 that Tyr369 is unable to replicate (Gordon et al. 2003; Corradi et al. 2007). Both the AnoACE5 and 6 models have a phenylalanine at the position of C-domain Phe391, suggesting that they could form an interaction with an aromatic group at P2. AnoACE5 and 6 also have a conserved negative charge at the position of Glu403 of the C-domain, which is replaced by Arg381 in the N-domain Fig. 7a. These two observations suggest that AnoACE5 and 6 and the C-domain of sACE may accommodate substrates with similar functionalities at the P2 position.

In contrast, the AnoACE2-4 models have a tyrosine at the position of N-domain Tyr369, indicating that they would not tolerate an aromatic group at P2, and may instead have a preference for a group to which they could form a hydrogen bond. Unlike the N-domain AnoACE2-4 do not have a large positive side chain at the position of Arg381. Like the C-domain AnoACE2, 3a and 3b have a glutamate, whilst AnoACE4 has a serine Fig. 7b. The models therefore suggest that AnoACE2-4 share some features of both the N- and C-domains of human sACE at the S2 subsite and this may well be reflected in their substrate and inhibitor binding properties.

Based on the models of AnoACE2-6, there appear to be some potentially significant differences in the S3 subsite compared to the two domains of sACE. For example, the N- and C-domain have a conserved phenylalanine residue (Phe490 and Phe512 respectively) which is replaced by tyrosine in AnoACE2-6 Fig. 7c. This could have potentially significant consequences on inhibitor and substrate interaction, as the stacking interaction between the phenyl moiety at P1 of both RXPA380 and RXP407 would not be maintained. Interestingly, a tyrosine is conserved at this position in *D. melanogaster* AnCE.

There are also interesting substitutions at the position equivalent to the C-domain Glu143. The negative charge here is retained in AnCE and the AnoACE2 model. In the N-domain and AnoACE3a, 3b, 5 and 6 it is lost and replaced by serine (N-domain) or glutamine. Most significantly though, in AnoACE4 Lys133 occupies this position Fig. 7c. The large positive lysine side chain is likely to have a significant effect on the type of groups that AnoACE4 can accommodate in the S1 subsite.

The AnoACE2-6 models also all have a conserved positive charge (either lysine or arginine) in the S1 subsite, which, although conserved as Lys62 in AnCE, is replaced by Gln54 and Leu81 in the N- and C-domains respectively Fig. 7c. The introduction of these large positive side chains into what is in both domains of sACE quite a large pocket of space is likely to influence the size and charge of functionalities that can be accommodated by AnoACE2-6 in the S1 subsite.

Based on our models, AnoACE5 and 6 have an additional negative charge in their S3 subsites compared to AnCE, the two domains of sACE and AnoACE2-4. Both have Glu54, which takes the place of a small, non-polar side chain in the other subsites Fig. 7c. Overall, the models suggest that the S1 subsites of AnoACE2-6 are quite polar environments compared to sACE. The models presented here are not sufficient to determine the effect that this may have on binding properties, further work will be needed to investigate this.
There is comparatively little variation in the S1' and S2' subsites of the AnoACE2-6 models compared to in the other subsites. A potentially significant difference is at the position of C-domain Val380. This residue has been implicated in the selectivity of RXPA380 for the C-domain. It contributes to a hydrophobic environment for the tryptophan moiety of the inhibitor that is lost in the N-domain as Val380 is replaced by Thr358. Interestingly, the valine at this position is conserved only in AnoACE2 and is replaced by a threonine in AnoACE3-6. This small difference, which has such a significant effect in determining the domain selectivity of RXPA380, highlights how subtle variations in the subsites of these enzymes can have significant effects on their biochemical properties.
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Fig. 7 Features of the active sites of the AnoACE2-6 models compared to the N- and C-domains of human sACE. In all images the N-domain selective inhibitor RXP407 is shown as sticks with green carbon atoms and the C-domain selective inhibitor RXPA380 as sticks with orange carbon atoms. The catalytic zinc ion is a grey sphere. (a) The S$_2$ subsite of AnoACE5 and 6. Key residues of AnoACE5 and 6 are shown as grey sticks using AnoACE5 numbering. The C-domain is shown as cyan sticks and the N-domain magenta. AnoACE5 and 6 share a conserved phenylalanine (Phe381) with the C-domain which is replaced by a tyrosine in the N-domain. This residue makes important contacts with the domain selective inhibitors and so suggests that AnoACE5 and 6 may share some binding preferences with the C-domain. There is also a conserved negative charge, Asp393 which is replaced by positive Arg381 in the N-domain, and a conserved valine, Val508, replaced by Thr496 in the N-domain. There is some similarity with the N-domain though, for example Ser566 which is replaced by Phe570 in the C-domain. (b) The S$_2$ subsite of AnoACE2-4. The S$_2$ subsites of the AnoACE2-4 models are very similar and so are represented here by AnoACE3a, shown as light orange sticks. There are some features shared with both the N- and C-domains of sACE; Tyr390 is conserved in the N-domain, but replaced by Phe391 in the C-domain, whilst Glu402 is conserved in the C-domain but replaced by the positive Arg381 in the N-domain. These residues are likely to have significant effects on substrate and inhibitor binding properties. (c) The S$_1$ subsite of AnoACE2-6. Common features of the AnoACE2-6 models are represented by AnoACE3a in light orange, with unique features of AnoACE4 and AnoACE5 and 6 shown as violet and grey sticks respectively. The AnoACE2-6 models share a conserved tyrosine at Tyr511 which is replaced by phenylalanine in both the N- and C-domain. All of the models have a unique positively charged residue not found in the N- or C-domains, shown here by Lys571 in AnoACE4 and Arg77 in AnoACE3. AnoACE4 has a further positively charged residue, Lys133, which is replaced by neutral or negatively charged residues in the N- and C-domains and the other models. In contract AnoACE5 and 6 have an additional negative charge introduced by Glu54. These substitutions of charged residues are likely to have a significant effect on substrate and inhibitor binding properties.

AnoACE1

AnoACE2-7 are clustered together on the same chromosome and thought to have arisen from a relatively recent gene duplication event. AnoACE1 is predicted to be considerably different to the other AnoACE homologues, belonging to a different functional grouping, and is likely to have acquired highly diverged functions (Burnham et al. 2005; Akif et al. 2012). It is perhaps not surprising then that AnoACE1 was much more challenging to model and so an experimentally determined crystal structure will surely be needed to study the mechanism of action of this enzyme in more detail. It seems that this enzyme will make an exceedingly interesting case study and could reveal more unknown roles of ACE homologues, aiding our understanding of this increasingly complex enzyme.

AnoACE9

The discovery of the AnoACE9 gene which appears to code for a protein with two homologous catalytic domains arranged in tandem provided the first evidence of a two domain ACE homologue in insects. This now means that this domain arrangement, also seen in sACE has been selected for on three separate occasions (Burnham et al. 2005). This organisation must surely then be in some way advantageous. Studies of human sACE have resulted in the suggestion that there may be some negative co-operativity between the two domains (Georgiadis et al. 2003; Binevski et al. 2003; Andújar-Sánchez et al. 2004; Burnham et al. 2005; Corradi et al. 2006) however more evidence is required before a conclusion can be reached. Studying the structure and function of AnoACE9 could potentially yield a lot of information about the benefits of this organisation.

Conclusion

Angiotensin converting enzyme has been known of and studied for half a century; however it is only in the last decade that we have truly begun to understand the diverse range of physiological functions of this enzyme and its homologues. We now appreciate that ACE and its homologues have extensive functions outside of blood pressure regulation.

Structural studies have greatly improved our understanding of the different biochemical properties of ACE homologues. The elucidation of the human tACE structure in 2003 was a huge breakthrough in the study of ACE and was followed by the report of the N-domain structure in 2006. The structure of the D. melanogaster homologue AnACE is also frequently used as a model of human ACE.

The discovery and subsequent study of ACE homologues from other organisms, most significantly D. melanogaster, has highlighted the diverse range of functions of this enzyme. ACE homologues are conserved in insects, where, amongst other proposed roles, there is substantial evidence for them having an essential role in reproduction.

Studying insect ACE homologues has already greatly improved our understanding of the mammalian enzyme, but these enzymes are also important to study in their own right. Where is this more evident than in the A. gambiae ACE homologues. The A. gambiae genome codes for more ACE homologues than has been seen in any other insect genome thus far and includes a two-domain enzyme comparable to sACE.

Initial genomic studies have indicated that these enzymes may be involved in metabolism, the immune response and reproduction, however much more evidence is required to confirm this. This could potentially be achieved by treating the mosquitoes with ACE inhibitors and observing the effect that this has. A similar approach has previously been taking with A. stephensi, which indicated that ACE homologues were involved in reproduction (Ekbote et al. 1999). However this is unlikely to distinguish between the functions of the different AnoACE homologues, hence a better approach may be to use RNAi to selectively knockdown the individual genes. Liao et al. recently used this approach successfully in their work on ACER (Liao et al. 2014).
As we have discussed, D. melanogaster AnCE has been successfully used as a model for inhibitor binding to human ACE. It is not unreasonable to consider that it may be possible to use AnoACE9 in a similar way as a model for human sACE in order to learn more about the rationale for the presence of two homologous domains arranged in tandem.

Here we have modelled the active sites of the A. gambiae ACE homologues based on the structure of D. melanogaster AnCE and compared them to the human and D. melanogaster enzymes. Whilst this is of course no substitute for experimentally determined crystal structures, it has indicated some key differences that could potentially be exploited in the design of insect specific ACE inhibitors; perhaps targeting their role in reproduction. In the longer term such inhibitors could be designed for use in insecticides, which would be particularly useful when considering A. gambiae as a vector for the transmission of the malaria causing parasite P. falciparum.

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