Interacting cogs in the machinery of the renin angiotensin system

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Abstract

Somatic angiotensin converting enzyme (sACE) is well-known for its role in blood pressure regulation and consequently, ACE inhibitors are widely prescribed for the treatment of hypertension. More than 60 years after the discovery of sACE, however, the molecular details of its substrate hydrolysis and inhibition are still poorly understood. Isothermal titration calorimetry, molecular dynamics simulations and fine epitope mapping suggest that substrate or inhibitor binding triggers a hinging motion between the two subdomains of each domain. Ligand binding to one domain further induces a conformational change in sACE to negatively affect the second domain's function and can also cause dimerization between sACE molecules. This has been linked to an increase in sACE expression via intracellular signalling. Inhibitor-induced dimerization could thus decrease the efficacy of hypertension treatment. At present, the only structural information available for sACE are crystal structures of the truncated domains in the closed conformation due to the presence of ligands. These structures do not provide any information regarding the open active site conformation prior to ligand binding, the relative orientation of the two domains in full-length sACE, or the dimerization interface. To guarantee effective therapeutic intervention, further research is required to investigate the hinging, negative cooperativity and dimerization of sACE. This review describes our current understanding of these interactions and proposes how recent advances in cryo-electron microscopy could enable structural elucidation of their mechanisms.

Keywords Zinc metalloprotease · Cooperativity · Dimerization · Molecular dynamics · Cryo-EM

Abbreviations

A β	Amyloid beta
ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme-2
AcSDKP	<i>N</i> -Acetyl serine-aspartate-lysine-proline
AngI	Angiotensin I
EM	Electron microscopy
FRET	Fluorescence resonance energy transfer
ITC	Isothermal titration calorimetry
MD	Molecular dynamics
sACE	Somatic ACE
SAXS	Small-angle X-ray scattering
tACE	Testis ACE

Introduction

Angiotensin converting enzyme (ACE; EC 3.4.15.1) is expressed as two distinct isoforms namely testis ACE (tACE) and somatic ACE (sACE) following transcription of the human *Ace* gene with tissue-specific promoters (Kessler et al. 2000). While tACE occurs exclusively in male germinal cells and is important for fertility (Hagaman et al. 1998), sACE is expressed in a variety of human tissues (Erdos 1990). The mature sACE is a type I transmembrane protein comprised of a single polypeptide chain. sACE also exists as a soluble form following cleavage at the juxtamembrane Arg1203-Ser1204 peptide bond and release (ectodomain shedding) from the membrane (Ehlers et al. 2012).

This zinc metalloprotease forms part of the M2 gluzincin family and was discovered in 1956 for its prominent role in blood pressure regulation via the renin-angiotensin aldosterone system (Skeggs Jr. et al. 1956). This led to development of the first clinically used ACE inhibitor, captopril, in 1977 without prior knowledge of the target protein's sequence or structure (Ondetti et al. 1977). sACE has a broad substrate specificity and cleaves a remarkable diversity of substrates

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ranging from 3 to 42 amino acids in length through both endo- and exopeptidase action. These include angiotensin I (AngI), enkephalins, kinins, neurotensin, formyl-Met-Leu-Phe, substance P, luteinizing hormone-releasing hormone (LH-RH), N-acetyl-Ser-Asp-Lys-Pro (AcSDKP) and the amyloid beta-peptide (A β) (Bernstein et al. 2013). Consequently, it plays a vital role in various processes apart from blood pressure regulation including myelopoiesis, erythropoiesis, haematopoiesis, reproduction, immunity, renal development, renal function and fibrosis. Its importance is underpinned by the developmental, haematological, cardiovascular and reproductive defects observed upon ACE knockout in mice (Shen et al. 2012).

Despite the early discovery of sACE, it was only realized in 1991 that it consists of two catalytically active domains separated by a linker region of 15 amino acids, each containing an HEMGH zinc-binding motif (Wei 1991). The N- and C-terminal domains share 60% sequence similarity, suggesting that they originated by an evolutionary gene duplication event (Cornell et al. 1995) and were conserved due to differences in their physiological functions (Soubrier 1988). Interestingly, despite the two active sites being 89% identical, the N- and C-domain differ in thermal stability (O'Neill et al. 2008; Voronov et al. 2002), post-translational modification (*N*-linked glycosylation), affinity for inhibitor binding, catalytic efficiency for certain substrates (Acharya et al. 2003) and optimal salt concentration for catalysis (Yates et al. 2014). The C-domain, for example, is the principal site for generation of the potent vasopressor angiotensin II (Fuchs et al. 2008), whereas the N-domain selectively catalyses inactivation of the antifibrotic peptide AcSDKP (Rousseau et al. 1995). Although clinically available ACE inhibitors show beneficial antifibrotic effects and are widely prescribed for the treatment of hypertension and cardiovascular disease, their use has been associated with side effects, such as cough and angioedema (Bicket 2002) due to non-selective inhibition of both domains. The development of domain-selective ACE inhibitors, however, requires insight into the architecture and properties of the active sites.

sACE is ~30% glycosylated by weight with ten N-domain and seven C-domain potential *N*-linked glycosylation sites (Yu et al. 1997). Since glycans at certain sites on each domain are required for correct folding, processing and thermal stability of the enzyme, this presented a major obstacle for X-ray crystallographic studies. A turning point in the design of ACE inhibitors came in 2003 when the first crystal structure was published by engineering sACE into a truncated C-domain and systematically determining the minimal glycosylation requirement for correct folding and processing (Gordon et al. 2003; Natesh et al. 2003). The N-domain structure was subsequently also solved (Anthony et al. 2010), and together, these structures enabled development of several domain-selective inhibitors over the last 15 years (Douglas et al.

2014; Fienberg et al. 2018; Watermeyer et al. 2008; Watermeyer et al. 2010). Unfortunately, these compounds are unsuitable for clinical use either due to a poor pharmacokinetic profile (Denti et al. 2014) or their peptidic nature. Recent findings suggest that indirect intramolecular interactions between the subdomains of each ACE domain are instrumental in conferring domain-selective inhibition (Lubbe et al. 2016). These are usually not apparent from the snapshots provided by crystal structures, making structure-based design of domain-selective inhibitors challenging. A further confounding factor is the lack of insight provided by the current crystal structures regarding intermolecular interactions of sACE. There is a great need to understand these events since interdomain cooperativity and sACE homodimerization could affect substrate hydrolysis, inhibitor binding, intracellular signalling, sACE expression and its release from the membrane. To guarantee not only effective, but also safe therapeutic intervention for diseases in which sACE is implicated, detailed investigations into the mechanisms of its inter- and intramolecular interactions are required, hence a shift from an active site-orientated perspective to a holistic view of the sACE molecule. This review will describe our current understanding of these interactions and highlight areas where further research is warranted.

The effect of intramolecular interactions on ACE activity and inhibitor binding

The overall topology of the two truncated domains is very similar, in line with their high sequence similarity (Corradi et al. 2006). Each ellipsoid domain is mainly composed of alpha helices with the active site zinc coordinated by the characteristic HEMGH motif and located within a large, occluded cleft (restricted to approximately 3 Å in diameter) (Fig. 1). This cleft divides each domain into two subdomains and is covered by a lid-like structure comprised of the first three N-terminal helices.

Based on the lid region's high B-factors and poor alignment between two chains within the N-domain crystal structure asymmetric unit, it has been proposed that hinging in this region could mediate opening of the active site cleft and enable entry of the large range of ACE substrates (Anthony et al. 2010). The lid was identified as one of the six hinge regions within each domain using normal mode analysis (Watermeyer et al. 2006). The ACE crystal structures to date, however, only represent the closed conformation, and thus visualization of active site closure via this technique required modelling of the open C-domain conformation onto the open ACE2 structure (~40% homology to the ACE domains). Nevertheless, the existence of such a hinging event is substantiated by experimental evidence of sACE in solution. It has been observed that certain monoclonal antibodies elicit an anticatalytic effect upon binding to the N- or C-domain surface

Fig. 1 Structure of the C-domain of ACE (PDB ID: 1O8A). Subdomain 1 (sky blue) and subdomain 2 (plum) are separated by the narrow active site cleft (dotted arc) which is covered by the N-terminal lid. The catalytic zinc ion (yellow sphere) is coordinated by two histidine residues and a glutamate residue (orange sticks) and is more proximal to chloride II than chloride I (green spheres)

by presumably preventing the conformational change required for substrate hydrolysis (Danilov et al. 2007; Naperova et al. 2008; Skirgello et al. 2006). Similarly, the observation that inhibitor presence alters binding of certain monoclonal antibodies to the sACE surface is supportive of a conformational change (Balyasnikova et al. 2007). Furthermore, isothermal titration calorimetry (ITC) studies revealed that substrate or inhibitor binding to the truncated N- or C-domain is largely entropically driven (Yates et al. 2014), suggesting that a significant conformational change (hinging) occurs between the two subdomains. Interestingly, this effect was dependent on the reaction's salt concentration with a greater entropy change being observed with increasing chloride concentration. Under these conditions, there was also a trend of increased substrate binding affinity (K_m) and efficiency of hydrolysis (k_{cat}) for both domains. The C-domain structure clearly shows two chloride binding sites (chloride I is found 20.7 Å and chloride II 10.4 Å away from the zinc), whereas the N-domain structure only contains a chloride at the second site. The chloride-induced conformational change is likely largely due to the presence of chloride II since mutagenesis of the C-domain chloride II-binding residues abolished the salt-induced increase in entropy. The molecular details of how chloride II binding affects the conformation and, subsequently, the activity of ACE are still unclear. There is a need to understand this process since it also occurs in membrane-bound sACE (Jaspard and Alhenc-Gelas 1995). It could thus act as a physiological regulator of the structure, dynamics and activity of sACE in different human tissues/plasma where chloride concentrations vary significantly (Yates et al. 2014). Furthermore, the fact that chloride concentration alters the binding affinity of ACE inhibitors (Wei

et al. 1992) opens up possibilities for the design of tissue-specific ACE inhibitors.

A detailed description of the hinging mechanism and elucidation of the open conformation of ACE is also crucial for the design of drug-like domain-selective inhibitors. Although ligand binding is entropically driven for both domains, the precise mechanism whereby these intradomain interactions occur appears to be dependent on intrinsic differences between the domains. Despite 89% identity between the two active sites, the inhibitor Compound 33RE binds with 1000-fold greater affinity to the N- than the C-domain (Douglas et al. 2014). Since N-selective inhibition shows potential for the treatment of fibrosis, this peptidomimetic inhibitor was co-crystallized with the N-domain to investigate its mechanism of selectivity. Surprisingly, all interactions with Compound 33RE were conserved except one hydrogen bond between the aminomethyl tetrazole and the N-domain Tyr369 (replaced by phenylalanine in the C-domain). Although Y369F mutation slightly increased the K_i , C-domain-like binding affinity was only observed upon combined mutation of the two unique S_2 residues as well as the entire S_2' subsite (Lubbe et al. 2016). The unique S_2 residue Arg381 and all six unique S_2' residues were distal to Compound 33RE but, nevertheless, synergistically influenced its binding. The N-domain has a melting temperature approximately 20 °C higher than that of the C-domain (O'Neill et al. 2008) and is less susceptible to inactivation by reactive oxygen species (Michel et al. 2001), suggesting that the C-domain active site might be more exposed prior to ligand binding. Since the Arg381 residue resides on hinge region 3 and the unique S_2' residues form a contact point between the two subdomains in the closed conformation, molecular dynamics (MD) simulations were employed to investigate whether differences in hinging between the two domains indirectly affect binding of Compound 33RE (Lubbe et al. 2016). Indeed, the N-domain displayed a concerted hinging motion where repulsion between Arg381 and the lid's N-terminus caused tipping of its C-terminus towards the cleft and formation of a subdomain interface between the unique S_2' residues (Fig. 2). The C-domain residues, in contrast, altered the lid's tipping, caused repulsion at the subdomain interface and decreased the stability of Compound 33RE in the active site, thus explaining the low binding affinity to this domain.

Recently, MD simulations were also used to study the binding of inhibitory peptides to the C-domain (Fang et al. 2019). Hinging between the C-domain subdomains was observed for the LIVT peptide ($IC_{50} = 110$ nM) but not YLVR ($IC_{50} = 15.42$ μM) nor YLVP ($IC_{50} = 151.0$ μM) strengthening the notion that potency is correlated with an inhibitor's ability to induce conformational change. Future efforts at designing domain-selective inhibitors with favourable pharmacokinetic properties might thus benefit from considering the indirect effects of protein dynamics on ligand binding. The recent advances in GPU computing would allow addition of MD

Fig. 2 Alignment between two conformations during the MD simulation demonstrate hinging between the N-domain subdomains in the presence of Compound 33RE (yellow balls and sticks). Unique residues (green sticks) in the N-domain active site create an interface for contact between opposing helices (orange pipes) and control the lid region's tipping towards the active site cleft (dotted arc). Coordinates for the conformations were taken from Lubbe et al. (2016).

simulations to the structure-based drug design pipeline to identify compounds capable of selectively inducing hinging in a particular domain. Hinging could further be exploited by designing allosteric inhibitors which lock a specific domain in the open conformation, thereby rendering it inactive. Compounds could potentially be designed against the large number of unique residues proximal to the lid to prevent its tipping motion, like the potent allosteric inhibitor designed against the ACE homologue neurolysin (Hines et al. 2014). This, however, requires structural insight into the open conformation of ACE.

Effect of intermolecular interactions on sACE activity and inhibitor binding

The two domains of sACE were initially thought to function independently since they are spatially separated by the linker region. Studies comparing substrate/inhibitor binding between bovine sACE and its truncated domains, however, revealed strong negative cooperativity where the turnover number of full-length sACE was less than the sum of the truncated domains' turnover numbers (Binevski et al. 2003). Negative cooperativity was also observed for human sACE in the hydrolysis of LH-RH (Jaspard et al. 1993), bradykinin analogues (Araujo et al. 2000), angiotensin I, angiotensin (1–7) (Rice et al. 2004), synthetic tripeptides (Skirgello et al. 2005), AcSDKP (Masuyer et al. 2015) and A β 1–16 (Larmuth et al. 2016) but not angiotensin (1–9) or bradykinin (Jaspard et al. 1993). It is not clear whether the latter substrates lack the ability to induce domain-domain

motion or whether their entry, binding and hydrolysis are simply not sensitive to the change being induced. Cooperativity is likely of physiological importance since synthetic as well as natural substrates display this behaviour. It is not known, however, whether it occurs in the membrane-bound form since all previous experiments were done using soluble sACE released from the membrane. ITC (Andujar-Sanchez et al. 2004) and active site titrations (Skirgello et al. 2005) showed that binding of a single molecule of non-domain selective ACE inhibitor (captopril or lisinopril) to full-length human sACE was sufficient to abolish activity of both domains. Cooperativity in inhibitor binding is thus an important factor which needs to be considered in the design of domain-selective inhibitors. Since such compounds are usually screened using truncated domains, future studies should validate promising compounds' domain selectivity in full-length sACE to pre-empt non-domain selective behaviour *in vivo*.

At present, the exact mechanism of inter-domain interaction and the basis for substrate-specificity is unknown. It does appear to rely on very specific interactions since cooperativity was abolished (the domains functioned independently) upon replacement of the N-domain in sACE by a second C-domain (Woodman et al. 2005). The two domains are likely brought into close proximity by considerable flexibility in the inter-domain linker region, but further clarification would require determination of the full-length sACE structure at high resolution.

The effect of *in vivo* conditions on the intermolecular interactions of sACE

In addition to the intradomain and interdomain interactions described, interactions between sACE molecules have also been observed under certain conditions. Kohlstedt et al. found that ACE inhibitors induced homodimerization of sACE on the cell membrane which, in turn, triggered intracellular signalling and increased *Ace* promoter activity (Kohlstedt et al. 2006). There is currently no consensus regarding the mechanism of dimerization with some studies suggesting importance of the N-domain (Kost et al. 2003), whereas others highlight the need for an active C-domain (Kohlstedt et al. 2006). Another study suggested that non-covalent N-domain interactions as well as disulphide-mediated C-domain interactions are involved (Gordon et al. 2010). Recently, sensitized emission fluorescence resonance energy transfer (FRET) in HEK293 cells showed homodimerization of tACE as well as sACE and confirmed the importance of disulphide interactions in C-domain-mediated dimerization (Abrie et al. 2018). Small angle X-ray scattering (SAXS) further revealed that homodimers can form in solution for sACE as well as the truncated N-domain. Interactions thus seem possible with either domain. Interestingly, an extended and compact

homodimer conformation was observed for both molecules, indicative of flexibility in the dimer interface. This might explain the discrepancies between earlier reports regarding the structural elements involved in dimerization. Since SAXS is a low-resolution technique, it did not allow differentiation between the two homologous domains meaning that the molecular details of the sACE dimerization interface are still unclear.

Dimerization is likely of physiological importance since patients treated with ACE inhibitors exhibit cardiovascular benefits which are independent of inhibiting catalytic activity and possibly a result of intracellular signalling (Ehlers et al. 2013). An interesting property of sACE is its ability to regulate itself in response to shear stress (Barauna et al. 2011). While ACE inhibitors increase dimerization and *Ace* promoter activity (Kohlstedt et al. 2006), shear stress reduces signalling via the cytoplasmic tail of sACE to decrease sACE expression. Increased blood flow likely alters the sACE conformation and disrupts the dimerization interface so that subsequent signalling does not occur. Understanding this process might allow for the design of novel antihypertensives which modulate sACE activity by disrupting dimerization. This approach might also benefit patients with increased plasma sACE levels due to mutation in the *Ace* gene. Though most missense *Ace* mutations are not associated with any detrimental side effects, patients with the Y465D mutation experience symptoms of nausea, vomiting, fatigue, pain and depression (Danilov et al. 2011). This N-domain mutation not only increases plasma sACE levels (5–7-fold), dimerization and shedding but also significantly alters the conformation of both domains as illustrated by detailed analyses of monoclonal antibody binding (Danilov et al. 2011). The hydrolysis of neuropeptides could thus perhaps be affected by the altered sACE conformation and elicit the symptoms experienced. Molecular insight is required, however, to understand the effect of mutation- and design-appropriate therapies for these patients.

Conclusion and future directions

Although sACE has been researched for more than 60 years and ACE inhibitors are widely prescribed, our current understanding of its substrate hydrolysis and inhibition is limited. The first step towards elucidating the intricate mechanisms of conformational change induced by substrate/inhibitor/chloride binding, shear stress or mutation would be to solve the full-length sACE structure at high resolution. Previous attempts at obtaining structural information on full-length sACE via X-ray crystallography were hampered by its high N-linked glycan content, interdomain linker flexibility and presence of the transmembrane domain. The molecular weight of sACE was also not suitable for electron microscopy (EM) as is evident from the low resolution (23 Å) of a 3D

reconstruction of porcine sACE using negative stain EM (Chen et al. 2010). Recent technological advances in microscopes, detectors and GPU computing have, however, led to a ‘resolution revolution’ where it is now possible to solve cryo-EM structures to near-atomic resolution (Bai et al. 2015). This is no longer limited to large molecules as demonstrated by the recent determination of high-resolution structures for sub-100 kDa proteins using conventional defocus-based cryo-EM methods (Herzik et al. 2019). Conformational heterogeneity and glycan flexibility (which are problematic for crystallography) also do not prevent structure determination via single particle analysis cryo-EM since it allows 3D classification of different conformational states and visualization of protein motion under near-native conditions (Murata and Wolf 2018). Cryo-EM thus appears appropriate for determining the structure of fully glycosylated human sACE (~180 kDa). sACE can be investigated under different chloride concentrations or complexed to different substrates, inhibitors or monoclonal antibody fragments. Since homodimerization has been demonstrated in solution, it could also be useful to identify residues constituting the dimerization interface. Although cryo-EM has traditionally not been part of the drug design repertoire, the dramatically improved resolution of structures solved via this technique now allows visualization of bound ligand atoms and structured water molecules (Ceska et al. 2019). It could thus provide valuable information for structure-based drug design of a protein such as sACE where conformational changes govern inhibitor potency and selectivity.

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Compliance with ethical standards

Conflict of interest Lizelle Lubbe declares that she has no conflict of interest. Edward D. Sturrock declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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