

Structure-Function Relationship of Angiotensin-Converting Enzyme: Glycosylation and Domain-Selectivity

Thesis presented for the Degree of DOCTOR OF PHILOSOPHY
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University of Cape Town

Structure-Function Relationship of Angiotensin-Converting Enzyme: Glycosylation and Domain-Selectivity

6th February, 2006

Somatic angiotensin I-converting enzyme (sACE), comprising structurally and functionally distinct N- and C domains, is the principal regulator of blood pressure, electrolyte and fluid homeostasis and is an important therapeutic target in the treatment of hypertension and cardiovascular disease. Testis ACE (tACE) is restricted to male germinal cells and plays an important role in fertility. Truncation of its unique 36-residue NH₂-terminus renders tACE identical to the C domain of sACE. The aim of the present study was to investigate the role of *N*-linked glycosylation as well as specific regions in the structure-function relationship of the N domain, the C domain (tACE) as well as the full-length somatic enzyme. Using glycosylation-deficient cells, a glucosidase inhibitor and site-directed mutagenesis of *N*-linked glycosylation sites, differences were observed in the processing- and functional dependence on *N*-linked glycosylation in the two domains. While glycosylation at the first (Asn⁷²) or third (Asn¹⁰⁹) *N*-linked glycosylation site of the C domain (tACE) was necessary and sufficient for maintaining normal processing and catalytic activity, this was abrogated by site-directed elimination of more than three of nine *N*-linked glycosylation sites in the N domain. Substitution of C domain (tACE) regions with corresponding N domain sequence resulted in the acquisition of N domain-like catalytic and thermal properties while within sACE, the two domains demonstrated evidence of negative cooperativity with respect to substrate-hydrolysis via close, thermally-stabilising intermolecular interactions irrespective of their relative orientation. However, when two C domains were duplicated in tandem, close intermolecular interactions resulted in positive cooperativity without increasing the thermal stability of the protein. The *in vitro* inhibitory activities of novel keto-ACE and lisinopril derivatives towards the N- and C domains were determined. A P₁' inhibitor functionality was an important determinant of inhibitory potency in a stereochemistry-dependent manner while the incorporation of bulky, hydrophobic P₂' and bulky aromatic P₂ inhibitor functionalities enhanced their C domain-selectivity.

CHAPTER 1

Review of the literature

1.1. Hypertension

'Essential hypertension' has a multi-factorial aetiology related to primary hyperaldosteronism, diet, environmental, and genetic factors (Fardella *et al.*, 2000). The renal origins of hypertension were first established in 1898 (Tigerstedt and Bergman, 1898) and confirmed by further studies in 1934 by Goldblatt and co-workers (Goldblatt *et al.*, 1934). Hypertension, which affects 26% of the adult population (Kearney *et al.*, 2005), has subsequently emerged as a major risk factor for cardiovascular and kidney disease resulting in an increased morbidity and a shorter life expectancy (Carretero *et al.*, 2000). Consequently, anti-hypertensive therapy, including vasodilators, diuretics, adrenergic antagonists and calcium channel blockers (Cushman, 2003) has become one of the cornerstones of modern medicine.

1.2. Renin-Angiotensin and Kallikrein-Kinin Systems

Arterial blood pressure and fluid homeostasis is regulated by the renin-angiotensin system (RAS) and the kallikrein-kinin system (KKS) (Figure 1.1.). Both systems comprise a complex, non-linear, interconnected cascade of vaso peptides generated by the action of vaso peptidases. Cardiovascular homeostasis is determined by the tightly regulated balance between these systems as well as amongst the various products of vaso peptidase activity.

1.2.1. Renin-Angiotensin System

The renal enzyme, renin (Houssay and Fasciolo, 1937), released from the juxtaglomerular cells of the kidney, restores perfusion pressure via tubuloglomerular feedback and by effecting a rate-limiting step in the RAS where liver-derived angiotensinogen is hydrolysed to angiotensin I (Ang I) (Page and Helmer, 1940; Skeggs *et al.*, 1954). Angiotensin I-converting enzyme (ACE) is primarily responsible for the subsequent hydrolysis of Ang I to angiotensin II (Ang II) (Figure 1.1.).

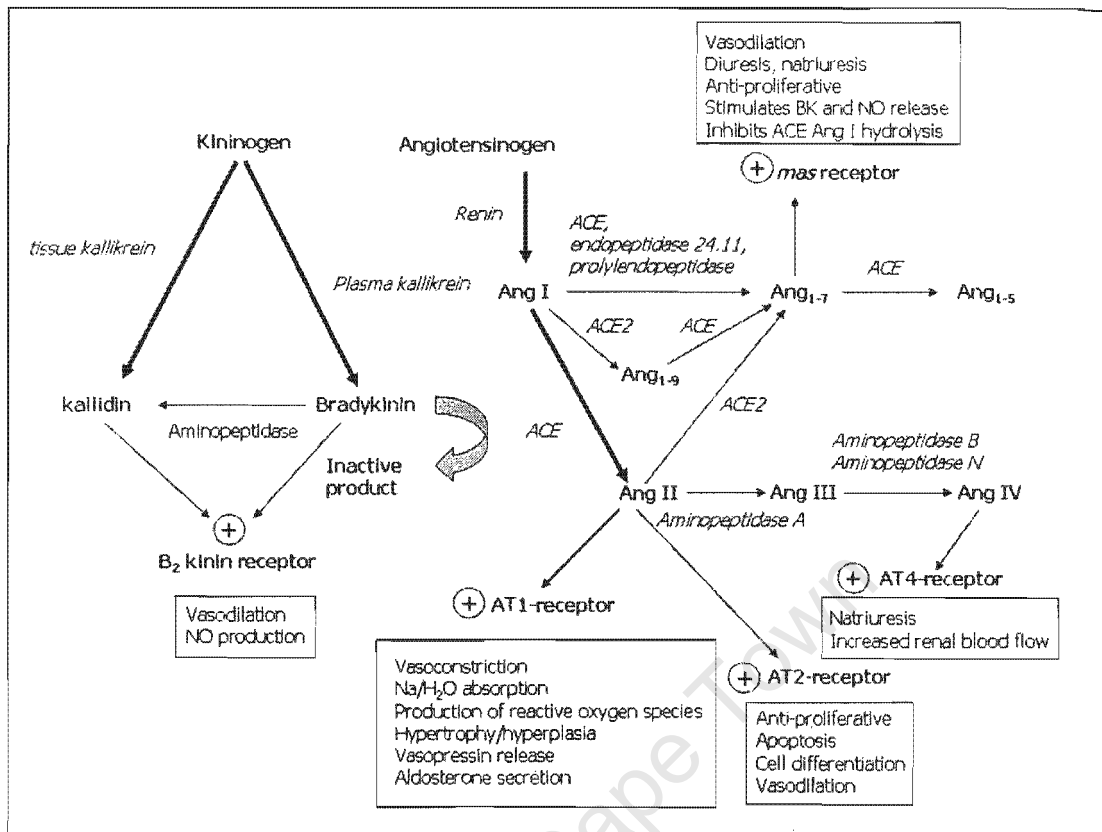


Figure 1.1. The renin-angiotensin and kallikrein-kinin systems.

1.2.1.1. Ang II and Ang₁₋₇: Effectors of cardiovascular homeostasis

Ang II is the principal effector of the RAS, acting in an endocrine, paracrine or autocrine manner, locally or systemically. It mediates its effects primarily via G-protein-coupled angiotensin type I (AT-1) or type II (AT-2) receptors (Bumpus *et al.*, 1991; Murphy *et al.*, 1991, Sasaki *et al.*, 1991; Richards *et al.*, 1999), the activation of which triggers opposing biological responses (Unger, 2002). AT-1 receptor signalling results in vasoconstriction, aldosterone production (Sancho *et al.*, 1976), sodium reabsorption, noradrenaline and vasopressin release as well as mitogenic, inflammatory and hypertrophic responses (de Gasparo *et al.*, 2000). The activation of AT-2 receptors counteracts these responses by inhibiting cell growth/proliferation and inducing cell differentiation, apoptosis, angiogenesis and vasodilation, effects largely mediated by bradykinin (BK) and nitric oxide (NO) (Meffert *et al.*, 1996; de Gasparo *et al.*, 2000; Brede and Hein, 2001) (Figure 1.1.).

Angiotensin-converting enzyme 2 (ACE 2) is an essential regulator of cardiac function acting as a negative regulator of the RAS (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000; Crackower *et al.*, 2002; Komatsu *et al.*, 2002). In this regard, ACE 2 mediates the hydrolysis of Ang II to Ang₁₋₇ (Ferrario *et al.*, 2005). Ang₁₋₇ is also generated via ACE-mediated hydrolysis of Ang I. Ang₁₋₇ elicits similar biological responses as that of AT-2 receptor activation. Mediating its actions via the *mas* receptor (Santos *et al.*, 2003), Ang₁₋₇ stimulates BK, prostaglandin (PG) and NO production (Jaiswal *et al.*, 1993 a,b; Porsti *et al.*, 1994; Brosnihan *et al.*, 1996; Ferrario *et al.*, 2005), inhibits growth and angiogenesis (Machado *et al.*, 2001) and facilitates natriuresis and diuresis (Ferrario *et al.*, 2005). Furthermore, Ang₁₋₇ is a vasodilator that potentiates BK (Deddish *et al.*, 1998; Fernandes *et al.*, 2005) and inhibits ACE Ang I hydrolysis (Deddish *et al.*, 1998) (Figure 1.1.).

Aminopeptidases degrade Angiotensin II into Ang III and Ang IV. Ang III binds to both AT-1 and AT-2 receptors, with similar effects as Ang II. Ang IV activates a novel AT-4 receptor, mediating effects such as natriuresis and increased renal blood (Wright and Harding, 1997; Ito *et al.*, 1995)(Figure 1.1.).

1.2.1.2. Local renin-angiotensin systems

In contrast to the circulating or endocrine RAS, which promotes rapid homeostatic responses, a local tissue specific autocrine or paracrine RAS generates local effects (Campbell, 1987). In the kidney (Taugner *et al.*, 1982a-d; Taugner and Ganten, 1982) a local RAS regulates renal function (Erdös, 1990) while in bone marrow, it enhances erythroid differentiation (Haznedaroglu and Öztürk, 2003) and in the pancreas it regulates flow circulation and insulin secretion (Leung and Carlsson, 2001).

1.2.2. Kallikrein-Kinin System

The predominantly tissue-associated kallikrein-kinin system (KKS), in addition to its physiological roles in blood pressure and sodium homeostasis, also mediates insulin-sensitivity, inflammation and renal responses to vasopressin (Campbell, 2003). In this pathway, hydrolysis of high molecular weight kininogen (HMWK) by plasma kallikrein generates the vasodilator, BK.

Tissue kallikrein acts on both high and low molecular weight forms of kininogen to generate kallidin while aminopeptidase-mediated cleavage of kallidin may also generate BK (Campbell, 2003) (Figure 1.1.).

BK and kallidin act predominantly on the type 2 (B_2) kinin-receptor with subsequent signalling involving secondary messengers such as prostaglandins and NO (Bhoola *et al.*, 1992; Campbell, 2003). Here, BK reverses the inhibitory interaction between the B_2 kinin receptor and endothelial NO synthase (Ju *et al.*, 1998; Campbell, 2003).

The RAS and KKS demonstrate a multilayered interaction where the plasma KKS counterbalances the RAS. For example, kallikrein is able to cleave angiotensinogen directly to Ang II and is implicated in the activation of pro-renin, while prolylcarboxypeptidase, a prekallikrein activator and Ang II inactivating enzyme, acts on both the RAS and KKS (Schmaier, 2003). Furthermore, ACE degrades BK and kallidin, abrogating their vasodilatory effects. ACE has also been linked to the upregulation of B_1 and B_2 kinin receptor expression (Ignjacev-Lazich *et al.*, 2005) while heterodimerisation between B_2 kinin and AT-1 receptors enhances AT-1 receptor signalling (AbdAlla *et al.*, 2001).

Activation of the systemic RAS blocks the vasodilatory KKS pathway and effects a systemic vasopressor response through the generation of Ang II primarily via ACE. Thus, increased activity of ACE is intimately linked to hypertension.

1.3. Angiotensin I-Converting Enzyme (ACE)

ACE was first isolated and characterised by Skeggs *et al.*, (1956) as hypertensin-converting enzyme. Subsequent studies indicated that the lungs, abundantly expressing ACE, were the most important sites for the generation of the vasopressor Ang II, thus placing ACE at a central position within the RAS (Ng and Vane, 1967, 1968; Cushman and Cheung, 1971; Ryan *et al.*, 1975; Caldwell *et al.*, 1976; Wigger and Stalcup, 1978; Lieberman and Sastre, 1983) where it is a key regulator of blood pressure, fluid and electrolyte homeostasis as well as myocardial remodelling, vascular and renal function (Ehlers and Riordan, 1989; Turner and Hooper, 2002).

Reinforcing these roles, ACE knockout mice present with a phenotype of low blood pressure, urine concentrating defects, underdevelopment of the renal medulla and papilla, mild anaemia, renal arteriolar thickening and male infertility (Krege *et al.*, 1995; Esther *et al.*, 1997; Tian *et al.*, 1997; Hagaman *et al.*, 1998; Kessler *et al.*, 2000; Xiao *et al.*, 2003; Xiao *et al.*, 2004).

A high level of sequence identity exists amongst ACE cDNA sequences from various mammals. Human and murine ACE are 83 % homologous (Bernstein *et al.*, 1988a,b) with even a higher degree of identity existing between human and rabbit ACE (Kumar *et al.*, 1989).

ACE is a chloride-dependent zinc dipeptidyl carboxypeptidase and member of the M2 gluzincin family of metalloproteases (Rawlings *et al.*, 2004). The larger somatic isoform of ACE (sACE) is a 1277 amino acid, 150-180 kDa (Soubrier *et al.*, 1988) type I transmembrane glycoprotein that is expressed in a variety of tissues including vascular endothelial cells, intestinal brush border cells and renal proximal tubule epithelial cells (Caldwell *et al.*, 1976; Bruneval *et al.*, 1986) (Figure 1.2.). Other locations of sACE include the choroid plexus (Arregui and Iversen, 1978), the prostate, epididymis (Cushman and Cheung, 1971; Yokoyama *et al.*, 1982) and mononuclear cells (Friedland *et al.*, 1978; Costerousse *et al.*, 1993). Its expression is upregulated by growth factors and glucocorticoids as well as β -adrenergic stimulation involving G proteins, protein kinases and adenylyl cyclase (Baudin, 2002).

sACE comprises a 28-residue C-terminal cytosolic tail, a 22-residue hydrophobic membrane-anchoring domain, a juxtamembrane stalk; and an ectodomain of 1227 residues consisting of two domains (N- and C) linked by a 15-residue inter-domain sequence. These two domains have resulted from internal duplication of an ancestral gene and display an overall amino acid similarity of 67.7% over 357 amino acids. In regions comprising essential active site residues, their identity increases to 89% (Soubrier *et al.*, 1988) (Figure 1.2.).

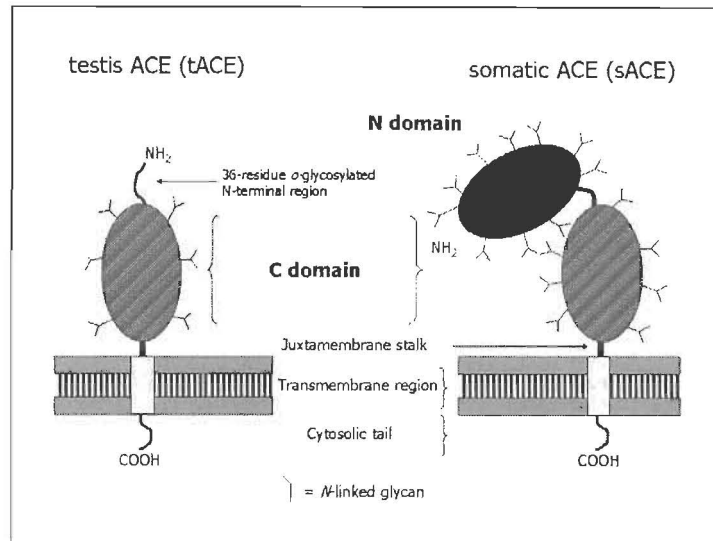


Figure 1.2. Diagrammatic representation of testis ACE (tACE) and sACE. tACE, with the exception of a 36-residue N-terminal region, is identical to the C domain of sACE.

Both domains contain a characteristic HEMGH zinc-coordinating motif, crucial for the peptidyl peptidase activity of the enzyme (Wei *et al.*, 1991; Jaspard *et al.*, 1993). Here, two histidines together with a glutamate, occurring 24-residues downstream, constitute the zinc coordinating ligands (Williams *et al.*, 1994)(Appendix 1, Figure 1.2).

The gene encoding sACE comprises 26 exons. With the exception of exon 13, which is removed via splicing of the primary 4.3 kb mRNA transcript, all are transcribed into mRNA (Hubert *et al.*, 1991). sACE is transcribed from exons 1-26, with exons 4-11 and 17-24 encoding the N- and C domains respectively (Figure 1.3.). The sACE promoter is located 5' to the first exon (Hubert *et al.*, 1991) and the region upstream of the transcription start site contains both positive and negative regulatory elements (Testut *et al.*, 1993). A soluble form of sACE, lacking the transmembrane region has been reported and is thought to arise from alternative mRNA splicing (Sugimura *et al.*, 1988).

Alternate transcription from exons 13-26 via the interaction of an internal promoter and intron 12 within the sACE gene (Howard *et al.*, 1990) has given rise to a smaller 701 amino acid, 90-110 kDa testicular isoform of the C domain, testis ACE (tACE) (Figure 1.2. and 1.3.). The tACE promoter, which contains a cAMP-responsive element, is active in a stage-specific manner in germinal cells (Nadaud *et al.*, 1992; Kessler *et al.*, 1998).

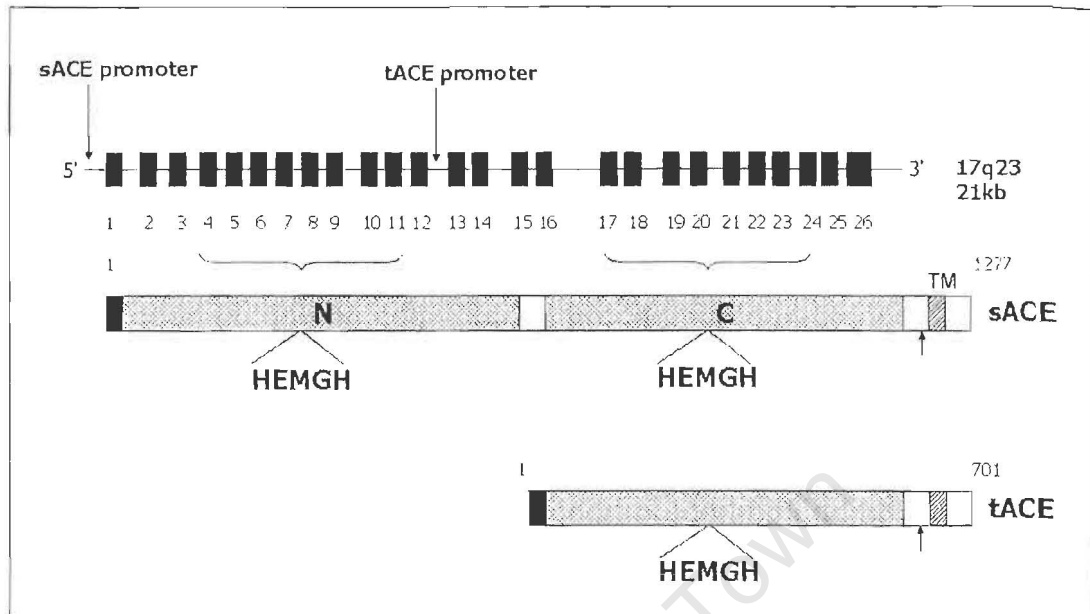


Figure 1.3. Alternate transcription from a somatic and germinal promoter within the ACE gene has given rise to two physiological isoforms, sACE and tACE. Arrows indicate the secretase cleavage site within the juxtamembrane stalk, TM: transmembrane region.

Like its larger somatic isoform, tACE is a type I transmembrane glycoprotein with a C-terminal cytosolic domain, a hydrophobic transmembrane region, a juxtamembrane stalk but with a single ectodomain which, with the exception of a 36-residue N-terminal region, is identical to the C domain of sACE (Kumar *et al.*, 1989; Ehlers *et al.*, 1989). Thus, tACE possesses only one active site per molecule (Ehlers *et al.*, 1989) (Figure 1.2.).

tACE is expressed exclusively in male spermatozoa (Langford *et al.*, 1991; Köhn *et al.*, 1998) and has been localised to the plasma membrane of the post-acrosomal region as well as the midpiece and neck of normal spermatozoa (Nikolaeva *et al.*, 2006). tACE expression is under hormonal control (Cushman and Cheung, 1971; Strittmatter *et al.*, 1985; Velletri *et al.*, 1985) with maximal expression taking place during the acrosome phase (Sibony *et al.*, 1994).

The reduced reproductive capacity of male mice lacking tACE (Xiao *et al.*, 2004) suggests that this isoform plays an important role in fertilization including sperm motility and capacitation (Hohlbrugger and Dahlheim, 1983; Foresta *et al.*, 1991; Hagaman *et al.* 1998; Siems *et al.*, 2003). tACE may also play a role in steroidogenesis and/or cell differentiation and growth (Millan and Aguilera, 1988; Isaac *et al.*, 1997). Recently, tACE was shown to be a releasing factor for glycosylphosphatidylinositol (GPI)-anchored proteins on the surface of sperm and zonae pellucidae (Kondoh *et al.*, 2005). However, another study by Leisle *et al.*, (2005) failed to demonstrate such a GPI-ase activity in ACE preparations from different sources.

1.3.1. ACE Ectodomain shedding

While the vast majority of sACE activity is cell-associated and membrane-bound (Xiao *et al.*, 2004), a relatively small fraction is shed into the systemic circulation as a soluble ectodomain. Cleavage occurs at the Arg¹²⁰³-Ser¹²⁰⁴ bond (Arg⁶²⁷-Ser⁶²⁸ in tACE) of the juxtamembrane stalk by a membrane-associated zinc metalloproteinase (secretase) in both human and rabbit ACE (Figure 1.3.) (Ehlers *et al.*, 1991; Sen *et al.*, 1991; Oppong *et al.*, 1993; Ramchandran *et al.*, 1994; Ramchandran and Sen, 1995; Ehlers *et al.*, 1996). ACE cleavage secretion requires a membrane-anchored substrate (Parvathy *et al.*, 1997; Balyasnikova *et al.*, 2002) as well as an accessible stalk, which is cleaved at a minimum distance from the ectodomain and the membrane (Ehlers *et al.*, 1996; Ehlers *et al.*, 1997; Schwager *et al.*, 2001).

The ectodomain cleavage secretion of sACE and tACE is regulated primarily via phosphorylation of the cytoplasmic domain via interactions with cellular proteins such as casein kinase II (CK-2) (Kohlstedt *et al.*, 2002), protein kinase C (PKC) (Ramchandran *et al.*, 1994; Kohlstedt *et al.*, 2002; Santhamma *et al.*, 2004), the chaperone BiP (Santhamma and Sen, 2000), calmodulin (Chattopadhyay *et al.*, 2005) and non-muscle myosin heavy chain IIA (Kohlstedt *et al.*, 2006). Glycosylation of the stalk region, while modulating shedding, does not inhibit cleavage secretion (Schwager *et al.*, 1999).

ACE solubilisation may be enhanced in response to phorbol esters such as phorbol-myristate-acetate (PMA) that stimulate the ACE secretase (Ehlers *et al.*, 1995; Peschon *et al.*, 1998; Balyasnikova *et al.*, 1998; Santhamma *et al.*, 2000) via a PKC-dependent mechanism (Ramchandran *et al.*, 1994; Kohlstedt *et al.*, 2002; Santhamma *et al.*, 2004). The shedding of sACE however is inefficient as compared with tACE (Beldent *et al.*, 1995; Woodman *et al.*, 2000) leading to the proposal of a C domain sheddase recognition motif which might be occluded by the N domain in sACE (Sadhukhan *et al.*, 1998; Pang *et al.*, 2001; Balyasnikova *et al.*, 2005; Woodman *et al.*, 2005).

Cleavage-secreted sACE is detected in biological fluids including plasma (Das *et al.*, 1977), cerebrospinal fluid (Schweissfurth and Schioberg-Schiegnitz, 1984), amniotic fluid (Yasui *et al.*, 1984) and semen (El-Dorry *et al.*, 1983). This soluble sACE, constituting a minor component of total ACE activity has as yet an unknown function although it is elevated in certain disease states including diabetes, hyperthyroidism and granulomatous disease (Lieberman, 1975). Furthermore, serum ACE levels may be modulated by a 250-bp insertion (I)/deletion (D) polymorphism within intron 16 of the ACE gene (Rigat *et al.*, 1990; Rigat *et al.*, 1992). Serum ACE levels are significantly higher in subjects homozygous for the D-allele as compared with II homozygotes (Rigat *et al.*, 1990).

1.3.2. ACE glycosylation

Both sACE and tACE are extensively *N*-glycosylated with carbohydrates such as *N*-acetylglucosamine, fucose, mannose, glucose and sialic acid contributing 18-30 % of their molecular masses (Ehlers *et al.*, 1992). Species- and tissue-dependent heterogeneity in glycosylation contributes to differences in the molecular mass and surface charge of various ACE forms (Hooper and Turner, 1987; Ehlers *et al.*, 1992). sACE contains 17 potential *N*-linked glycosylation sites (Soubrier *et al.*, 1988; Ripka *et al.*, 1993), of which ten are located in the N domain (Soubrier *et al.*, 1988). In human seminal plasma sACE, only 7 of these sites are glycosylated primarily with complex (N-acetyl-lactosaminyl), oligomanosidyl and hybrid-type glycans (Ripka *et al.*, 1993).

The soluble form of sACE has a higher sialic acid content contributing to its lower pI as compared with the membrane-bound form (Baudin *et al.*, 1997). There is no Ser/Thr-rich region to suggest *O*-glycosylation.

In contrast, tACE has a unique 36-residue serine- and threonine-rich N-terminal region, which is heavily *O*-glycosylated (Ehlers *et al.*, 1992). Yu *et al.*, (1997) demonstrated that of the seven potential *N*-linked glycosylation sites in tACE, Asn⁷², Asn⁹⁰ and Asn¹⁰⁹ were fully glycosylated, Asn¹⁵⁵, Asn³³⁷ and Asn⁵⁸⁶ were partially glycosylated and Asn⁶²⁰ was unglycosylated.

1.3.3. Structure-function relationships of ACE

The first three-dimensional structure of ACE was described by Natesh *et al.*, (2003) and was that of native human tACE and its complex with the inhibitor, lisinopril. The X-ray structure revealed significant homology with that of neurolysin (Brown *et al.*, 2001) as well as a *P. furiosus* carboxypeptidase (Arndt *et al.*, 2002). The crystal structure of *Drosophila* AnCE, which has a 42% sequence identity with human tACE, was determined soon thereafter (Kim *et al.*, 2003). Neurolysin, *P. furiosus* carboxypeptidase and *Drosophila* AnCE have similar secondary and core structures as tACE with active sites located in deep narrow grooves that can accommodate only small peptide substrates (Figure 1.4.). The ACE 2 crystal structure has similarly revealed the existence of two subdomains defined by a large hinge-bending motion upon the binding of inhibitors (Towler *et al.*, 2004).

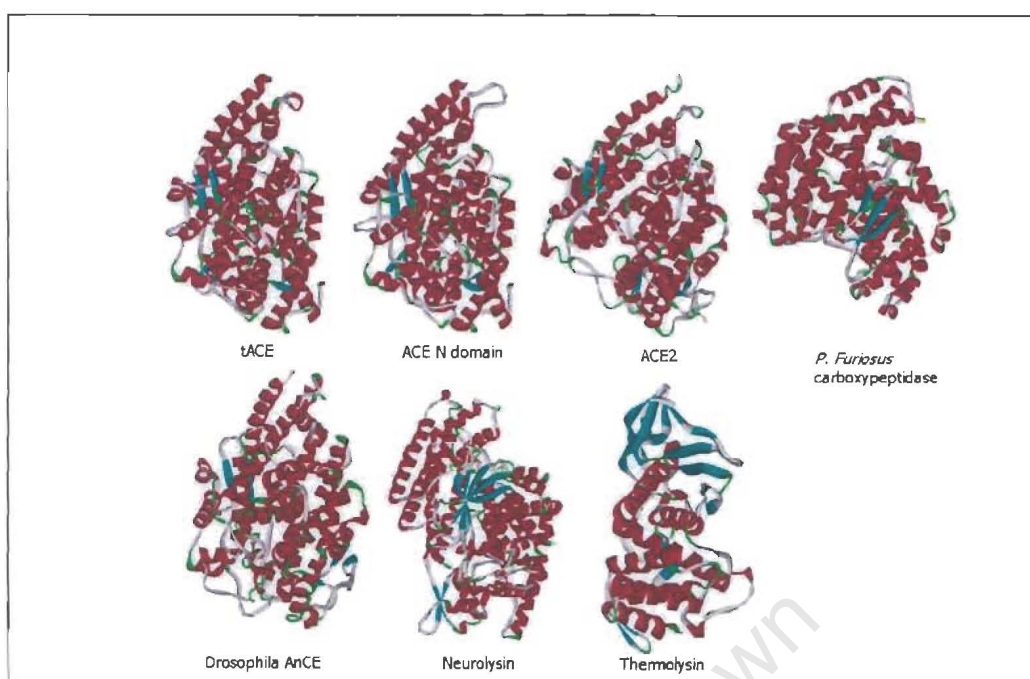


Figure 1.4. Crystal structures of tACE (PDB:108A; Natesh *et al.*, 2003); ACE2 (PDB:1R42; Towler *et al.*, 2004); *P. furiosus* carboxypeptidase (PDB: 1KA4; Arndt *et al.*, 2002); Drosophila AnCE (PDB: 1J38; Kim *et al.*, 2003); Neurolysin (PDB: 1I1I; Brown *et al.*, 2001); Thermolysin (PDB: 1THL; Holland *et al.*, 1995) as well as a homology model of the sACE N domain.

The ~ 60% sequence identity between tACE and the N domain has enabled homology modelling of the N domain structure (Tzakos *et al.*, 2003). Furthermore, the three-dimensional N domain crystal structure has also been recently determined (Corradi *et al.*, in press). Primary sequence differences are well distributed over the length of the two mainly helical proteins and their overall structures are very similar (Appendix 1, Figure 1.2). Both have an ellipsoid shape with a central groove that extends into the protein molecule, separating it into two subdomains I and II. In tACE, there are seven cysteine-residues, six of which form three adjacent disulfide linkages in an *aabbcc* pattern. An unpaired cysteine residue, Cys⁴⁹⁶ is present in the reduced form and is located between the second and C-terminal disulfide bonds (Sturrock *et al.*, 1996). The number and position of these cysteine residues are conserved both within the N domain as well as across species suggesting that they play an important role in enzyme structure and function (Sturrock *et al.*, 1996). Moreover, the formation of disulfide linkages in both tACE (C domain) and the N domain is suggestive of independent folding within the sACE molecule.

Helices α 1-3 form an N-terminal lid partially concealing the active site channel (Figure 1.5.). This lid, comprising several charged residues, may regulate access of inhibitors and polypeptide substrates to the active site cleft. Notable differences exist in the charge and hydrophobicity of residues comprising the lid in tACE and the N domain, this region being more negatively charged in the N domain (Acharya *et al.*, 2003).

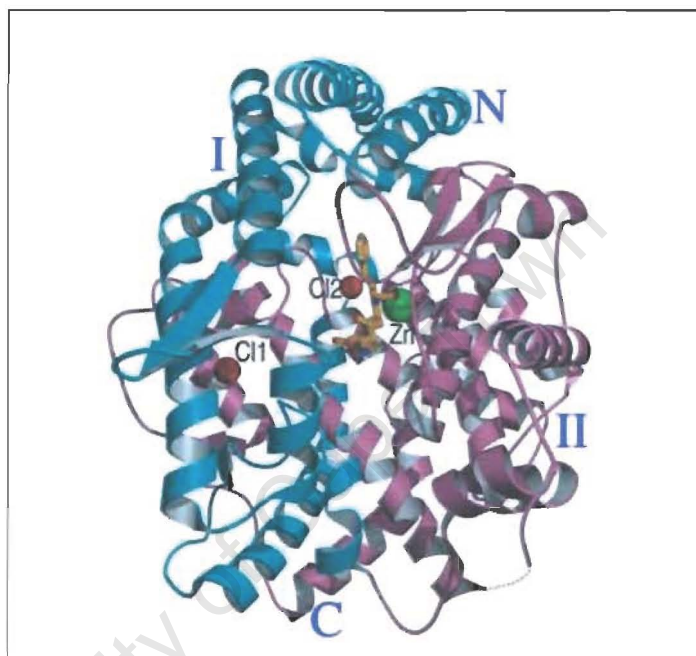


Figure 1.5. The three-dimensional crystal structure of tACE in complex with the inhibitor, lisinopril [From Natesh *et al.*, 2003]. The structure shows the two subdomains I and II, represented in green and cyan, the two bound chloride ions in red, the catalytic zinc in light green and the bound inhibitor in yellow.

The majority of sequence differences in the substrate-binding channels of the N- and C domains occur in non-helical areas (Tzakos *et al.*, 2003) and these differences are explored in more detail in **chapters 2-4** with respect to their influence on the substrate and inhibitor specificity of the two domains. Notably, significant differences are observed in the charge of the binding grooves with the N-domain binding groove being more positively charged than that in tACE (C domain).

Both tACE and the N domain possess two gluzincin zinc-coordinating motifs, HEXXH and EXXXD separated by a 23-residue spacer containing two proline residues. In tACE, the zinc-coordinating motif (H³⁸³EMGH³⁸⁷) is located on helix α 13 while the additional zinc-coordinating Glu⁴¹¹ is located on helix α 14.

There are two buried chloride ions 20.3 Å apart and these are bound to ligands from both subdomains I and II (Figure 1.5.) The first chloride ion, Cl1 (20.7 Å away from the catalytic zinc) is bound to four ligands Arg⁴⁸⁹, Arg¹⁸⁶, Trp⁴⁸⁵ and water and is surrounded by four additional tryptophans forming a hydrophobic shell. These residues are also common binding sites for substrates and inhibitors. Moreover, they are in close proximity to Lys⁵¹¹, which stabilises the C-terminal carboxylate of lisinopril (Tzakos *et al.*, 2003). Thus, Cl1, through structural orientation of this lysine residue may be the anion of importance in high-affinity substrate and inhibitor binding.

The second chloride ion, Cl2 is located closer to the catalytic zinc (10.4 Å) and is bound to Arg⁵²² and Tyr²²⁴ (Arg¹⁰⁹⁸ in sACE) (Natesh *et al.*, 2003; Acharya *et al.*, 2003) (Figure 1.5.). Substitution of Arg¹⁰⁹⁸ for Gln eliminates the chloride-dependence of the C domain for the hydrolysis of tetrapeptide substrates (Liu *et al.*, 2001). It is likely that Cl2 interacts with substrates and inhibitors by virtue of its position on helix $\alpha 17$ along with Tyr⁵²⁰ and Tyr⁵²³ which interact with substrate and inhibitor (Natesh *et al.*, 2003). It has been suggested that in the absence of Cl2, Arg⁵²², which acts as a carrier of neighbouring Tyr⁵²³, forms a salt-bridge with Asp⁴⁶⁵. Here, Tyr⁵²³, which is away from the zinc-binding site, cannot stabilise the substrate-enzyme transition state (Tzakos *et al.*, 2003).

A chloride channel filled with a network of five hydrogen-bonded water molecules connects the external solution with the inner part of the protein. This channel and its water network may provide a pore for the entrance of Cl2 from the external solution (Tzakos *et al.*, 2003). Furthermore, chloride activation may induce conformational changes in the protein that ultimately permits access of peptide substrates to the active site channel via its 3 Å aperture (Acharya *et al.*, 2003).

1.3.3.1 Mechanism of ACE-catalysed substrate hydrolysis

Tyrosyl, glutamyl, arginyl and lysyl residues have been identified as essential components of the ACE active site and important for its catalytic activity (Bünning *et al.*, 1978; Chen and Riordan, 1990).

Both domains of sACE as well as tACE display a general base-type mechanism of substrate hydrolysis where the catalytic zinc stabilizes a bound water/hydroxide and/or activates the scissile carbonyl of the substrate (Figure 1.6.). Glu⁹⁶⁰ of the C domain HEMGII motif assists in the nucleophilic attack of the activated water/hydroxide on the carbonyl forming a tetrahedral intermediate.

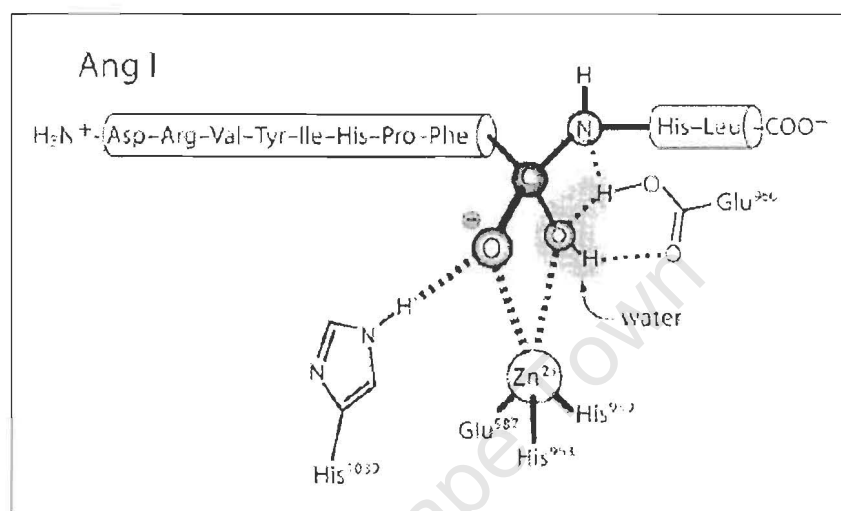


Figure 1.6. The mechanism of of Ang I hydrolysis by the C domain of sACE [From Fernandez *et al.*, 2001].

This Glu-residue thus acts as a general acid/base shuttling the hydrogen from the activated water to the scissile amide nitrogen of the substrate making it a better leaving group and facilitating cleavage of the amide bond. His¹⁰⁸⁹(C domain) stabilises the scissile carbonyl bond during transition state binding (Fernandez *et al.*, 2001). The tetrahedral intermediate is further stabilised by Tyr¹⁰⁹⁶ in the presence of chloride ions, which orientates Arg¹⁰⁹⁸ away from Asp¹⁰⁴¹ resulting in decomposition of the intermediate into product (Matthews, 1988).

ACE cleaves oligopeptides with free carboxyl groups, with the exception of Pro at the P₁' position and Asp or Glu at the P₂' position. The enzyme demonstrates broad substrate selectivity (Rohrbach *et al.*, 1981) acting both as an exo- and endocarboxypeptidase (Figure 1.7.). For substrates like Substance P (SP) and luteinizing hormone-releasing hormone (LHRH) also known as gonadotropin releasing hormone (GnRH), where the C-termini are amidated, sACE not only acts as a dipeptidyl carboxypeptidase but also as an endopeptidase (Erdős, 1990: Hooper, 1991). Naqvi *et al.*, (2005) recently characterised the molecular basis of the exopeptidase activity of the sACE C domain.

This involves interactions between the substrate C-terminal P₂' side-chain and the S₂' pocket of the C domain that act together with carboxylate-docking interactions with Lys¹⁰⁸⁷ and Tyr¹⁰⁹⁶. Such carboxylate docking interactions are thought to stabilise the ground state, restricting the registration of substrates with a C-terminal carboxylate and limiting their processing to the cleavage of a C-terminal dipeptide.

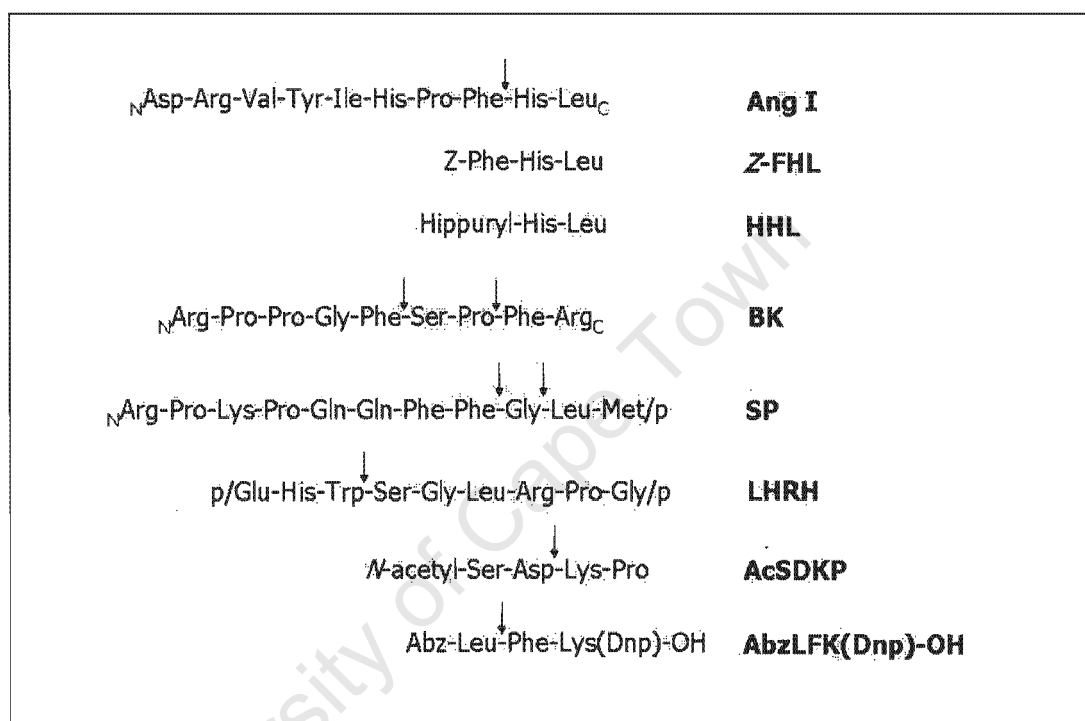


Figure 1.7. Some physiological and synthetic ACE substrates showing the position of peptide bond cleavage (arrow).

Despite the high degree of structural homology between the N- and C domains of sACE as well as the ~89% amino acid sequence identity in regions comprising essential active site residues (Soubrier *et al.*, 1988), each domain demonstrates distinct substrate specificities (Ehlers and Riordan, 1991). Both are catalytically-active, depend on zinc for their activity, are activated by chloride and are sensitive to competitive ACE inhibitors (Wei *et al.*, 1991). Regions of sequence within the active centre, regions surrounding the active centre (Williams *et al.*, 1996) as well as unique structural elements (Marcic *et al.*, 2000) are the primary determinants of domain substrate-specificity.

1.3.3.2. Physiological substrates of ACE

In vitro, both N- and C domains catalyse the hydrolysis of the decapeptide Ang I to the active vasopressor octapeptide Ang II via removal of a C-terminal dipeptide, His-Leu. *In vivo*, the C domain of membrane-bound sACE is primarily responsible for Ang I hydrolysis and thus by itself, sufficient for blood pressure regulation and cardiovascular homeostasis (Esther *et al.*, 1997; Kessler *et al.*, 2003; Fuchs *et al.*, 2004; Van Esch *et al.*, 2005). Furthermore, both domains are responsible for the inactivating hydrolysis of the vasodilator BK, which is a more favoured substrate than Ang I. BK has a substantially lower K_m and a greater k_{cat}/K_m as compared with Ang I (Jaspard *et al.*, 1993). BK is inactivated via sequential hydrolysis of two carboxy-terminal dipeptides at Pro⁷-Phe⁸ and Phe⁵-Ser⁶ (Figure 1.7.) (Ehlers and Riordan, 1990).

SP, a neuropeptide co-localising with neuronal ACE in the striatonigral pathway (Defendini *et al.*, 1983; Strittmatter *et al.*, 1984; Matsas *et al.*, 1984; Barnes *et al.*, 1988; Oblin *et al.*, 1988) is hydrolysed into a C-terminal di- and tripeptide amide, the rate constant of which is higher for the C domain where hydrolysis is more readily activated by chloride (Jaspard *et al.*, 1993). On the other hand, the N domain is primarily responsible for the N-terminal endoproteolytic cleavage of LHRH (Jaspard *et al.*, 1993) as well as the preferential hydrolysis of the bradykinin-potentiating peptide Ang₁₋₇ (Junot *et al.*, 2001).

Another highly N domain-specific substrate is the haemoregulatory peptide N-acetyl-Ser-Asp-Lys-Pro (AcSDKP) (Junot *et al.*, 2001). The N domain hydrolyses AcSDKP 50-fold faster than does the C domain and this substrate is the only known N domain-specific physiological substrate (Michaud *et al.*, 1997). AcSDKP is generated from the N-terminus of thymosin β 4 via cleavage by prolyl oligopeptidase (Cavasin *et al.*, 2004), is a negative regulator of stem cell proliferation, blocking cell entry into the S-phase (Lombard *et al.*, 1990; Volkov *et al.*, 1996; Michaud *et al.*, 1997) and has antifibrotic effects in the kidney and heart (Pokharel *et al.*, 2002; Kanasaki *et al.*, 2003; Pokharel *et al.*, 2004), indicating that the physiological role of sACE is not limited to cardiovascular homeostasis (Rieger *et al.*, 1993; Rousseau *et al.*, 1995; Azizi *et al.*, 1996).

N domain isoforms of ACE which may arise as a result of limited intracellular proteolysis or post-transcriptional modifications (Andrade *et al.*, 1998; Ronchi *et al.*, 2005), have been detected in human ileal fluid (Deddish *et al.*, 1994) as well as in the urine of healthy and hypertensive human subjects and rats, where they may act as a marker for hypertension (Casarini *et al.*, 1995; Casarini *et al.*, 2001; Marques *et al.*, 2003). Their detection in kidney, lung, aorta, heart, liver, adrenal and testicular tissues of spontaneously hypertensive rats (Bueno *et al.*, 2004; Ronchi *et al.*, 2005) suggests that they may play a role in local tissue-specific renin-angiotensin systems.

In this regard, the N domain was recently found to be responsible for the degradation of Alzheimer amyloid β -peptide, inhibiting its aggregation and cytotoxicity (Oba *et al.*, 2005). Other studies have suggested that both N- and C domains contribute to the clearance of amyloid β -protein and that this clearance is inhibited by the ACE inhibitor, captopril (Hemming and Selkoe, 2005). Thus, ACE may play a role in the pathogenesis of Alzheimer's Disease (AD)

1.3.3.3. Synthetic peptide substrates of ACE

A variety of synthetic peptide ACE substrates have been designed that have in common with physiological substrates a free carboxylic C-terminal group and a non-prolyl residue in the penultimate position. The presence of a proline residue at the P_2' carboxy-terminal position increases affinity for both domains of ACE whereas a histidine at the penultimate P_1' position appears to confer C domain substrate-selectivity (Wei *et al.*, 1991).

HHL, which resembles the C-terminus of Ang I, is hydrolysed by ACE to liberate a hippurate and HL (Friedland and Silverstein, 1976). Here, a hippuryl (benzoyl glycine) functionality at the P_1 position increases C domain selectivity (Wei *et al.*, 1991; Marcic *et al.*, 2000). Substitution for a Phe at the P_1 position in Z-Phe-His-Leu (Z-FHL) results in equal domain selectivity (Danilov *et al.*, 1994; Michaud *et al.*, 1997). Thus, the Z-FHL/HHL k_{cat} ratio is characteristic of a particular domain (Williams *et al.*, 1996).

Furylacryloyl-phenylalanyl-glycyl-glycine (FAPGG) resembles the C-terminus of BK and ACE-mediated cleavage liberates a FAP and GG (Holmquist *et al.*, 1979; Bénéteau-Burnat and Baudin, 1986). Like Ang I, both HHL and FAPGG have a strong chloride-dependence and an optimum pH of 8.3 (Baudin, 2002).

Due to its more efficient hydrolysis than Ang I (Jaspard *et al.*, 1993), BK has been used as a model peptide in the generation of a series of internally quenched fluorogenic substrates (Fluorescence Resonance Energy Transfer (FRET) peptides). These peptides contain an ortho-amino benzoic acid (donor) at the N-terminus and EDDnp (2,4-dinitrophenyl ethylene diamine) (acceptor) at the C-terminus occupying the S_2' subsite (Araujo *et al.*, 1999). Cleavage at any of the peptide bonds located between the donor-acceptor pair liberates fluorescence. Internally-quenched fluorogenic peptides based upon the highly N domain-selective substrate AcSDKP have been designed with blocked (Abz-peptidyl-EDDnp) or free (Abz-peptidyl-K(Dnp)-P-OH) C-terminal carboxyl groups (K(Dnp): 2,4-dinitrophenyl-lysine). These substrates are hydrolysed more efficiently than the previously mentioned BK-analogues and they include amongst others Abz-YRK(Dnp)P (hydrolysed equally by both domains), Abz-LFK(Dnp)-OH (more C domain-specific) and Abz-SDK(Dnp)P-OH (more N domain-specific) (Araujo *et al.*, 2000; Bersanetti *et al.*, 2004). The structural basis for the high C domain-selectivity of Abz-LFK(Dnp)-OH was recently investigated via molecular docking with the tACE structure (Natesh *et al.*, 2003) and shown to arise primarily from differences in the S_1' pockets in the N- and C domains (Bersanetti *et al.*, 2004).

1.3.3.4. Chloride-dependence of substrate hydrolysis

Despite the relatively constant plasma concentration of chloride, substrate hydrolysis by the C domain of ACE is highly dependent on the chloride concentration and is strongly activated by chloride while the N domain is not (Bünning and Riordan, 1983; Shapiro *et al.*, 1983; Wei *et al.*, 1991; Jaspard *et al.*, 1993; Moiseeva *et al.*, 2005). The lower chloride-dependence of the N domain may be related to the presence of only one chloride-binding pocket (Arg⁵⁰⁰ and Arg⁵⁰²). The second chloride-binding pocket is lost due to substitution of Arg¹⁸⁶ (tACE) with His¹⁶⁴ in the N domain (Acharya *et al.*, 2003).

Chloride activation is both substrate- and pH-dependent (Shapiro and Riordan, 1984; Ehlers and Kirsch, 1988). For Ang I it is high and for BK it is low while for Abz-peptidyl-EDDnp substrates the chloride-dependence is higher than for Abz-peptidyl-K(Dnp)P-OH substrates (Araujo *et al.*, 2000).

1.3.4. ACE inhibitors

BK potentiating peptides (BPPs) were the first naturally occurring ACE inhibitors, isolated from the venom of the Brazilian snake *Bothrops jararaca* (Ferreira and Rocha e Silva, 1965; Ferreira, 1965). BPPs are pyroglutamyl, proline-rich oligopeptides characterised by their ability to potentiate the effects of BK (Murayama *et al.*, 1997; Cotton *et al.*, 2002; Hayashi and Camargo, 2005).

The discovery of BPPs lead to the development of the first non-peptidic ACE inhibitor, captopril (D-3-mercapto-2-methyl-propanoyl-L-proline) in 1977 (Cushman *et al.*, 1977; Ondetti *et al.*, 1977; Cushman *et al.*, 1999). Captopril is an *N*-thioalkyl derivative mimicking the two carboxy-terminal Ala-Pro residues of the substrate with its thiol functionality coordinating the zinc atom in the ACE active site (Tzakos *et al.*, 2005)(Figure 1.8.).

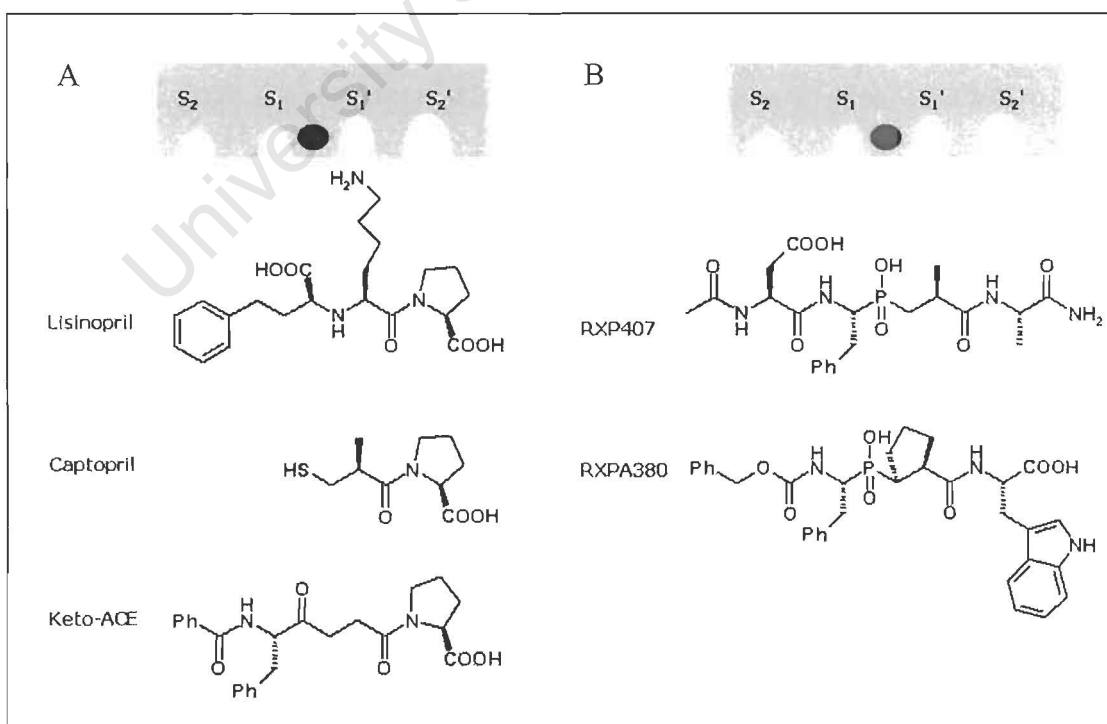


Figure 1.8. ACE inhibitors lisinopril, captopril and the ketomethylene derivative, keto-ACE and their binding to the ACE active site (A). N domain-selective RXP407 (Dive *et al.*, 1999) and C domain-selective RXPA380 (Georgiadis *et al.*, 2004) phosphinic peptide inhibitors and their binding to the ACE active site (B). The blue oval represents the catalytic zinc ion.

In overcoming the side-effects associated with the thiol group such as taste disturbances, skin rashes, nephritic syndrome and neutropenia, compounds with zinc-coordinating carboxyalkyl moieties were developed. These have included lisinopril ($\{N-[(S)-1\text{-carboxy-3-phenylpropyl}]\text{-L-lysyl-L-proline}\}$) (Figure 1.8.) and enalaprilat ($\{N-[(S)-1\text{-carboxy-3-phenylpropyl}]\text{-L-alanyl-L-proline}\}$) (Patchett *et al.*, 1980; Ehlers and Riordan, 1989). Other ACE inhibitors incorporating a carboxylate zinc-binding group include benazepril, perindopril, moexipril, ramipril, quinapril and trandolapril. Fosinopril is the only commercial ACE inhibitor with a phosphinyl zinc-binding functionality (Piepho, 2000).

In tissues where ACE occurs behind tissue-blood barriers, such as tACE in the testes and sACE in certain regions of the brain, the enzyme is not inhibited (Vollmer and Boccagno, 1977; Waites and Gladwell, 1982; Sakaguchi *et al.*, 1988a-c). The ease with which ACE inhibitors are able to penetrate these lipid barriers is governed by their lipophilicity. While lisinopril is the least lipophilic, fosinopril is the most lipophilic (Piepho, 2000). In order to increase their lipophilicity, most ACE inhibitors are administered as esterified pro-drugs, which enhances intestinal uptake via passive diffusion, thus improving absorption and bioavailability (Toutain and Lefebvre, 2004). Lisinopril is the only ACE inhibitor that does not require hepatic de-esterification while ramipril is the only ACE inhibitor that has been shown to inhibit tissue ACE, a feature related to its high lipophilicity conferred by the incorporation of a pentane ring (Bender *et al.*, 1990).

ACE inhibitors are slow, tight-binding competitive or mixed-type inhibitors with K_i values in the subnanomolar range (Shapiro and Riordan, 1984; Goli and Galardy, 1986; Baudin and Bénétteau-Burnat, 1999). They bind to sACE at a 1:1 stoichiometry, indicating binding to only one of the two active sites (Ehlers and Riordan, 1991; Michaud *et al.*, 1997; Andujar-Sanchez *et al.*, 2004; Skirgello *et al.*, 2005). Thus, a degree of cooperativity exists with respect to inhibitor binding. Binding is complex and substrate-dependent and follows a two-step mechanism where the initial EI complex undergoes a chloride-dependent slow isomerization to a more tightly bound EI* complex (Shapiro and Riordan, 1984).

For example, the high potency and long duration of action of ramipril as compared with other inhibitors is related to its ability to rapidly form a slowly dissociating EI complex (Bünning, 1987; Vasmant and Bender, 1989).

1.3.4.1. Efficacy of current generation ACE inhibitors

While the efficacy of orally-active ACE inhibitors is similar to other antihypertensive agents, they have a favourable metabolic profile, better tolerability and limited side effects (Ibrahim, 2006). Consequently, they are the most commonly prescribed drugs for the treatment of hypertension. Furthermore, they are widely used in the successful treatment and management of congestive heart failure, myocardial infarction, diabetic nephropathies (Fernandez *et al.*, 2003) and hypertension accompanied by cardiometabolic syndrome and type II diabetes (McFarlane *et al.*, 2003). Their beneficial effects are also partly due to their ability to prevent events downstream of Ang II formation such as heart and vessel remodelling, salt and water retention and elevated vascular tone (Der Sarkissian *et al.*, 2005).

ACE inhibitors may also potentiate the antifibrotic effects of AcSDKP in the kidney and heart by inhibiting its hydrolysis (Pokharel *et al.*, 2002; Kanasaki *et al.*, 2003; Peng *et al.*, 2005). They are beneficial in insulin-resistant conditions occurring concomitantly with hypertension where they enhance systemic glucose disposal and glucose transport activity in skeletal muscle through a NO-dependent effect of BK as well as antagonism of the effects of Ang II on skeletal muscle (Henriksen and Jacob, 2003). ACE inhibitors have been shown to attenuate the development of atherosclerosis via activation of the NO pathway in the vascular endothelium (Scribner *et al.*, 2003). Moreover, they enhance the survival of vascular endothelial cells by up-regulating proteins that promote cell survival (Hamdi and Castellon, 2003).

ACE inhibitors increase ACE expression (Costerousse *et al.*, 1998) and together with BK, increase the activity of protein kinase CK2 and JNK as well as the phosphorylation of ACE within its cytosolic tail (Kohlstedt *et al.*, 2002; Kohlstedt *et al.* 2004). This outside-in signalling is related to the increased expression of COX-2 and subsequently also prostacyclin and prostaglandin E₂ (Kohlstedt *et al.*, 2005).

ACE inhibitors reactivate desensitised BK B₂ kinin receptors, potentiating the effects of ACE-resistant BK analogues (Hecker *et al.*, 1997). This involves cross-talk between ACE and the B₂ kinin receptor (Marcic *et al.*, 1999).

1.3.4.2. Domain-selectivity of current generation ACE inhibitors

In addition to distinct differences in the specificity of the N- and C domains of sACE for various substrates, they also demonstrate different inhibitor selectivities which may be organ/tissue specific (Bevilacqua *et al.*, 1996). The order of potency for captopril, enalaprilat and lisinopril is: lisinopril>enalaprilat>captopril for the C domain while this order is reversed for the N domain (Wei *et al.*, 1992).

Lisinopril is thus a more C domain-selective ACE inhibitor (Natesh *et al.*, 2004; Tzakos and Gerothanassis, 2005) while captopril is about 15-fold more N domain-selective (Michaud *et al.*, 1997), inhibiting the hydrolysis of N domain-specific substrates such as AcSDKP (Rousseau *et al.*, 1995; Azizi *et al.*, 1996; Michaud *et al.*, 1997). The N domain-selectivity of captopril is due to the substitution of V³⁸⁰ (tACE) for T³⁵⁸ in the N domain S₁' pocket accommodating the inhibitor alanyl functionality (Natesh *et al.*, 2004; Tzakos and Gerothanassis, 2005). The structural basis for the C domain-selectivity of lisinopril is addressed in detail in **Chapter 4**.

Current generation ACE inhibitors were designed without knowledge of the three-dimensional structure of ACE (Acharya *et al.*, 2003; Natesh *et al.*, 2003). The design of captopril was based upon the structure of bovine pancreas carboxypeptidase A, a zinc-dependent carboxypeptidase thought to have similar catalytic mechanisms to that of ACE (Cushman *et al.*, 1977; Ondetti *et al.*, 1977; Cushman *et al.*, 1999). Lisinopril and enalaprilat were developed based upon their inhibition of thermolysin (Patchett *et al.*, 1980; Patchett *et al.*, 1985). Furthermore, the design of most ACE inhibitors has focussed on the incorporation of strong zinc-binding functionalities such as thiolates or carboxylates. However, a disadvantage has been poor inhibitor selectivity since binding is determined by the strength of the interaction with the zinc atom (Dive *et al.*, 2004).

The poor domain-selectivity of ACE inhibitors may be the underlying cause of their adverse side effects. Since both the N- and C domains are involved in BK hydrolysis, non-specific inhibition of both domains may be linked to the phenomena of persistent cough (5-20% of patients) and potentially life-threatening angioedema (0.1-0.5% of patients)(Bickett, 2002; Dickstein *et al.*, 2002; Adam *et al.*, 2002; Morimoto *et al.*, 2004).

Since BK is hydrolysed more readily by ACE than Ang I, ACE inhibition may not only reduce Ang II levels but may also lead to the accumulation of BK. This together with the accumulation of SP, an effector of bronchoconstriction (Morice *et al.*, 1987; Ehlers and Riordan, 1989), may be the underlying cause of cough most likely via the stimulation of vagal C fibre afferents controlling the cough reflex (Semple, 1995). BK has also been implicated in the induction of airways hyper-responsiveness in asthma (Godat *et al.*, 2004). Hyperkalemia (<1%) occurs most often in patients with renal insufficiency (Piepho, 2000; Morimoto *et al.*, 2004) while hypotensive reaction and/or deterioration in kidney function has been reported in elderly patients or in patients who are either volume depleted, receiving large doses of diuretics or are in heart failure (Ibrahim, 2006).

1.3.4.3. Development of novel domain-selective ACE inhibitors

The recently elucidated structure of native testis ACE, tACE in complex with lisinopril (Natesh *et al.*, 2003) as well as subsequent structures of ACE-inhibitor complexes (Kim *et al.*, 2003 and Natesh *et al.*, 2004) has provided valuable insights into the molecular basis of the specific interactions between the ACE substrate-binding pockets and ACE inhibitors. This has permitted the development of approaches that aim to increase the domain-selectivity of ACE inhibitors, improving their efficacy and reducing the prevalence of adverse side-effects. Several classes of compounds have been developed in this regard including ketomethylene derivatives and phosphinic peptides .

1.3.4.3.1. Keto-ACE

Keto-ACE, a ketomethylene derivative of the blocked tripeptide substrate, Bz-Phe-Gly-Pro is 37-48 times more C-selective as compared with the N domain (Almquist *et al.*, 1980; Deddish *et al.*, 1998) (Figure 1.8.).

Keto-ACE contains a ketomethylene isostere replacement at the scissile bond that mimics the tetrahedral transition state of the proteolytic reaction (Meyer *et al.*, 1981). The structural basis for its C domain-selectivity is discussed in **Chapter 4**.

1.3.4.3.2. Phosphinic peptides

Phosphinic peptides, incorporating a weaker zinc-binding phosphinic moiety ($\text{PO}_2\text{-CH}_2$) mimic the structure of substrates in the transition state (Dive *et al.*, 2004). The first highly selective N domain-selective phosphinic peptide ACE inhibitor RXP407, is about 1000-fold more N-selective (Dive *et al.*, 1999; Junot *et al.*, 2001; Dive *et al.*, 2004) (Figure 1.8.). The rationale behind the development of RXP407 was based upon the specific hydrolysis of AcSDKP by the N domain. Selective inhibition of the N domain could maintain normal undifferentiated cells in the quiescent phase during chemotherapy while permitting normal Ang I metabolism by the C domain (Michaud *et al.*, 1997). Dive and co-workers have also developed a highly C domain-selective inhibitor, RXPA380 which is 3000-fold more selective for the C domain of ACE (Georgiadis *et al.*, 2003) (Figure 1.8.). Both RXP407 and RXPA380 are stable *in vivo* and are resistant to rapid hepatic metabolism, this being most likely the result of the strong negative charge of the phosphinic functionality which limits hepatic uptake and non-specific interactions with peptidases (Dive *et al.*, 2004). The structural basis for the domain-selectivity of these compounds is discussed in **Chapter 4**.

Rationale and Objectives

Recent advances in the structural elucidation of vasopeptidases involved in the renin-angiotensin/kallikrein-kinin systems have made a significant contribution to our understanding of the structure-function relationships of these enzymes. In particular, the elucidation of the structure-function relationship of ACE, a critical regulator of cardiovascular homeostasis, has permitted the development of domain-selective inhibitors. However, while the structural and functional characterisation of ACE has been extensive, many aspects have required further investigation.

The glycosylation heterogeneity of ACE has hampered attempts at its crystallisation and structure determination. Moreover, the exact role of glycosylation in the structure-function relationship of ACE and in particular the role of *N*-linked glycosylation, has not been determined.

While large strides have been made with respect to the functional characterisation of physiological and synthetic ACE substrates, the solution of its three-dimensional crystal structure has enabled the development of approaches aimed at identifying regions and residues that contribute to the thermal stability, substrate- and inhibitor-specificity of the N- and C domains.

Differences in the ectodomain cleavage secretion of sACE and tACE may suggest some form of interaction between the independently folded N- and C domains, further reinforced by observations of interdomain cooperativity with respect to inhibition and substrate hydrolysis. However, the structural basis for these interactions has not been determined.

In examining these aspects, the aims of the present work were:

1. To determine the role of *N*-linked glycosylation in the structure, function and processing of testis ACE and the N domain of sACE (**Chapter 2**).
2. To develop strategies in the production of minimally-glycosylated ACE that is amenable to crystallisation and structure determination (**Chapter 2**).
3. To delineate regions of the N- and C domains which confer domain-specific substrate-selectivity and structural stability (**Chapter 3**).
4. To assess the nature of the interactions between the N- and C domains within sACE with respect to substrate hydrolysis given the hypothesis that they are closely associated and that this association governs the ectodomain cleavage secretion of sACE (**Chapter 3**).
5. To determine the basis for the domain-selectivity of the N- and C domains using novel analogues of lisinopril and ketomethylene inhibitors (**Chapter 4**).

CHAPTER 2

The role of *N*-linked glycosylation.

2.1. Introduction

Protein glycosylation, an important post-translational modification, plays a critical role in protein folding, expression, secretion, stability, recognition and activity (Varki, 1993; Imperiali and Rickert, 1995). *N*-linked protein glycosylation involves the transfer of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ from a dolichol precursor to the Asn-residue of an Asn-Xaa-Ser/Thr glycosylation sequon by the oligosaccharyltransferase complex (OST). Subsequent sequential trimming and processing of the oligosaccharide by various glycosidases in the Endoplasmic Reticulum (ER) is followed by the development of complex and hybrid-type structures in the Golgi apparatus (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985; Helenius and Aebi, 2001) (Figure 2.1.). Glucosidase I removes the outer α 1,2-linked glucose residue from the oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$. This is followed by the removal of the remaining two glucose residues in $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ and $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ by glucosidase II (Petrescu *et al.*, 1997). Subsequent recognition of $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ by calreticulin and calnexin results in chaperone-dependent folding in the ER (Ou *et al.*, 1993; Hebert *et al.*, 1995; Ware *et al.*, 1995; Petrescu *et al.*, 1997). Further trimming occurs in the Golgi apparatus with the subsequent formation of complex and hybrid-type oligosaccharides (Figure 2.1.).

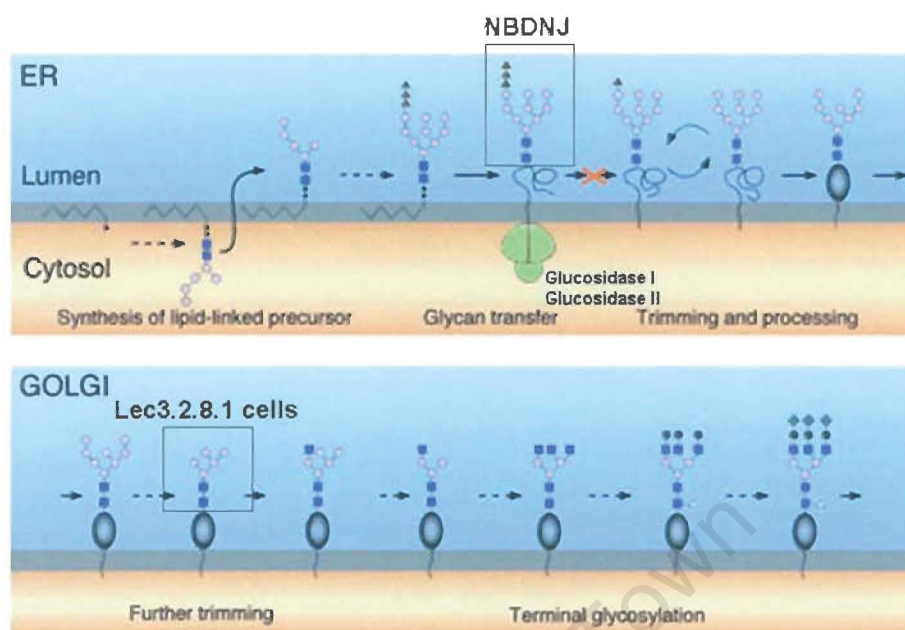


Figure 2.1. The mammalian *N*-linked glycosylation pathway occurs in the ER and Golgi apparatus. Processing of *N*-linked glycans to complex and hybrid-type structures may be inhibited in the ER by glucosidase inhibitors such as NBDNJ and in the Golgi apparatus of glycosylation-deficient *Lec3.2.8.1* cells [Figure adapted from Helenius and Aebi, 2001].

Glycoform heterogeneity is dependent upon local conformational properties of the polypeptide, the availability of monosaccharide precursors and the expression pattern of various modifying enzymes in the glycosylation pathway (Paulson and Colley, 1989). The glycosylation heterogeneity of ACE has hampered attempts at its crystallisation and structure determination. Partial or complete deglycosylation has thus been considered an appropriate strategy in producing proteins amenable to crystallisation and various approaches in this regard have been attempted. While the expression of rabbit tACE in *E.coli* generated completely deglycosylated protein that was catalytically-inactive (Sadhukhan and Sen, 1996), expression of partially glycosylated ACE in both yeast and *HeLa* cells generated active enzyme.

Protein expression in glycosylation-deficient cells or in the presence of glucosidase inhibitors limits glycan processing to simple forms, which are sensitive to *endo H* cleavage between the two GlcNAc residues.

Lec3.2.8.1 cells are deficient in sialic acid metabolism, have a disrupted *N*-acetylglucosamine (GlcNAc) transferase I and defective UDP-galactose and CMP-sialic acid translocation (Butters *et al.*, 1999). Here, protein *N*-glycan processing in the Golgi apparatus is limited to the Man₅GlcNAc₂ stage (Stanley, 1981) (Figure 2.1.).

The *N*-alkylated imino sugar *N*-butyl deoxynojirimycin (NBDNJ) is a glucosidase I-inhibitor that limits *N*-glycan processing in the ER to the Glc₃Man₇₋₉GlcNAc₂ stage, resulting in oligomannose forms attached to GlcNAc (Gross *et al.*, 1983; Foddy and Hughes, 1988; Fleet *et al.*, 1988; Mellor *et al.*, 2004) (Figure 2.1.). Expression of a truncated form of tACE (tACEΔ36NJ) lacking the 36-residue *N*-terminal region as well as the transmembrane and cytosolic regions, in the presence of NBDNJ yielded electrophoretically-homogeneous enzyme that retained the catalytic activity of wild-type tACE (Yu *et al.*, 1997). This protein, expressed using pEE14, a high level GS-MSX mammalian expression vector, was subsequently crystallised and has yielded the first three-dimensional tACE structure (Natesh *et al.*, 2003). This structure has subsequently served as a homology modelling template for the N domain (Tzakos *et al.*, 2003).

Site-directed mutagenesis of *N*-linked glycosylation sites in tACE has been another strategy for generating protein amenable to crystallisation and structural determination (Gordon *et al.*, 2003). Moreover, this approach has permitted the determination of the roles of individual *N*-linked glycosylation sites in enzyme processing and catalytic activity of tACE (C domain).

The role of *N*-linked glycosylation in the processing and catalytic activity of the N domain as a separate functional entity within sACE has not been extensively investigated. Moreover, the successful determination of the tACE crystal structure (Natesh *et al.*, 2003) using the aforementioned expression and deglycosylation strategies has opened the way for the determination of the three-dimensional crystal structure of the N domain using similar approaches (Corradi *et al.*, in press).

The aims of the present study were:

1. To assess the effect of deglycosylation on the catalytic properties of tACE, the kinetic parameters of tACE *N*-linked glycosylation site mutants as well as tACE expressed in the presence of NBDNJ, were determined.
2. To produce N domain protein that is amenable to crystallisation using site-directed mutagenesis of *N*-linked glycosylation sites, protein expression in the presence of the glucosidase-inhibitor, NBDNJ and protein expression in glycosylation-deficient *Lec3.2.8.1* cells.
3. To elucidate the role of *N*-linked glycosylation in the processing and catalytic activity of the N domain.

2.2. Materials and Methods

2.2.1. Kinetic characterisation of tACE glycoforms

tACE Δ 36 is a truncated form of tACE lacking the heavily *O*-glycosylated 36 residue N-terminal region (Ehlers *et al.*, 1992). This construct has been used as a template in the generation of a series of glycosylation mutants in which six of the seven potential *N*-linked glycosylation sites of tACE (Asn-residues 72, 90, 109, 155, 337 and 586) have been mutated in a step-wise site-directed manner to Gln-residues (Figure 2.2.). The construction, expression and purification of these mutants have been described previously (Gordon *et al.*, 2003; Gordon, K., M.Sc. dissertation, 2002).

Kinetic constants for the hydrolysis of HHL by tACE site-directed glycosylation mutants and tACE Δ 36NJ expressed in CHO-*K1* cells with or without 1.5 mM NBDNJ were determined according to the method described by Friedland and Silverstein (1976). Assays were conducted in triplicate under initial rate conditions at 37°C such that substrate hydrolysis was < 10%. Hydrolysis reactions were performed in 100 mM potassium phosphate buffer, pH 8.3, containing 300 mM NaCl with 4 nM protein over a 0 mM-7 mM substrate concentration range. Reactions were stopped with 180 μ l 0.28*N* NaOH followed by derivatisation of liberated HL with 12 μ l of 150 mM *o*-phthaldialdehyde. Derivatisation was stopped with 26 μ l 3*N* HCl (Appendix 2.1.).

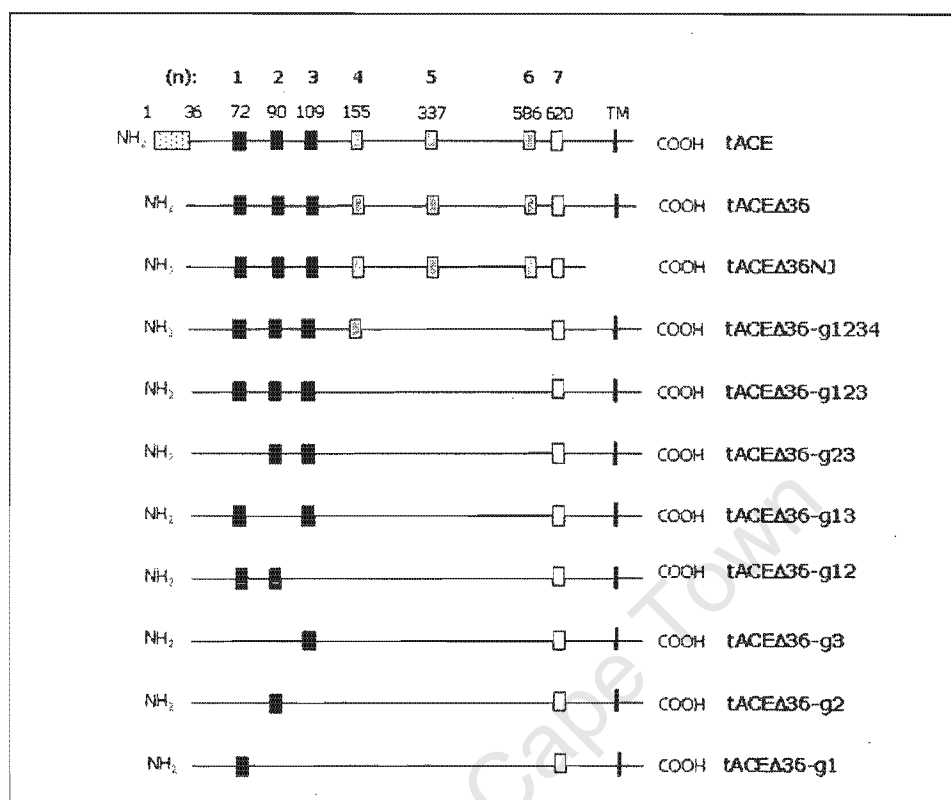


Figure 2.2. Site-directed glycosylation mutants of tACE. Squares in black are fully glycosylated sites, squares in grey are partially glycosylated while Asn⁶²⁰ is unglycosylated. TM: Transmembrane region. Residues 1-36 at the N-terminus, which comprise the *O*-glycosylated region, are deleted in tACEΔ36. tACEΔ36NJ is a soluble form of tACEΔ36 lacking the transmembrane and cytosolic regions. Its construction, expression and purification have been described previously (Yu *et al.*, 1997). [Figure adapted from Gordon *et al.*, 2003].

Fluorescence was measured at $\lambda_{\text{Ex}}=360\text{nm}$ and $\lambda_{\text{Em}}=486\text{nm}$ on a Cary Eclipse fluorometric plate reader (Varian). Kinetic constants were calculated via non-linear regression analysis of Michaelis-Menten plots (GraphPad Prism 4.01) together with a HL standard curve. Turnover numbers (k_{cat}) and specificity constants (k_{cat}/K_m) were determined using a calculated molecular mass of 100 kDa for tACE (Appendix 2.1.).

2.2.2. Homology modelling of the N domain and prediction of *N*-linked glycosylation sites

N domain sequence D²¹-P⁶⁰¹ (aligned as D¹-P⁵⁸³) was modelled via SWISS-MODEL using as template the crystal structure of tACE in complex with lisinopril (D⁴⁰-P⁶¹⁷)(PDB: 1O86A) (Natesh *et al.*, 2003)(Schwede *et al.*, 2003).

The homology modelled N domain structure was viewed using SwissPDBViewer (Schwede *et al.*, 2003), PyMOL (DeLano Scientific LLC) and DS Viewer Pro (Accelrys) and compared to that of the tACE-lisinopril complex (PDB: 1O86A)(Natesh *et al.*, 2003). The presence of *N*-linked glycosylation sites in the N domain was predicted using the NetNGlyc1.0 server (Gupta, in preparation). A threshold of 0.50 was used for *N*-glycosylation potential.

2.2.3. Cloning of a truncated soluble N domain

The plasmid pECE-ACED629 encoding soluble N domain plus linker region and part of the C domain (L¹-D⁶²⁹) was a gift from Prof. Sergei Danilov, University of Illinois, Chicago (Balyasnikova *et al.*, 2003). ACED629 cDNA (1.994 kb) was excised from the vector pECE using *Xba*I and *Eco*RI and subcloned into Bluescript II SK+ (Stratagene). Sequenced ACED629 cDNA was then subcloned into the expression vector pcDNA3.1(+)(Invitrogen) using *Xba*I and *Eco*RI. ACED629 was then subcloned from pcDNA3.1(+) into the GS-MSX amplification vector pEE14 (Celltech), using *Hind*III and *Eco*RI (Figure 2.3.) (Appendix 2.4.).

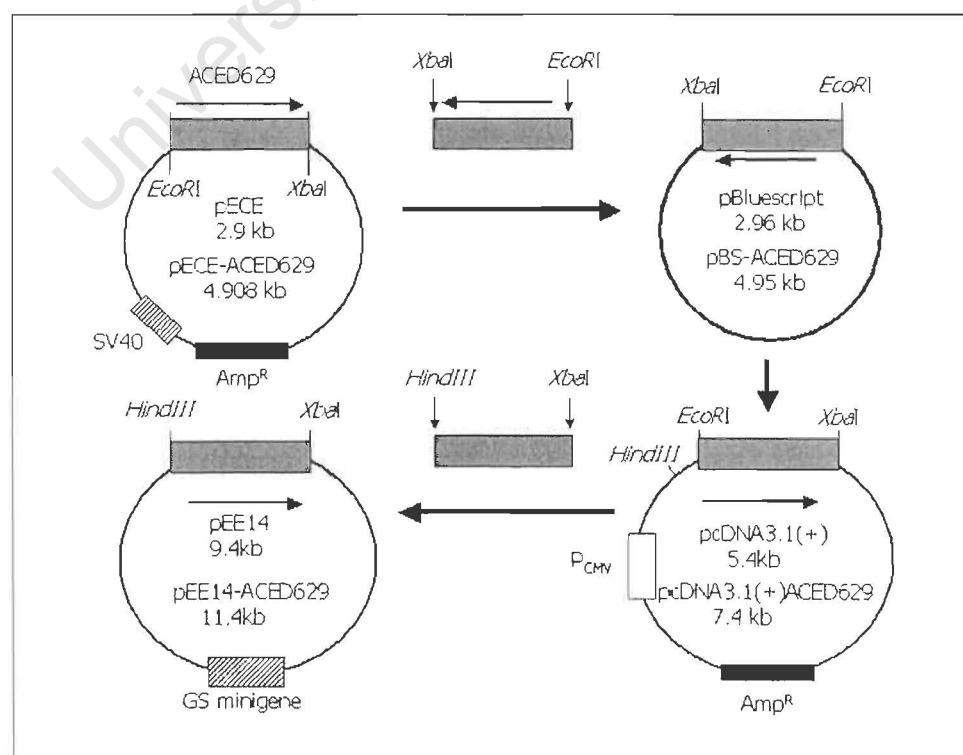


Figure 2.3. Subcloning ACED629 cDNA into mammalian expression vectors pcDNA3.1(+) and pEF14.

2.2.4. Site-directed mutagenesis of N domain *N*-linked glycosylation sites

Based upon the localisation of predicted *N*-linked glycosylation sites in the N domain homology model, a site-directed mutagenesis approach was employed in the step-wise mutation of Asn⁹; Asn²⁵; Asn⁴⁵; Asn⁸²; Asn¹¹⁷; Asn¹³¹; Asn²⁸⁹; Asn⁴¹⁶ and Asn⁴⁸⁰ to corresponding Gln residues. Asn⁴⁹⁴, which was predicted to be non-glycosylated, was not mutated (Figure 2.4.). 100 ng of pBS-ACED629 DNA template was amplified with 0.4 mM of each dNTP, 200 nM of forward and reverse mutagenic primer and 3U *Pfu* DNA Polymerase (Promega)(Appendix 2.5.).

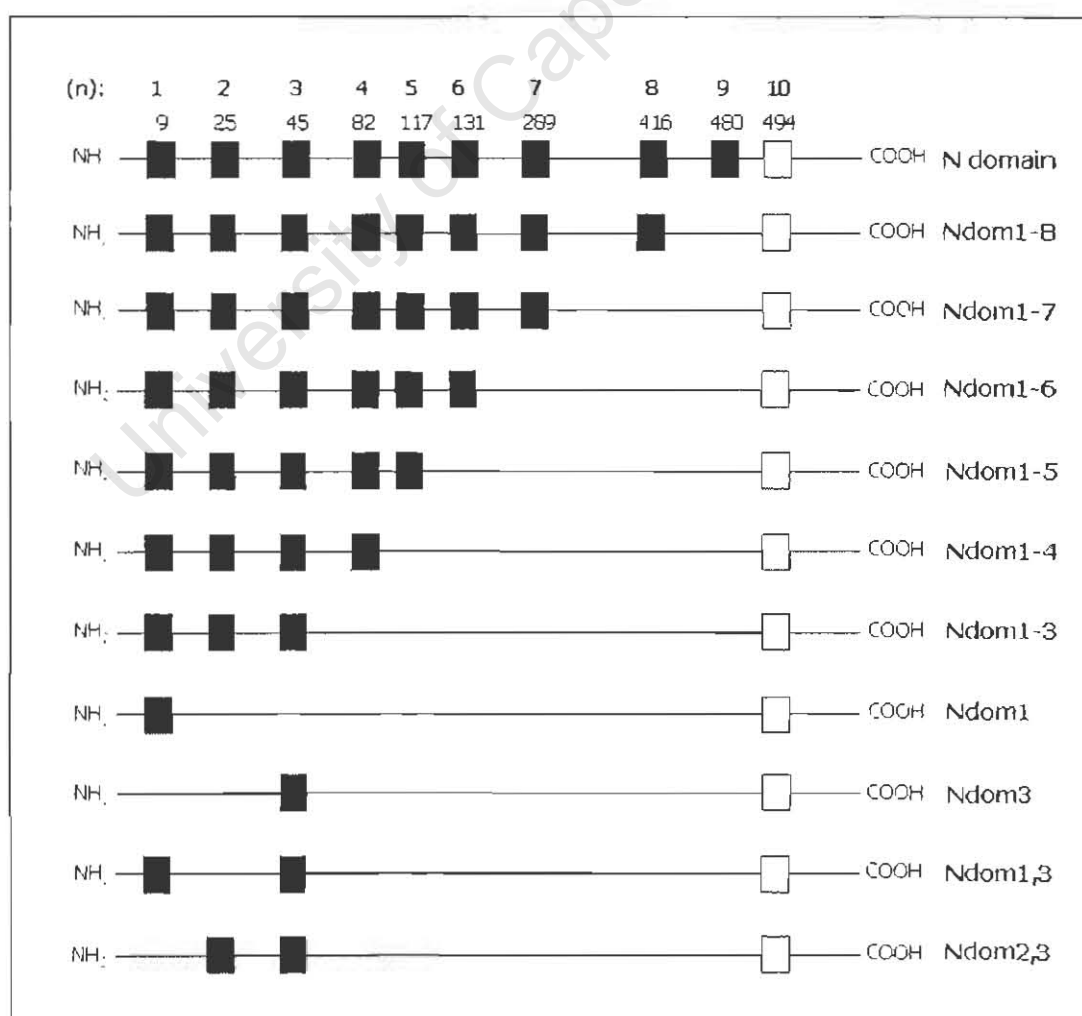


Figure 2.4. Site-directed glycosylation mutants of the N domain. Asn⁴⁹⁴ is not glycosylated and was not mutated based upon NetNGlyc1.0 prediction data.

Reactions were conducted in 20 mM Tris-HCl, pH 8.8 containing 10 mM KCl, 10 mM (NH₄)₂ SO₄, 2.0 mM MgSO₄, 0.1 % Triton X-100, 0.1 mg/ml Bovine Serum Albumin (BSA), 5 % dimethylsulfoxide (DMSO) and 5 mM MgCl₂. Cycling parameters: 95°C, 5 minutes followed by 16 cycles of 95°C, 30 sec; 55°C, 30 sec and 75°C, 12 minutes and then a final step at 75°C for 20 minutes. Amplicons were visualised on 1% agarose gels and digested with 20U of *DpnI* (Promega) at 37°C for 60 minutes (Appendix 2.5.).

Competent *E.coli* XL-1 Blue cells were transformed with 5µl/100µl *DpnI*-treated DNA. DNA was isolated and purified from bacterial cultures and digested with the relevant restriction enzymes encoded by the mutagenic primers in order to identify mutants. Mutated N domain ACED629 was sequenced in pBS and subcloned into the expression vector pcDNA3.1+ using *XbaI* and *EcoRI* (Appendix 2.4.).

2.2.5. Expression and activity of N domain site-directed glycosylation mutants

CHO-K1 cells were stably-transfected with N domain site-directed glycosylation mutants in pcDNA3.1(+) using the calcium phosphate method (Promega). Positive clones were selected with Geneticin G418 in 10 % FCS. Following 36 hours incubation in OPTIMEM (Gibco BRL), culture medium was removed and concentrated ten-fold. Enzyme activity was determined following incubation of 10 µl culture medium with 120 µl 1.0 mM Z-FHL(Bachem) at 37°C for 30 minutes. Reactions were stopped with 725 µl 0.28N NaOH. Liberated HL was derivatised for 10 minutes at ambient temperature with 50 µl 150 mM *o*-phthaldialdehyde (Sigma). Derivatisation was terminated by adding 100 µl 3N HCl. Fluorescence was measured from 200 µl at $\lambda_{Ex}=360\text{nm}$ and $\lambda_{Em}=486\text{nm}$ on a Cary Eclipse fluorimetric plate reader (Varian). Activities in mU/ml were calculated using a HL (Sigma) standard curve (Appendix 2.1.).

Cell lysates and culture media were analysed via Western blot using an ACE polyclonal antibody and horseradish- peroxidase-conjugated goat anti-rabbit IgG (Appendix 2.8.).

2.2.6. N domain expression in CHO-*K1* and *Lec3.2.8.1* cells with or without NBDNJ.

pECE-ACED629 was stably transfected into CHO-*K1* cells and *Lec3.2.8.1* cells (A gift from Simon J. Davis) (Butters *et al.*, 1999). All transfections were conducted along with pSV2Neo (10:1) using the calcium phosphate method (Promega). Positive clones were selected with Geneticin G418 (Sigma) in 10% FCS. Transfected cells were bulk cultured in 2% FCS with or without 1.5 mM NBDNJ (Toronto Research Chemicals Inc.) for CHO-*K1* cells and 0.5 mM NBDNJ for *Lec3.2.8.1* cells.

The effects of *Lec3.2.8.1* cells and NBDNJ on the expression of N domain was assessed by determining enzyme activity in culture media using 5.7 mM HHL (Sigma) and 1.0 mM Z-FHL as substrates. Activity was determined from 10 μ l culture medium as described previously (Appendix 2.1.).

2.2.7. Purification and kinetic characterisation of N domain glycoforms

N domain expressed in CHO-*K1* and *Lec3.2.8.1* cells with or without NBDNJ was purified from culture medium supernatants by immunoaffinity chromatography over a protein-G agarose column coupled to an N domain specific monoclonal antibody (5C5) (a gift from Prof. Sergei Danilov). Protein was eluted with 50mM ethanolamine pH 11.5 and dialysed against 5mM HEPES pH 7.5, 0.1 mM PMSF. Purified proteins were concentrated in an Amicon concentrator (30kDa cut-off) (Millipore) at 1000-2000 g and 4°C. Protein concentrations were determined using the Bradford method (BioRad) (Appendix 2.10.) and subjected to 10% SDS-PAGE (Appendix 2.7.).

Kinetic constants for the hydrolysis of HHL and Z-FHL by purified N domain were determined as described previously over a 0 mM-2.5 mM (Z-FHL) and 0 mM-7 mM (HHL) substrate concentration range.

Based upon an observed K_m of 0.93 mM for HHL by the N domain, k_{cat} values for its hydrolysis were subsequently determined following incubation with 2.0 mM ($\sim 2 \times 0.93$ mM) substrate for 30 minutes. Turnover numbers (k_{cat})(Z-FHL and HHL) and specificity constants (k_{cat}/K_m)(Z-FHL) were determined using a calculated molecular mass of 100 kDa for the N domain (Appendix 2.1.).

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2.3. Results

2.3.1. Kinetic characterisation of tACE site-directed glycosylation mutants and tACE expressed in the presence and absence of NBDNJ.

In order to determine the effects of site-directed deglycosylation as well as expression in the presence of NBDNJ on the catalytic properties of tACE, the kinetic parameters of purified protein were determined using the synthetic tripeptide substrate HHL. All tACE site-directed glycosylation mutants, with the exception of tACE Δ 36 -g2, which had a single intact glycosylation site (Asn⁹⁰) were catalytically active and demonstrated similar kinetic parameters as that of wild-type tACE with all *N*-linked glycosylation sites intact (Table 2.1). tACE Δ 36NJ expressed in the presence of NBDNJ displayed similar catalytic properties to tACE Δ 36NJ expressed in the absence of this inhibitor (Table 2.2).

Table 2.1 Kinetic parameters for the hydrolysis of HHL by site-directed glycosylation mutants of tACE

	K_m (mM)	k_{cat} (sec ⁻¹)	k_{cat}/K_m (sec ⁻¹ mM ⁻¹)
<i>tACE</i>	2.7±0.67	196±23	69
<i>tACE</i> Δ 36 -g1	1.6±0.40	128±11	79
<i>tACE</i> Δ 36 -g3	2.5±0.67	115±14	45
<i>tACE</i> Δ 36 -g13	2.7±0.82	170±25	63
<i>tACE</i> Δ 36 -g2	-	-	-
<i>tACE</i> Δ 36 -g12	2.1±0.41	195±15	94
<i>tACE</i> Δ 36 -g123	2.9±1.03	210±37	71
<i>tACE</i> Δ 36 -g1234	1.5±0.18	85±3.2	56

Data expressed as mean ±SEM.

Table 2.2 Kinetic parameters for the hydrolysis of HHL by tACEΔ36- NJ expressed in the presence and absence of the glucosidase inhibitor, NBDNJ.

	K_m (mM)	k_{cat} (sec ⁻¹)	k_{cat}/K_m (sec ⁻¹ mM ⁻¹)
tACEΔ36NJ	4.1±0.47	310±18	76
tACEΔ36NJ+1.5 mM NBDNJ	2.6±0.37	253±15	99

Data expressed as mean ±SEM.

2.3.2. *N*-linked glycosylation potential of the N domain

Asn-residues 9, 25, 45, 82, 117, 131, 289 and 480 in the N domain were predicted to be *N*-glycosylated with *N*-glycosylation potentials exceeding the 0.50 threshold. Asn-residues 416 and 494 were predicted to be unglycosylated. Furthermore, NetNGlyc 1.0 predictions indicated a trend of greater *N*-glycosylation potential towards the N-terminus of the N domain (Table 2.3).

Table 2.3 NetNGlyc1.0 prediction of N domain *N*-glycosylation potential.

(Threshold=0.5)				
SeqName	Position	Potential	Jury agreement	N-Glyc result

N domain	9 NFSA	0.7222	(9/9)	++
	25 NSSA	0.6104	(8/9)	+
	45 NITA	0.6823	(9/9)	++
	82 NFTD	0.7781	(9/9)	+++
	117 NMSR	0.7067	(9/9)	++
	131 NKTA	0.6436	(8/9)	+
	289 NATH	0.6022	(8/9)	+
	416 NDTE	0.4987	(5/9)	-
	480 NETH	0.5877	(6/9)	+
	494 NVTP	0.1544	(9/9)	---

2.3.3. The N domain homology model.

Primary sequence alignment of the N domain and tACE revealed that while certain *N*-linked glycosylation sites occurred at corresponding positions, other *N*-linked glycosylation sites occurred at unique positions both within the N domain as well as in tACE (Figure 2.5.).

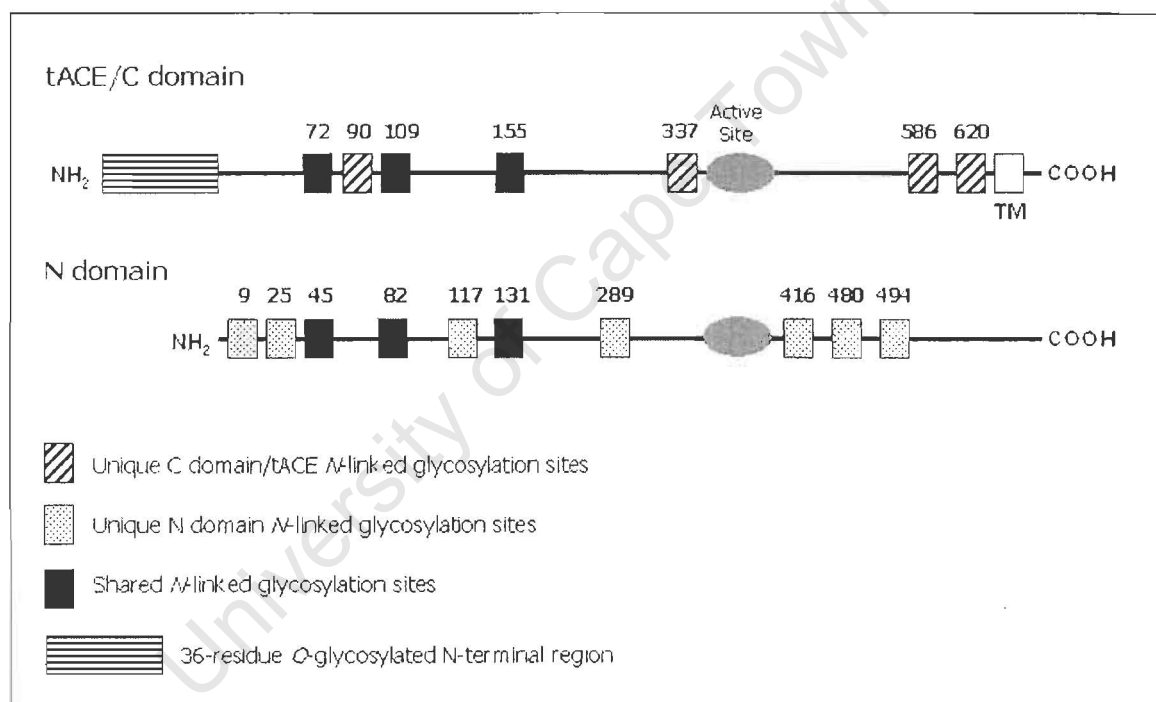


Figure 2.5. Distribution of *N*-linked glycosylation sites in tACE and the N domain (primary sequence numbering). Active site: HEMGH motif; TM: transmembrane region.

Alignment of the N domain (D¹-P⁵⁸³) with the crystal structure of tACE in complex with lisinopril (D⁴⁰-P⁶¹⁷)(PDB:1O86A) generated a homology model of RMSD= 0.15Å (Figure 2.6.).

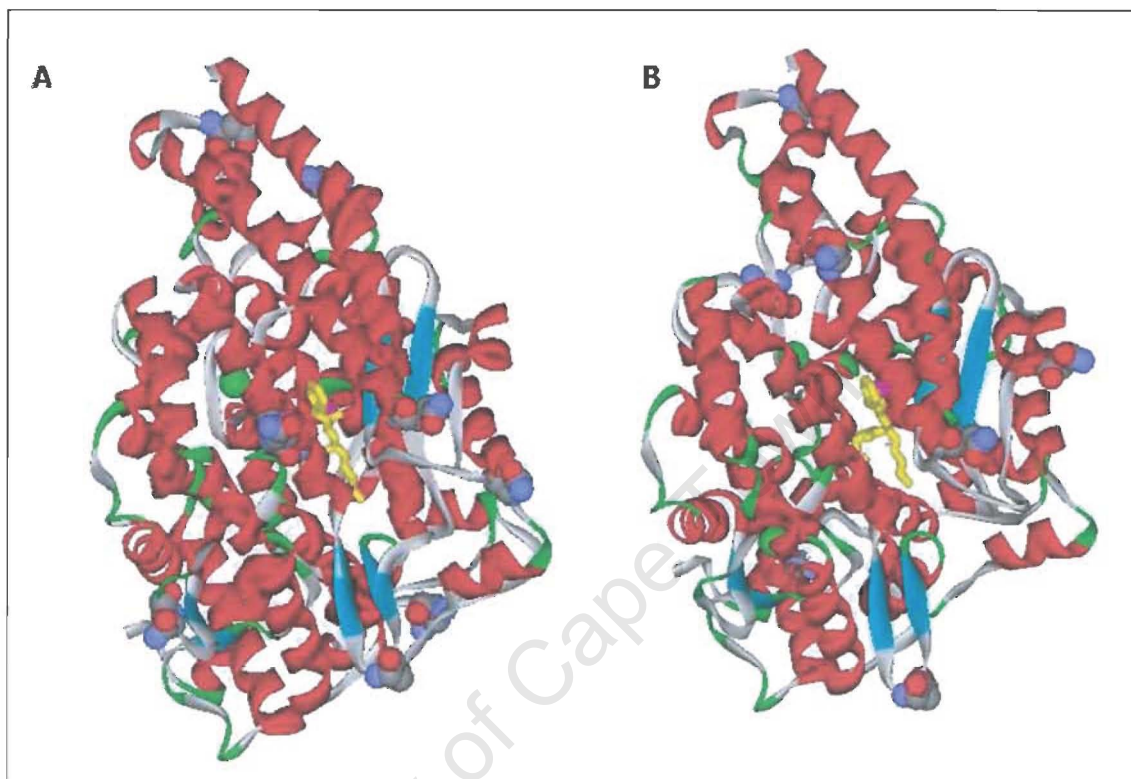


Figure 2.6. Homology modelled structure of the N domain (A) and the crystal structure of the tACE-lisinopril complex (PDB 1O86A)(B) showing the surface localisation of *N*-linked glycosylation sites (spheres in red, grey and purple). Lisinopril (yellow), zinc atom (magenta) and chloride ions (green).

The structure of tACE and the homology-model of the N domain indicated that all of the predicted *N*-linked glycosylation sites were surface localised (Figure 2.6.). Asn⁴⁹⁴ and Thr⁴⁹⁶ of the N domain (primary sequence numbering), which formed part of the *N*-linked glycosylation sequon N⁴⁹⁴VT⁴⁹⁶P predicted to be non-glycosylated, occurred within 6 Å (hydrogen-bonding distance) of the P₁ phenylalanine functionality of the lisinopril molecule (Figure 2.7. A). The corresponding residues forming part of the tACE S₁ subsite, Ser⁵¹⁶ and Val⁵¹⁸, occurred at similar distances from the lisinopril P₁ group (Figure 2.7. B).

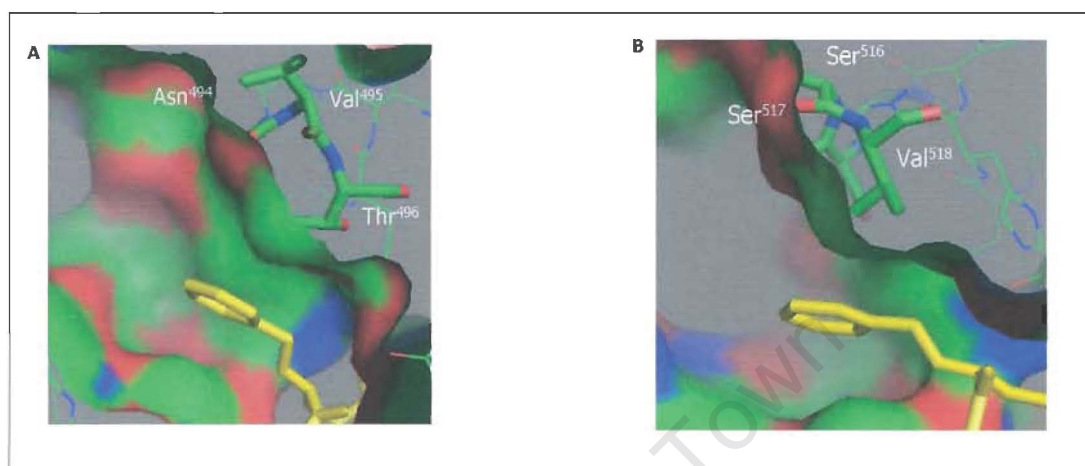


Figure 2.7. Interactions of the P₁ Phenylalanine functionality of lisinopril (yellow) with the N⁴⁹⁴VT sequon of the N domain (A) and corresponding residues of the tACE S₁ subsite pocket (B).

2.3.4. Expression and activity of N domain site-directed glycosylation mutants

Based upon the localisation of predicted *N*-linked glycosylation sites in the N domain homology model, *N*-linked glycosylation sequons were mutated in a step-wise manner to Gln residues. Asn⁴⁹⁴, predicted to be non-glycosylated, was not mutated. The expression and activity of mutant proteins was assessed via Western blot and the hydrolysis of Z-FHL. Western blot analysis detected the presence of all N domain glycosylation mutants in CHO-*K1* cell lysates, where a correlation existed between electrophoretic mobility and the extent of site-directed deglycosylation (Figure 2.8.A). A doublet occurring below each mutant protein band in the cell lysate samples and a single protein band with a higher mobility than the N domain in the culture medium was taken to be the result of non-specific binding of the ACE polyclonal antibody.

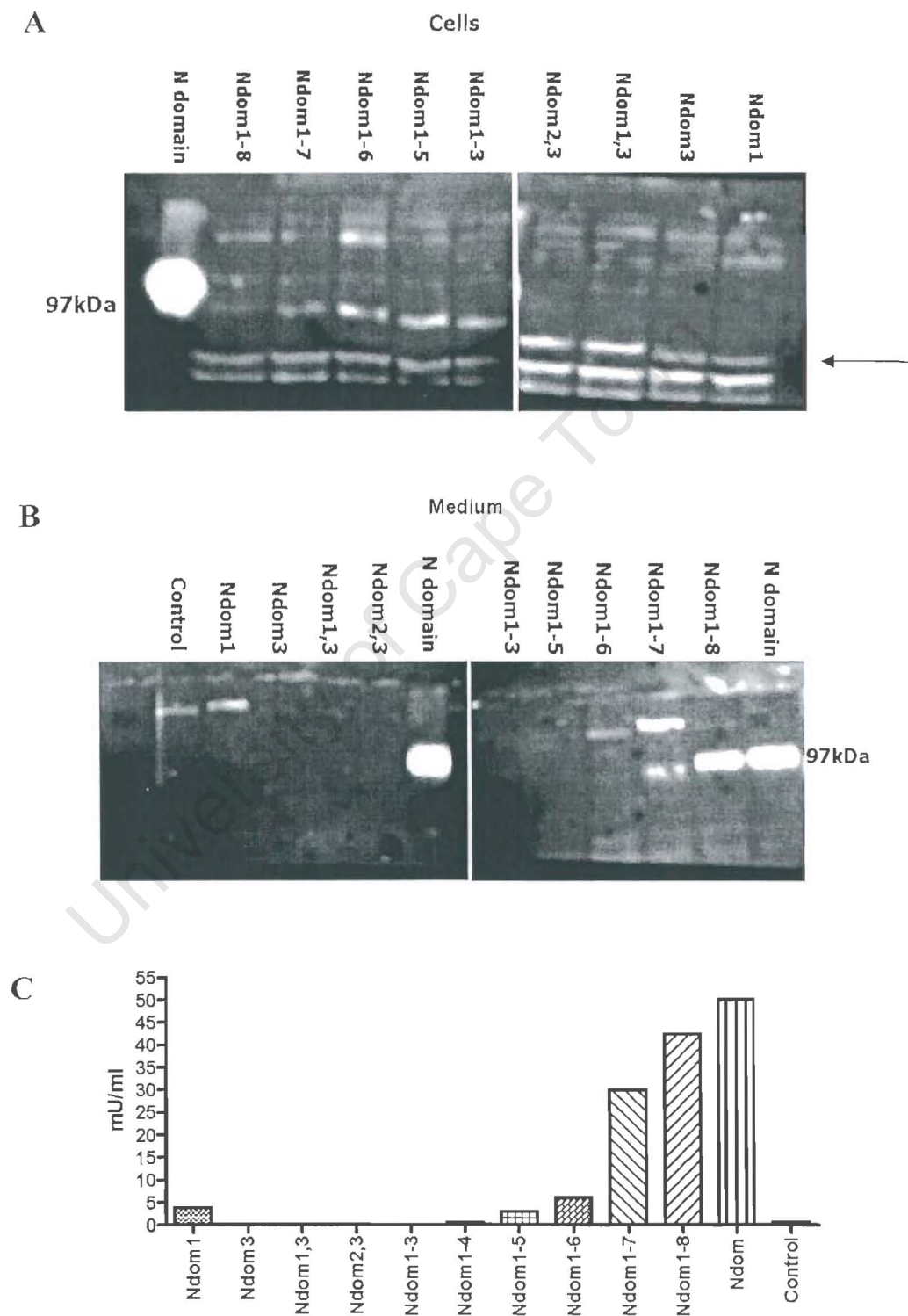


Figure 2.8. Western blot of N domain glycosylation mutants in cell lysates (A) and culture media (B). Activities of N domain glycosylation mutants in culture media of CHO-K1 cells were determined via hydrolysis with 1.0 mM Z-FHL (C). Arrow indicates the position of N domain glycosylation mutants.

Ndom 1 with only an intact Asn⁹ residue had the greatest mobility, whereas Ndom1-8, with Asn residues 9,25,45,82,117,131,289 and 416 intact, had the lowest mobility as compared with fully glycosylated wild-type N domain (Figure 2.8.A). While all N domain glycosylation mutants were detected in CHO-*K1* cell lysates, Ndom1 to Ndom1-6 were absent from the culture medium as shown by the lack of ~97kDa bands and the absence of activity (Figure 2.8.B and C). This was taken to indicate that these proteins underwent defective intracellular processing to the cell surface. The detection of N domain glycoforms Ndom1-7 and Ndom1-8 in both cell lysates and media as well as their activity in culture medium suggested normal folding and intracellular processing (Figure 2.8.A-C).

2.3.5. Expression of N domain in CHO-*K1* and *Lec3.2.8.1* cells with or without NBDNJ.

10% SDS PAGE of N domain purified from the culture medium of CHO-*K1* cells yielded a single protein band corresponding to the molecular weight of the N domain (Figure 2.9.). N domain expression in CHO-*K1* cells in the presence of 1.5 mM NBDNJ resulted in a sharper protein band with a similar electrophoretic mobility to that of N domain expressed in these cells without NBDNJ. This was consistent with the action of NBDNJ in the early ER *N*-linked glycosylation pathway where oligosaccharides are limited to the Glc₃Man₇₋₉GlcNAc₂ stage. In *Lec3.2.8.1* cells, where oligosaccharides are limited to the smaller Man₅GlcNAc₂ stage later in the *N*-linked glycosylation pathway, N domain electrophoretic mobility was greater than for CHO-*K1* cells with or without NBDNJ.

Here, the resulting protein band was sharper and electrophoretically more homogeneous. N domain expression in *Lec3.2.8.1* cells together with 0.5 mM NBDNJ caused a reduction in electrophoretic mobility. NBDNJ results in an oligosaccharide with 7 additional glycans as compared with *Lec3.2.8.1* cells alone (See Figure 2.1.). Thus, the resultant protein band migrated intermediate between that of N domain expressed in CHO-*K1* cells (with or without NBDNJ) and *Lec3.2.8.1* cells without NBDNJ (Figure 2.9.).

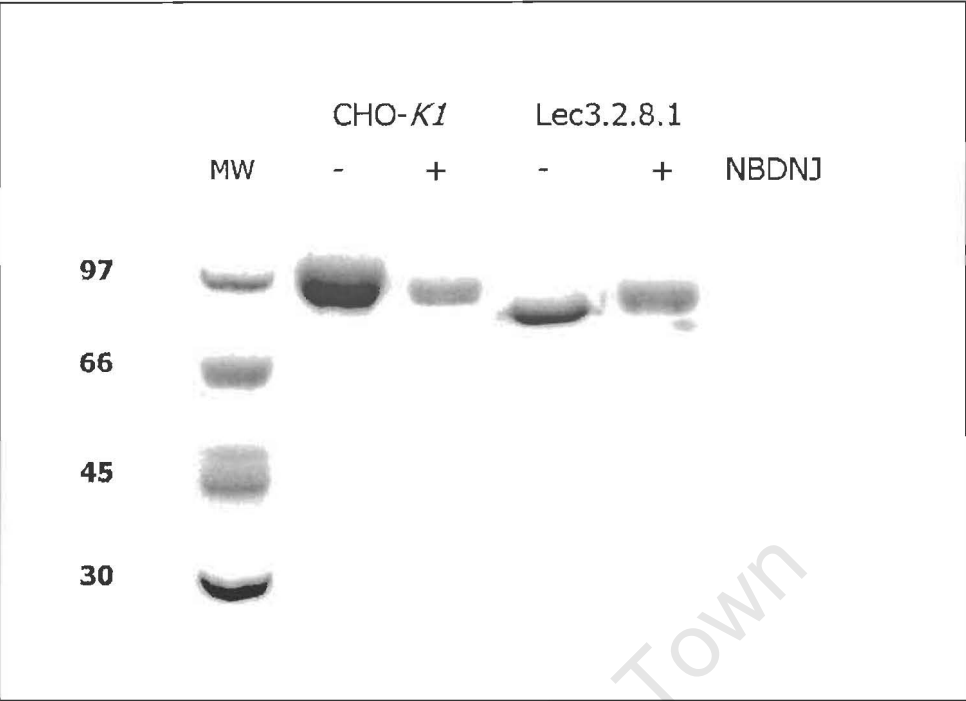


Figure 2.9. 10% SDS-PAGE of N domain expressed in CHO-*K1* cells and *Lec3.2.8.1* cells in the presence of (+) 1.5 mM NBDNJ (CHO-*K1* cells) or 0.5 mM NBDNJ (*Lec3.2.8.1* cells). MW: molecular weight marker.

In the presence of 1.5 mM NBDNJ, N domain expression in CHO-*K1* cells was reduced by ~63 % (Table 2.4). When N domain was expressed in *Lec3.2.8.1* cells in the presence of 0.5 mM NBDNJ, no such reduction in expression was observed (data not shown). The Z-FHL/HHL ratio of ~6.5 was characteristic of N domain activity and was in the same range as that previously reported in the literature (Danilov *et al.*, 1994).

Table 2.4 Effect of 1.5 mM NBDNJ on the expression of the N domain in CHO-*K1* cells.

<i>N domain in CHO-K1 cells</i>	<i>Z-FHL</i>	<i>HHL</i>	<i>Z-FHL/HHL</i> *
	<i>% activity</i>	<i>% activity</i>	<i>mU/ml ratio</i>
2% FCS	100	100	6.4±1.8
2% FCS + 1.5 mM NBDNJ	36±1.7	38±12.1	6.5±2.9

* Z-FHL/HHL ratio was calculated based on N domain activity (mU/ml) determined in culture medium using Z-FHL and HHL as substrates.

2.3.6. Kinetic characterization of N domain expressed in CHO-*K1* and *Lec3.2.8.1* cells with or without NBDNJ.

In order to determine the effects of NBDNJ- and *Lec3.2.8.1* cell-mediated deglycosylation on the catalytic activity of the N domain, the kinetic parameters of purified N domain expressed in CHO-*K1* and *Lec3.2.8.1* cells in the presence and absence of NBDNJ were determined (Table 2.5). Z-FHL is hydrolysed equally by both the N and C domains of sACE while HHL is more C domain-specific. The $k_{\text{cat}}\text{Z-FHL}/k_{\text{cat}}\text{HHL}$ ratio obtained in the present study was characteristic of the N domain (Danilov *et al.*, 1994). N domain expression in the presence of NBDNJ or in *Lec3.2.8.1* cells with or without NBDNJ did not significantly alter this ratio nor did it alter the k_{cat} , K_m and k_{cat}/K_m values relative to fully glycosylated N domain expressed in CHO-*K1* cells.

Table 2.5 Kinetic parameters for the hydrolysis of HHL and Z-FHL by N domain expressed in CHO-*K1* and *Lec3.2.8.1* cells with or without NBDNJ.

<i>N Domain</i>	K_m (Z-FHL) (mM)	k_{cat} (Z-FHL) (sec ⁻¹)	k_{cat}/K_m (Z-FHL) (sec ⁻¹ mM ⁻¹)	k_{cat} (HHL) (sec ⁻¹)	$k_{\text{cat}}\text{Z-FHL}/$ $k_{\text{cat}}\text{HHL}$
CHO-<i>K1</i> cells	0.36±0.04	39.4±13.3	110.8±42.3	1.8±0.13	21.9
CHO-<i>K1</i>+ 1.5 mM NBDNJ	0.45±0.04	54.9±4.9	123.0±2.2	5.5±0.16	10.0
<i>Lec3.2.8.1</i> cells	0.33±0.08	39.7±12.0	127.3±56.7	3.8±0.14	10.5
<i>Lec3.2.8.1</i>+0.5 mM NBDNJ	0.30±0.05	31.2±5.8	106.4±32.5	3.3±0.12	9.5

Data expressed are the means ± SEM.

2.4. Discussion

The extensive glycosylation of ACE has, until recently, been an impediment in its crystallisation and structural elucidation. Various strategies aimed at producing partially or completely deglycosylated protein, have been employed. These have included site-directed mutagenesis, protein expression in glycosylation-deficient cells and the use of glycosylation inhibitors. In the present study, a combination of these approaches was employed in the production of partially deglycosylated N domain. The effects of deglycosylation, as well as the roles of individual *N*-linked glycosylation sites on the processing and catalytic activity of N domain as well as tACE, were investigated.

Of the seven potential *N*-linked glycosylation sites in tACE, the first three sites (Asn⁷², Asn⁹⁰ and Asn¹⁰⁹) are fully glycosylated while Asn¹⁵⁵, Asn³³⁷ and Asn⁵⁸⁶ are partially glycosylated (Yu *et al.*, 1997). In order to investigate the significance of these sites in the catalytic activity of tACE, the kinetic parameters of a series of previously constructed tACE site-directed glycosylation mutants (Gordon *et al.*, 2003) were determined. *N*-linked glycosylation at the first (Asn⁷²) or third (Asn¹⁰⁹) site from the N-terminus was necessary and sufficient in maintaining the processing and catalytic activity of the protein. However, *N*-linked glycosylation at the second (Asn⁹⁰) site from the N-terminus alone resulted in catalytically-inactive protein that underwent defective intracellular processing.

In rabbit tACE, *N*-linked glycosylation at the first (Asn¹⁰⁸) or the second (Asn¹²⁶) site from the N-terminus was sufficient for normal processing and activity. However, in contrast to human tACE, glycosylation of the third site (Asn¹⁴⁵) alone resulted in inactive, rapidly degraded protein (Sadhukhan and Sen, 1996). Thus, a species-dependent difference exists with respect to the role of individual N-terminal *N*-linked glycosylation sites in tACE. This extends to sACE, where *N*-linked glycosylation shows a species and tissue-dependent redundancy. Of the 17 potential *N*-linked sites of sACE, only 7 are glycosylated in human seminal plasma (Ryan *et al.*, 1993) while the first, most N-terminal residue in rat pulmonary ACE (Asn⁹) is not glycosylated (Ripka *et al.*, 1993).

Moreover, murine ACE has two fewer *N*-linked glycosylation sites with some sites having no counterparts in human ACE (Soubrier *et al.*, 1988; Bernstein *et al.*, 1989).

The most C-terminal *N*-linked glycosylation site in human tACE (Asn⁶²⁰) is not glycosylated (Yu *et al.*, 1997). The sequon N⁶²⁰WT occurs in close proximity to the secretase cleavage site Arg⁶²⁷-Ser⁶²⁸ and glycosylation of this sequon may potentially interfere with cleavage secretion of tACE from the cell surface via steric hindrance. Similarly, the equivalent residue Asn¹¹⁹⁶ in sACE is also not glycosylated while in rabbit and murine tACE, the corresponding site is absent.

Thus, it would appear that the reduced glycosylation potential of more C-terminal sites in ACE may be of physiological relevance in permitting ectodomain cleavage secretion of these membrane bound proteins. This is supported by Kawaoka and Webster (1989), who demonstrated that oligosaccharide side-chains in the stalk of the viral glycoprotein, hemagglutinin interfered with its cleavage. Interestingly, while the disruption of all three *N*-linked glycosylation sites in *Drosophila melanogaster* AnCE did not influence its intracellular transport, secretion or C domain-like catalytic activity, its stability relative to the wild-type enzyme was reduced indicating additional roles for *N*-linked glycans in maintaining stability and possibly rendering the protein more resistant to proteolysis (Williams *et al.*, 1996).

As a template for the site-directed mutagenesis of tACE *N*-linked glycosylation sites, a truncated form of tACE lacking the 36-residue N-terminal region was used (tACEΔ36) (Gordon *et al.*, 2003). This region, contributing 15 kDa to the molecular weight of tACE (100 kDa), is heavily *O*-glycosylated. In the present study, the kinetic parameters of tACEΔ36NJ were no different to that of wild-type tACE and their expression levels were comparable. This was in agreement with previous observations that the region is neither required for processing nor catalytic activity (Ehlers *et al.*, 1992). The exact function of this region remains unclear but it has been suggested to play an important role in targeting of the enzyme to vesicular elements within the cytoplasm of spermatids (Yotsumoto *et al.*, 1984; Ehlers *et al.*, 1992).

Moreover, it may also play a role in receptor-mediated clearance of the enzyme by the epididymal epithelium following degradation of cytoplasmic droplets shed by maturing spermatozoa (Hermo *et al.*, 1988).

Homology modelling of the N domain indicated that all *N*-linked glycosylation sites, with the exception of N⁴⁹⁴VT, were localised on the surface of the protein. Similar to previous observations with tACE, the N domain displayed a tendency towards increased *N*-linked glycosylation potential towards its N-terminus. An important determinant of this *N*-linked glycosylation potential is the sequence of the Asn-X-Ser/Thr sequon (Shakin-Eshleman *et al.*, 1996). In the present study, more C-terminal sequons of the N domain demonstrated a lower *N*-linked glycosylation potential. These included N⁴¹⁶DTE and N⁴⁸⁰ETH where negatively-charged Glu and Asp residues might inhibit glycosylation by influencing the ability of the OST complex to simultaneously bind to the negatively-charged dolichol-PP-oligosaccharide precursor and the sequon. Large hydrophobic amino acids such as Trp in the unglycosylated N⁶²⁰WTP sequon of tACE may inhibit core glycosylation by generating an unfavourable protein conformation. Furthermore, the presence of Pro immediately following a sequon (as in N⁶²⁰WTP of tACE and N⁴⁹⁴VTP of the N domain) usually has an inhibitory effect on glycosylation efficiency.

The preferential glycosylation of more N-terminal sequons is not limited to ACE. Suppression of *N*-linked glycosylation sites towards the N-terminus of recombinant monomeric butylcholinesterase (BchE) resulted in reduced relative expression levels and intracellular retention of the enzyme (Nachon *et al.*, 2002). Other glycoproteins that show a similar preference for *N*-linked glycosylation at the N-terminus include vasoactive intestinal peptide I receptor (Couvineau *et al.*, 1996) and parathyroid-related peptide receptor (Zhou *et al.*, 2000).

Employing a similar site-directed mutagenesis approach as for tACE (Gordon *et al.*, 2003), the N domain revealed a different dependence on *N*-linked glycosylation for processing and catalytic activity.

With the exception of more C-terminal residues Asn²⁸⁹, Asn⁴¹⁶ and Asn⁴⁸⁰, step-wise site-directed elimination of the first six most N-terminal residues Asn⁹, Asn²⁵, Asn⁴⁵, Asn⁸², Asn¹¹⁷ and Asn¹³¹ resulted in protein that was retained intracellularly most likely due to defective intracellular processing. As in the case of both tACE and sACE, the most C-terminal *N*-linked glycosylation sequon of the N domain (Asn⁴⁹⁴) was predicted to be unglycosylated. This was supported by MALDI-TOF MS analysis of Lys-C and tryptic N domain digests (data not shown).

The homology model of the N domain revealed that unlike other *N*-linked sequons, N⁴⁹⁴VT occurred in the N domain S₁ active site pocket within hydrogen bonding distance of the P₁ phenylalanine functionality of lisinopril. These residues, replacing S⁵¹⁶SV in the tACE (C domain) S₁ pocket may contribute to the observed functional differences between the N- and C domains. This is examined in further detail in **Chapter 3**. N⁴⁹⁴ in the N domain S₁ pocket results in an amino-aromatic interaction with Phe (P₁) of the N domain-favoured substrate, BK as well as electrostatic interactions with Asp (P₁) of the highly N domain-specific substrate, Abz-SDK(Dnp)P-OH (Jaspard *et al.*, 1993; Araujo *et al.*, 2000; Tzakos *et al.*, 2003). Therefore, attachment of an *N*-linked glycan at N⁴⁹⁴VT would occlude the S₁ pocket, interfering with or preventing substrate hydrolysis.

Another strategy employed in the generation of minimally glycosylated N domain was the use of glycosylation-deficient *Lec3.2.8.1* cells as well as the glucosidase I-inhibitor, NBDNJ. Expression of N domain in *Lec3.2.8.1* cells neither influenced the kinetic parameters nor processing and secretion of the enzyme. However, while these cells have been used successfully in generating proteins amenable to crystallisation, they may not fully inhibit oligosaccharide processing beyond simple *endo H*-sensitive oligomannoses (Butters *et al.*, 1999). Moreover, a minor amount of fucosylated oligosaccharides may be produced in a site- or protein-specific manner via an alternate pathway (Lin *et al.*, 1994).

In agreement with the data on a truncated form of tACE (Yu *et al.*, 1997), N domain expression in CHO-*K1* cells in the presence of 1.5 mM NBDNJ did not influence its kinetic parameters.

Thus, the attachment of simple oligomannose *N*-glycans (Glc₃Man_{7.9}GlcNAc₂) to the proteins is sufficient in maintaining their catalytic activity. However, as has been previously observed for tACE (Yu *et al.*, 1997), N domain expression in CHO-*K1* cells in the presence of NBDNJ was significantly reduced although sufficient amounts were produced for functional studies.

Naim (1993) observed that deoxynojirimycin delayed transport of human small intestinal ACE to the cell surface and its subsequent secretion into the extracellular milieu while the expression of the cell-surface transferrin receptor in the presence of NBDNJ is reduced in a dose-dependent manner (Platt *et al.*, 1992). Thus, NBDNJ-mediated glycan modification within or close to structural motifs involved in intracellular transport in the ER and Golgi apparatus, may affect the structure or folding of the protein and thus its transport competency (Naim, 1993). Moreover, protein expression in the presence of NBDNJ also does not fully prevent the synthesis of *endo H*-resistant complex-type glycans (Butters *et al.*, 1999).

In overcoming the partial suppression of complex oligosaccharide-formation by *Lec3.2.8.1* cells or NBDNJ and exploiting the compartmental separation of the early and late stages of *N*-glycan processing, a combined approach of protein expression in *Lec3.2.8.1* cells along with reduced NBDNJ concentrations has been suggested. >95% of expressed proteins with *endo H*-resistant glycans remaining after expression in NBDNJ are rendered *endo H*-sensitive by the deficient glycosylation machinery of *Lec3.2.8.1* cells (Butters *et al.*, 1999). Following this approach, the expression of N domain in *Lec3.2.8.1* cells in the presence of 0.5 mM NBDNJ resulted in protein that possessed the same catalytic activity as compared with N domain produced in CHO-*K1* cells alone. Thus, the N domain is able to maintain its processing and catalytic activity with even simpler oligomannose *N*-glycans (Man₅GlcNAc₂).

The use of a reduced NBDNJ concentration in *Lec3.2.8.1* cells also abrogated the suppressive effects of higher NBDNJ concentrations on N domain expression in CHO-*K1* cells (data not shown).

Interestingly, N domain expressed in *Lec3.2.8.1* cells without NBDNJ appeared to be electrophoretically more homogeneous as compared with N domain expressed in CHO-*K1* cells or *Lec3.2.8.1* cells with NBDNJ. This suggested that this system might be suitable for generating protein amenable to crystallisation provided that sufficient levels of expression are attained.

In order to improve the modest expression levels of N domain in CHO-*K1* and *Lec3.2.8.1* cells, N domain was cloned into the high level GS-MSX expression vector pEE14. This vector has been used successfully in the expression of tACEΔ36NJ in CHO-*K1* cells in the presence of NBDNJ, resulting in protein that was successfully crystallised (Yu *et al.*, 1997; Natesh *et al.*, 2003). In the present study, expression levels of N domain (pEE14-ACED629) in CHO-*K1* cells, despite the presence of 1.5 mM NBDNJ, were substantially increased resulting in a final yield of purified protein suitable for crystallisation purposes (data not shown)(Corradi *et al.*, in press). A pEE14-based expression system may thus also be a suitable means of producing homogeneous proteins in *Lec3.2.8.1* cells with or without NBDNJ.

The exact role of *N*-linked glycosylation in the biosynthesis and activity of ACE is poorly understood. Hartley and Soffer (1978) suggested that by increasing the hydrophilicity of the molecule, glycosylation might mediate a process between the blood and endothelium. Glycosylation may also play a role in targeting and biosynthesis of the enzyme where more selective *N*-linked glycosylation at the N-termini may serve as signals in trafficking to membranes (Guan, *et al.*, 1985; Yu *et al.*, 1997). *N*-linked glycans may affect the folding of the polypeptide chain and the recognition of the protein by enzymes involved in folding and transport (Naim, 1993; Kasturi *et al.*, 1994) as well as its interaction with lectin-like receptors (Sliker and Lane, 1985; von Figura and Hasilik, 1986). Kost *et al.*, (1998) proposed that ACE could exist as an active dimer and that dimerisation between ACE molecules via their glycan moieties may be mediated by a lectin-like carbohydrate-recognising centre on the ACE molecule (Kost *et al.*, 2000).

This carbohydrate recognising domain is suggested to reside on the N domain of sACE (Kost *et al.*, 2003). It has been previously suggested that homodimerisation of secretory and membrane glycoproteins influences the rate at which proteins are transported from the ER to the Golgi apparatus. Monomeric ACE may fold into transport-competent pseudodimers that leave the ER for the Golgi system. Carbohydrate modifications of ACE by NBDNJ delay its transport, reinforcing the proposed role of glycans in ACE dimerisation (Naim, 1993). However, the increased basal shedding of ACE in the presence of NBDNJ suggests that carbohydrate-mediated dimerisation on the cell surface plays a role in ectodomain cleavage secretion (Kost *et al.*, 2003).

The present study has shed more light on the roles of *N*-linked glycosylation in the processing and catalytic activity of ACE. A multi-pronged approach was employed in the generation of partially glycosylated N domain that included the use of the glucosidase I-inhibitor, NBDNJ and glycosylation deficient *Lec3.2.8.1* cells. This approach, reducing the glycosylation heterogeneity of the N domain, has proven to be a successful strategy in producing protein amenable to crystallisation (Corradi *et al.*, in press). A similar strategy may be followed in the development of a pEE14-based system for sACE expression in the presence of NBDNJ and/or in *Lec3.2.8.1* cells. These approaches have furthermore suggested that the presence of simple oligomannose-type *N*-glycans at *N*-linked glycosylation sites of the N domain are sufficient in maintaining the processing and catalytic activity of the protein. Thus, an N domain crystal structure generated using these methodologies will be a true reflection of the native protein.

Furthering the objective to produce partially deglycosylated protein, site-directed mutagenesis has been used to determine the minimal site-specific glycosylation requirements of tACE and the N domain in terms of protein structure and function. The N- and C domains have demonstrated differences in their requirements for *N*-linked glycosylation. In tACE, *N*-linked glycosylation at only one of three more N-terminal sequons is sufficient in maintaining normal processing and activity. In the N domain the reliance on *N*-linked glycosylation appears to be more extensive, with the elimination of only a few C-terminal sequons rendering the protein defective in its intracellular processing.

The different glycosylation requirements of tACE (C domain) and the N domain may partly be influenced by the residue utilisation of their glycosylation sequons. Moreover, in tACE, where ectodomain cleavage secretion is an *in vivo* phenomenon, reduced glycosylation at its C-terminus may serve to maintain secretion by removing any steric constraints which may be imposed by *N*-linked oligosaccharides. The different dependence of the N domain on *N*-linked glycosylation may be attributed to the fact that this domain is neither naturally occurring nor does it undergo ectodomain cleavage secretion (Pang *et al.*, 2001). More extensive glycosylation may be the result of its normal occurrence as a folding-, transport- and processing-dependent N-terminal region of a larger somatic ACE molecule. Here, it has been shown that 7 of the 17 potential *N*-linked sites of seminal plasma sACE are glycosylated (Ryan *et al.*, 1993). These would include Asn⁹-Asn²⁸⁹ of the N domain (Ndom1-7) shown by step-wise mutagenesis in the present study to be the minimal requirement for producing processing-competent, active N domain. In this context it would be appropriate to extend N domain mutagenesis studies in order to ascertain exactly which individual sites or site combinations (if any) are crucial for processing and activity. Furthermore, the role of individual *N*-linked glycosylation sites in full length sACE should be investigated as well as the effect of glucosidase inhibitors and glycosylation deficient cells on the catalytic activity of the enzyme and its passage through the various compartments of the mammalian glycosylation apparatus.

CHAPTER 3

Structural and functional differences between the N- and C domains and their interactions within sACE.

3.1. Introduction

Although the N- and C domains of sACE have ~60% sequence identity, they display distinct substrate and inhibitor specificities (Reviewed in **Chapter 1**). The basis for these functional differences has been greatly aided by the recently elucidated structures of ACE (Natesh *et al.*, 2003, Kim *et al.*, 2003, Corradi *et al.*, in press). In the present study, structural, kinetic and thermal inactivation approaches were employed to:

1. Explore the interactions between the N- and C domains of sACE by using constructs in which the relative orientations of the N- and C domains were “swopped” or in which two C domains were repeated in tandem.
2. Probe the role of specific regions of tACE (C domain) and the N domain in conferring domain selectivity. The regions required for domain-specific catalytic activity of tACE were delineated by making use of four chimeric ACE constructs where regions of tACE were replaced with the corresponding protein sequence of the N domain.

3.2. Materials and Methods

3.2.1. Construction of soluble sACE

Full-length, membrane-bound sACE is poorly shed (Woodman *et al.*, 2005). In order to facilitate purification of sufficient quantities of sACE from CHO cell culture medium, a soluble sACE construct was generated as follows: A *NotI* site at position 3742 within the human sACE cDNA sequence was selected in order to achieve truncation of the cDNA at Ser¹²¹¹. This truncation, 7 residues downstream of the R-S (Arg¹²⁰³-Ser¹²⁰⁴, human sACE numbering) secretase cleavage site, occurs within the juxtamembrane stalk region thereby eliminating the transmembrane and cytosolic regions. Full-length sACE (4.020 kb) sequenced in pBluescript II SK+/- (Stratagene) was digested with *XbaI* and *NotI* and sub-cloned into similarly-digested expression vector, pcDNA3.1(-)(5.428 kb)(Invitrogen). The identity and correct orientation of the construct pcDNA3.1-sACE¹²¹¹ was checked by restriction mapping using *XbaI*, *EcoRI* and *BglII* (Figure 3.1.) (Appendix 2.4.).

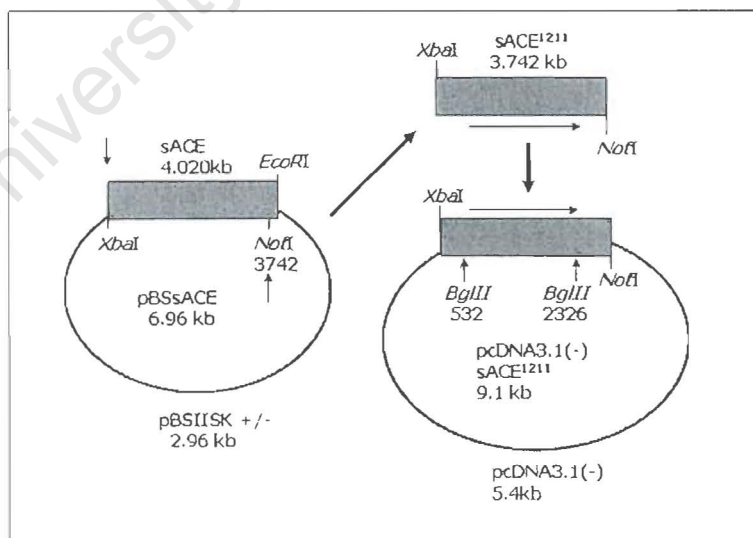


Figure 3.1. Generation of soluble sACE¹²¹¹ and cloning into the expression vector pcDNA3.1(-).

3.2.2. Domain swap-over mutants

Domain swap-over mutants, in which the relative NH₂- and COOH-terminal orientations of the C- and N domains are swapped (CNdom-ACE) or in which two C domains are duplicated in tandem (CCdom-ACE) have been described previously by Woodman *et al.*, (2005) and they are shown in Figure 3.2.

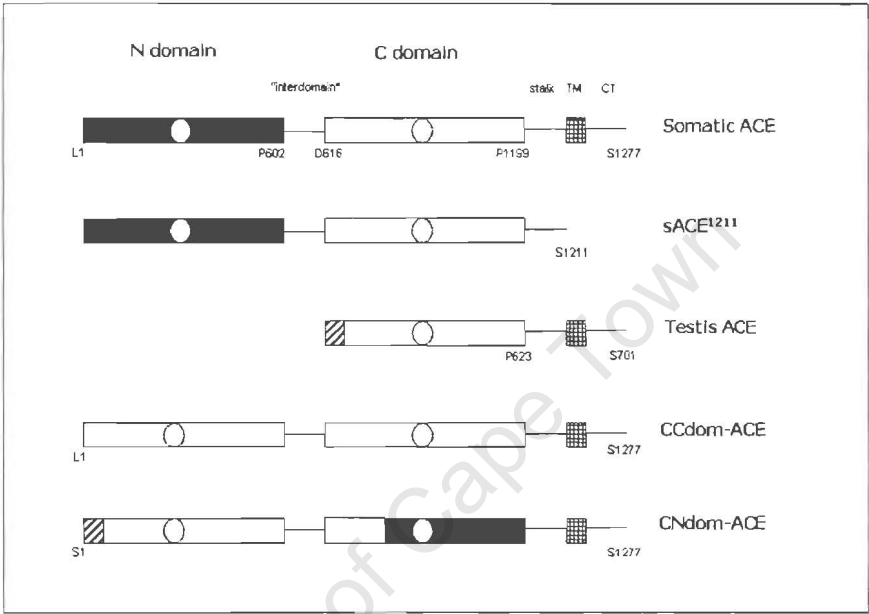


Figure 3.2. CCdom-ACE and CNdom-ACE constructs. TM: Transmembrane region; CT: cytosolic tail.

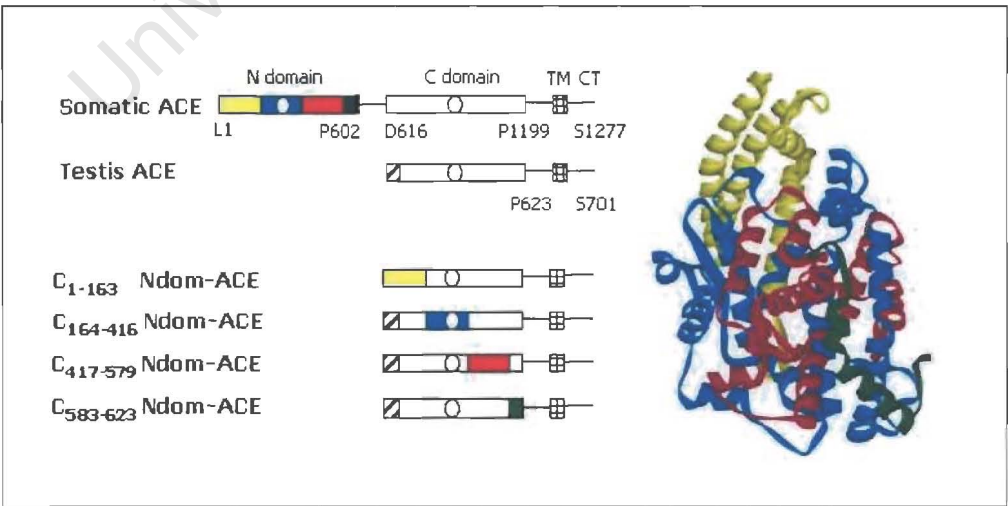


Figure 3.3. tACE-N domain chimeras were generated by substituting regions of tACE (white box) with corresponding N domain sequence (coloured boxes). The substituted regions (tACE numbering) are highlighted in colour on a ribbon representation of the three-dimensional structure of tACE (Natesh *et al.*, 2003).

3.2.3. testis ACE-N domain chimeras

tACE-N domain chimeras, in which specific regions of tACE are substituted with corresponding N domain sequence have been described previously (Woodman, PhD thesis 2003) and they are shown in Figure 3.3.

3.2.4. Protein expression and purification

CCdom-ACE, CNdom-ACE and the four chimeric constructs, C₁₋₁₆₃Ndom-ACE, C₁₆₄₋₄₁₆Ndom-ACE, C₄₁₇₋₅₇₉Ndom-ACE, and C₅₈₃₋₆₂₃Ndom-ACE were co-transfected with pSV2-Neo into CHO cells and stably expressed as previously outlined (Ehlers *et al.*, 1991b). The soluble sACE construct pcDNA3.1-sACE¹²¹¹ was transfected into CHO cells and stably expressed as above. Positive clones were grown to confluence and ACE expression was induced overnight in 2% FCS complete medium supplemented with 10 μ M ZnCl₂ (Appendix 2.6.). Soluble proteins in the culture medium were purified via lisinopril-sepharose affinity chromatography, as described previously (Ehlers *et al.*, 1991b) (Appendix 2.9.). Protein concentrations were determined according to the Bradford method (Appendix 2.10.).

3.2.5. Kinetic characterisation

Kinetic constants for the hydrolysis of HHL and Z-FHL were determined as described in Chapter 2 (Appendix 2.1.). The hydrolysis of the C domain-specific FRET peptide AbzLFK(Dnp)-OH was determined in a continuous fluorometric assay at 37°C under initial rate conditions in 0.1 M Tris-HCl buffer, pH 7.0, containing 50 mM NaCl and 10 μ M ZnCl₂ (Araujo *et al.*, 2000; Bersanetti *et al.*, 2004) with a protein concentration of 1.0 nM over a substrate concentration range of 0 μ M-8 μ M. Fluorescence was measured at λ_{Ex} =320nm and λ_{Em} =420nm on a Cary Eclipse fluorescent cuvette reader (Varian) (Appendix 2.2.). All assays were conducted in triplicate.

Kinetic constants were calculated via non-linear regression analysis of Michaelis-Menten plots (GraphPad Prism 4.01). For Z-FHL and HHL, a HL standard curve was used while for AbzLFK(Dnp)-OH, a standard calibration curve was generated by total peptide hydrolysis. Turnover numbers (k_{cat}) and specificity constants (k_{cat}/K_m) were determined using a calculated molecular mass of 170 kDa for sACE¹²¹¹, CCdom-ACE and CNdom-ACE and 100 kDa for tACE, N domain and the chimeric proteins.

3.2.6. Homology modelling of chimeric proteins.

C₁₋₁₆₃ Ndom-ACE, C₁₆₄₋₄₁₆ Ndom-ACE, C₄₁₇₋₅₇₉ Ndom-ACE, and C₅₈₃₋₆₂₃ Ndom-ACE were modelled using the tACE-lisinopril complex as a template (Natesh *et al.*, 2003)(PDB:1086A). Modelling was performed using SWISSMODEL (<http://swissmodel.expasy.org/>) (Schwede *et al.*, 2003) and structures were aligned and visualised using DeepView/Swiss-PDB Viewer, DS Viewer Pro (Accelrys) and PyMOL (DeLano Scientific). Secondary structure comparisons were performed via the PredictProtein Server (Rost and Liu, 2003; <http://cubic.bioc.columbia.edu/predictprotein>).

3.2.7. Lisinopril inhibition of CCdom-ACE, CNdom-ACE and sACE¹²¹¹

Inhibition constants (K_i 's) for the commercial inhibitor lisinopril were determined by co-incubating 0.8 µg/ml (CCdom-ACE and sACE¹²¹¹) and 2.0 µg/ml (CNdom-ACE) enzyme with increasing concentrations of inhibitor (0nM-500nM) for 60 minutes at ambient temperature. Residual enzyme activities were determined at 0.1 mM and 0.5 mM Z-FHL in 100 mM potassium phosphate buffer, pH 8.3, containing 300 mM NaCl at 37°C for 30 minutes. The remainder of the assays were conducted as described in **Chapter 2**. Fluorescence was measured at λ_{Ex} =360nm and λ_{Em} =486nm and K_i values determined from Dixon plots of $1/V$ vs $[I]$ (GraphPad Prism 4.01). Here, V =rate of hydrolysis and $[I]$ = lisinopril concentration in nM. Assays were conducted in triplicate (Appendix 2.3.).

3.2.8. Determination of thermal stabilities

Thermal stabilities were determined by incubating 10 µg/ml enzyme at 55°C for 0-60 minutes. Residual activities were determined from 8 ng/ml enzyme with 8.0 µM Abz-YRK(Dnp)P, a FRET peptide hydrolysed equally by both N- and C domains. Assays were conducted in triplicate and as described previously for AbzLFLK(Dnp)-OH. % residual enzyme activity = rate of reaction following thermal denaturation/rate of reaction of native enzyme at 4°C x 100.

3.3. Results

3.3.1. Kinetic characterisation of tACE-N domain chimeras and domain swop-over mutants.

In order to determine the effects of a C- and N domain swop-over and a C domain duplication on the catalytic properties of sACE, the kinetic parameters of CCdom-ACE, CNdom-ACE and sACE¹²¹¹ were determined using the synthetic tripeptide substrate Z-FHL (hydrolysed equally by both domains)(Danilov *et al.*, 1994) and a highly C domain-specific FRET peptide, Abz-LFK(Dnp)-OH (Bersanetti *et al.*, 2004). The k_{cat} of CCdom-ACE was greater than two-fold the k_{cat} for tACE using Z-FHL as substrate (Table 3.1, Figure 3.4.). For the hydrolysis of AbzLFK(Dnp)-OH, the k_{cat} of CCdom-ACE was also greater than twice the k_{cat} for tACE (Table 3.1, Figure 3.4.). This suggested that the two domains of CCdom-ACE exhibited positive cooperativity. In contrast, in the case of both sACE¹²¹¹ and CNdom-ACE, the k_{cat} values for both Z-FHL and AbzLFK(Dnp)-OH were less than the sum of the k_{cat} values for the individual N and C (tACE) domains (Table 3.1, Figure 3.4.). These results are suggestive of negative cooperativity between the C and N domains irrespective of their relative NH₂ or COOH positions.

Table 3.1. Kinetic parameters for the hydrolysis of Z-FHL and AbzLFK(Dnp)-OH (LFK)

	Z-FHL	LFK	Z-FHL	LFK	Z-FHL	LFK
	K_m (mM)	K_m (μM)	k_{cat} (sec ⁻¹)		k_{cat}/K_m	
<i>N domain</i>	1180.7±118.6	5.0±0.5	73.2±5.8	1.0±0.1	0.06±0.01	0.2±0.0
<i>tACE</i>	318.35±11.8	3.3±0.5	107.0±3.1	20.6±0.5	0.34±0.02	6.3±1.0
<i>sACE</i> ¹²¹¹	406.8±75.2	3.6±0.3	116.9±3.4	13.3±0.49	0.29±0.06	3.7±1.7
<i>CNdom-ACE</i>	158.4±16.4	4.7±1.4	106.6±2.6	19.2±2.8	0.68±0.05	4.1±2.0
<i>CCdom-ACE</i>	357.6±89.7	5.0±0.8	255.3±113	69.4±5.7	0.71±1.3	13.9±7.1

Data expressed as mean ± SEM.

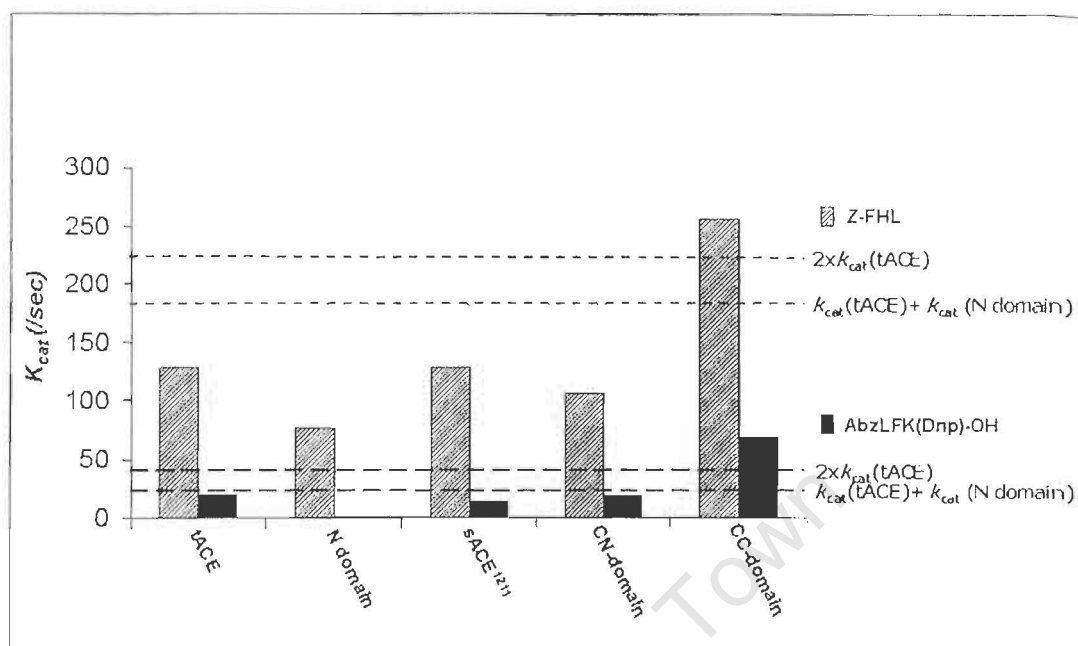


Figure 3.4. Comparative rates of hydrolysis of Z-FHL (hatched bars) and AbzLFK(Dnp)-OH (black bars). For sACE¹²¹¹ and CNdom-ACE, threshold of independent hydrolysis = $k_{cat}tACE + k_{cat}Ndomain$ (dashed lines) for both substrates. Negative cooperativity: $k_{cat} < k_{cat}tACE + k_{cat}Ndomain$. For CCdom-ACE, threshold of independent hydrolysis = $2 \times k_{cat}tACE$ (dashed lines) for both substrates. Positive cooperativity: $k_{cat} > 2 \times k_{cat}tACE$.

To investigate the effect of the replacement of specific regions of tACE with corresponding N domain sequence, the kinetic parameters of tACE-N domain chimeras were determined with respect to the hydrolysis of Z-FHL and AbzLFK(Dnp)-OH as well as HHL, which is preferentially hydrolysed by the C domain (Wei *et al.*, 1991). The Z-FHL/HHL k_{cat} ratio, characteristic of the N domain or C domain, was consistent with that previously reported (Williams *et al.*, 1996) (Table 3.2). C₄₁₇₋₅₇₉Ndom-ACE demonstrated an elevated K_m and reduced k_{cat} for both these substrates suggesting its acquisition of N-domain-like catalytic properties (Table 3.2). C₁₋₁₆₃ NdomACE and C₅₈₃₋₆₂₃ NdomACE exhibited rates of Z-FHL hydrolysis tending to that of the N domain, with HHL specificity constants in the same range as that of the N domain. In order to confirm these observed shifts in substrate specificity, hydrolysis of the highly C domain-specific FRET peptide AbzLFK(Dnp)-OH was assessed.

Table 3.2. Kinetic parameters for the hydrolysis of Z-FHL and HHL by chimeric proteins

	K_m (mM)		k_{cat} (sec ⁻¹)		k_{cat}/K_m (mM ⁻¹ sec ⁻¹)		
	Z-FHL	HHL	Z-FHL	HHL	Z-FHL	HHL	
<i>N</i> domain	1200±120	1100±190	73.2±5.8	24.5±1.3	0.06±0.05	0.02±0.01	3.0
<i>t</i> ACE	300±60	500±50	128.0±28	262.5±5.6	0.43±0.47	0.53±0.11	0.5
<i>C</i> ₄₁₇₋₅₇₉ <i>N</i> dom-ACE	2800±900	2700±900	34.4±8.1	11.8±4.8	0.01±0.01	0.004±0.005	3.0
<i>C</i> ₁₋₁₆₃ <i>N</i> dom-ACE	400±30	1100±300	97.5±29.2	15.1±5.4	0.24±0.97	0.014±0.02	6.5
<i>C</i> ₅₈₃₋₆₂₃ <i>N</i> dom-ACE	300±20	1100±30	36.8±4.0	12.9±2.3	0.12±0.2	0.01±0.08	3.0

Data shown are the mean values ± SEM.

The hydrolysis of AbzLFK(Dnp)-OH by *C*₁₋₁₆₃*N*dom-ACE, *C*₄₁₇₋₅₇₉*N*dom-ACE, and *C*₅₈₃₋₆₂₃*N*dom-ACE, as shown by k_{cat} and k_{cat}/K_m values, was more N domain-like supporting observations with HHL and Z-FHL (Table 3.3)(Figure 3.5.).

Table 3.3. Kinetic parameters for the hydrolysis of AbzLFK(Dnp)-OH by chimeric proteins.

	K_m (uM)	k_{cat} (sec ⁻¹)	k_{cat}/K_m (uM ⁻¹ sec ⁻¹)
<i>t</i> ACE	3.3±0.5	20.6±0.5	6.2±1.0
<i>N</i> domain	5.0±0.5	1.0±0.05	0.2±0.1
<i>C</i> ₄₁₇₋₅₇₉ <i>N</i> dom-ACE	3.1±0.6	0.5±0.04	0.2±0.07
<i>C</i> ₁₋₁₆₃ <i>N</i> dom-ACE	13.6±0.9	12.0±0.6	0.9±0.6
<i>C</i> ₅₈₃₋₆₂₃ <i>N</i> dom-ACE	3.6±0.4	3.3±0.2	0.9±0.4

Data shown are the mean values ± SEM.

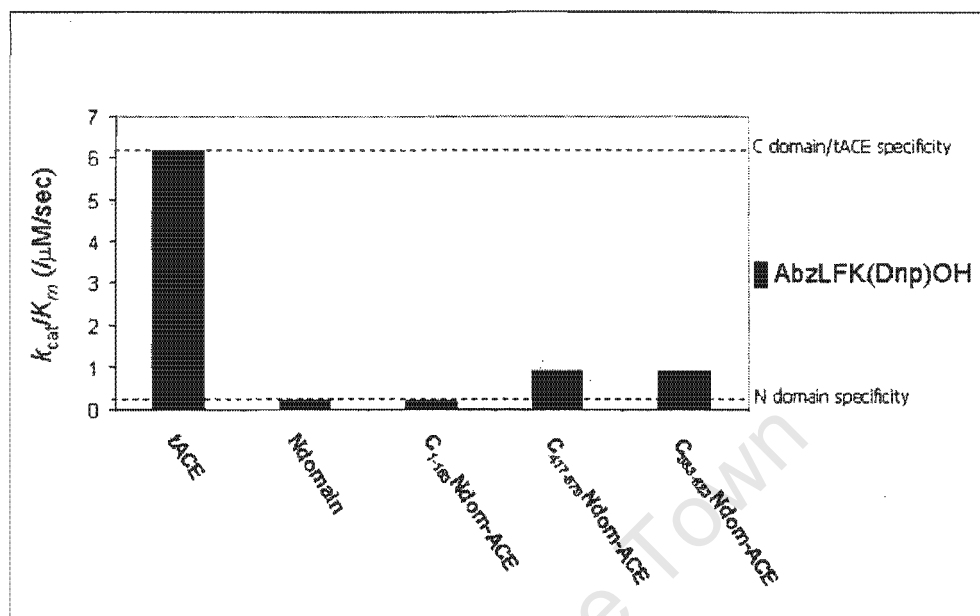


Figure 3.5. Specificity of tACE (C domain), N domain and chimeric proteins for AbzLFK(Dnp)-OH. Dashed lines represent the specificity constants (k_{cat}/K_m) for tACE (upper line) and N domain (lower line).

3.3.2. Homology modelling and secondary structure prediction of tACE-N domain chimeric proteins

Chimeras C₁₋₁₆₃Ndom-ACE, C₄₁₇₋₅₇₉Ndom-ACE, and C₅₈₃₋₆₂₃Ndom-ACE were shed into the CHO culture medium but demonstrated altered kinetic parameters, while C₁₆₄₋₄₁₆Ndom-ACE was inactive (Woodman, PhD thesis, 2003). In order to determine the structural basis for these changes, homology modelling was performed. Fits of 0.0 Å to 0.16 Å RMS were obtained for all four chimeras using the tACE-lisinopril complex as a template (PDB 1086A).

C₁₋₁₆₃Ndom-ACE: The region replaced in C₁₋₁₆₃Ndom-ACE with N domain residues L¹ – D¹⁴⁰ comprises α-helices α1, α2, α3, and α4, and β-sheets β1 and β2 (Natesh *et al.*, 2003) (Figure 3.6.). The sequence identity between the N- and C domains in this region was only 25% with significant differences in charge and hydrophobicity.

Moreover, the substituted N domain sequence contained two additional *N*-linked glycosylation sites at N⁹ and N²⁵ as well as a significantly higher proline content (4.3%) and helix content (67.6%) as compared with the corresponding tACE sequence (1.9% and 51.9% respectively).

In C₁₆₄₋₄₁₆ Ndom-ACE, the substituted region spanned the two sub-domains of testis ACE including the active site (Figure 3.6.). Although secondary structure predictions did not reveal significant differences in proline or helix content, an *N*-linked glycosylation site at N³³⁷ within helix α 12 was eliminated and an *N*-linked glycosylation site was introduced at N²⁸⁹ in helix α 11. Homology modelling revealed that N²⁸⁹ occurred at the N terminus of the C-terminal sub-domain and that the S₁' pocket residues D³⁷⁷, V³⁷⁹, and V³⁸⁰ were changed to Q³⁵⁵, S³⁵⁷, and T³⁵⁸ respectively (Figure 3.7A and B). Furthermore, the chloride-binding residue R¹⁸⁶ was replaced by H¹⁶⁴.

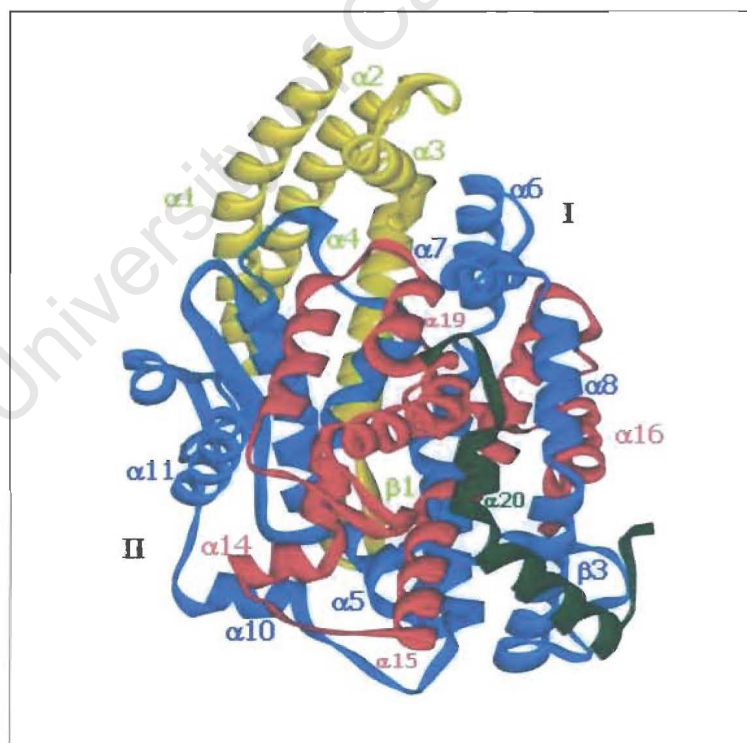


Figure 3.6. Ribbon representation of the tACE-lisinopril crystal structure showing substituted N domain regions. C₁₋₁₆₃NdomACE (yellow); C₁₆₄₋₄₁₆Ndom-ACE (blue); C₄₁₇₋₅₇₉Ndom-ACE (red) and C₅₈₃₋₆₂₃Ndom-ACE (green).

C₄₁₇₋₅₇₉Ndom-ACE: A region 30 residues distal to the active site was replaced with N domain sequence in the C₄₁₇₋₅₇₉Ndom-ACE chimera (Figure 3.6.). The tACE region replaced in this chimera shared 62% sequence identity with the N domain. The residues in this region that interact with the chloride ions, zinc atom, and lisinopril were all conserved, including R⁵²² (R⁵⁰⁰ in the N domain) which was identified as essential for chloride activation (Liu *et al.*, 2001). However, in C₄₁₇₋₅₇₉Ndom-ACE three *N*-linked glycosylation sequons were introduced at N⁴¹⁶, N⁴⁸⁰, and N⁴⁹⁴. Both N⁴¹⁶ and N⁴⁸⁰ are surface localized *N*-linked glycosylation sites (**Chapter 2**), whereas N⁴⁹⁴, together with V⁴⁹⁵ and T⁴⁹⁶ (forming a non-glycosylated sequon, **Chapter 2**) occurs in close proximity to α -helices α 15, α 16, and α 17, and the 3_{10} helix H6. These N domain residues, occupying the S₁ subsite of tACE replaced S⁵¹⁶, S⁵¹⁷, and V⁵¹⁸ resulting in alterations in charge and hydrophobicity as well as the protrusion of N⁴⁹⁴ into the S₁ pocket (Figure 3.7 C and D). The substituted N domain sequence in C₄₁₇₋₅₇₉Ndom-ACE was predicted to have a slightly higher helix content (52.3%) as compared with the corresponding tACE sequence (51.6%).

C₅₈₃₋₆₂₃Ndom-ACE: While the substituted region of this mutant (Figure 3.6.) did not include key active site residues, *N*-linked glycosylation sites at N⁵⁸⁶ and N⁶²⁰ in tACE were lost. Secondary structure comparison of the substituted N domain region with that of the corresponding tACE sequence revealed a slightly higher proline content (9.8% vs 9.5%) and helix content (51.2% vs 50%).

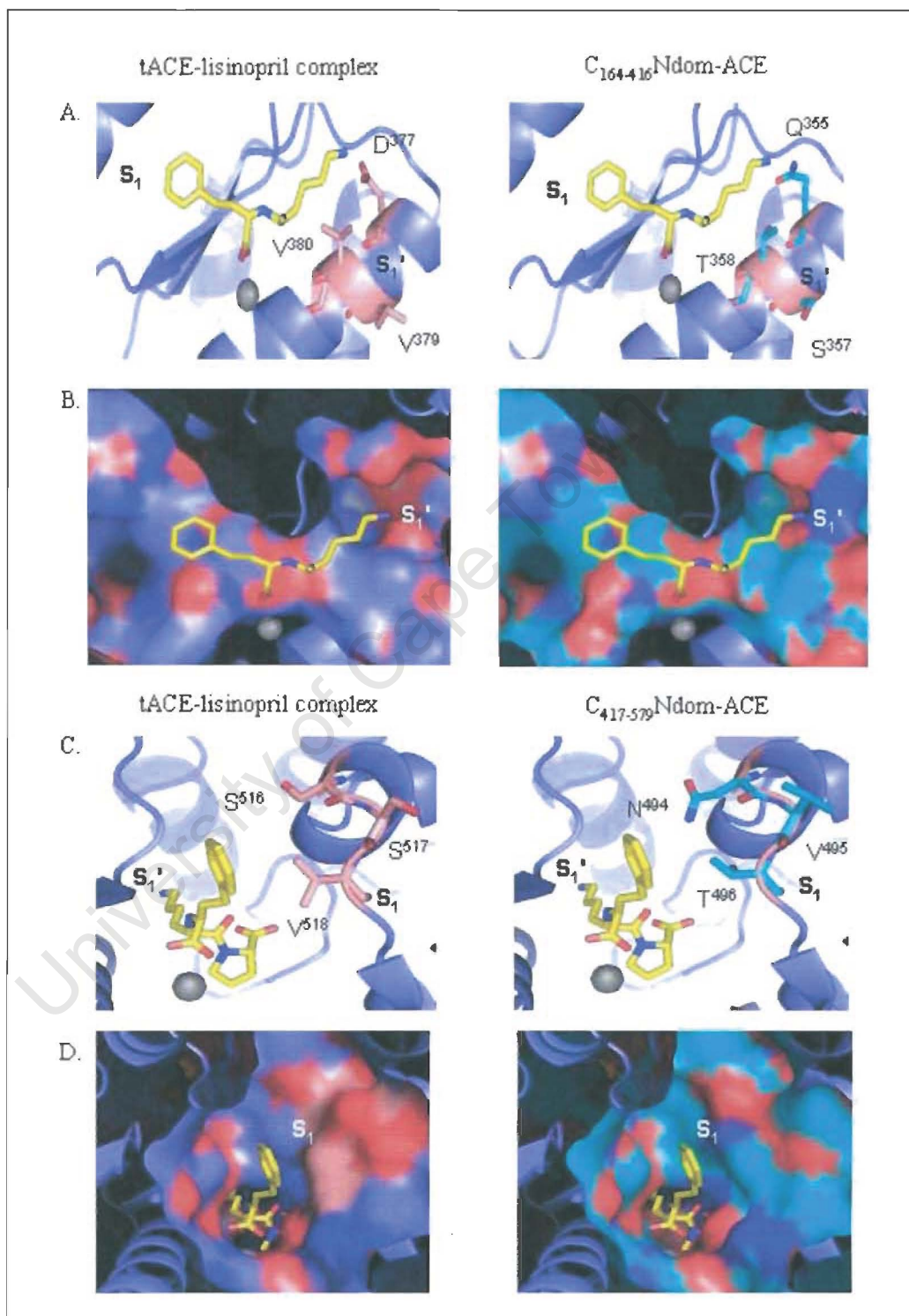


Figure 3.7. S_1 and S_1' subsite pockets of C₁₆₄₋₄₁₆Ndom-ACE (A, B right) and C₄₁₇₋₅₇₉Ndom-ACE (C, D right) respectively showing alterations in their S_1' and S_1 residues respectively as compared with the tACE-lisinopril complex (A-D left). Substituted N domain residues are according to N domain numbering.

3.3.3. Lisinopril inhibition of CCdom-ACE, CNdom-ACE and sACE¹²¹¹

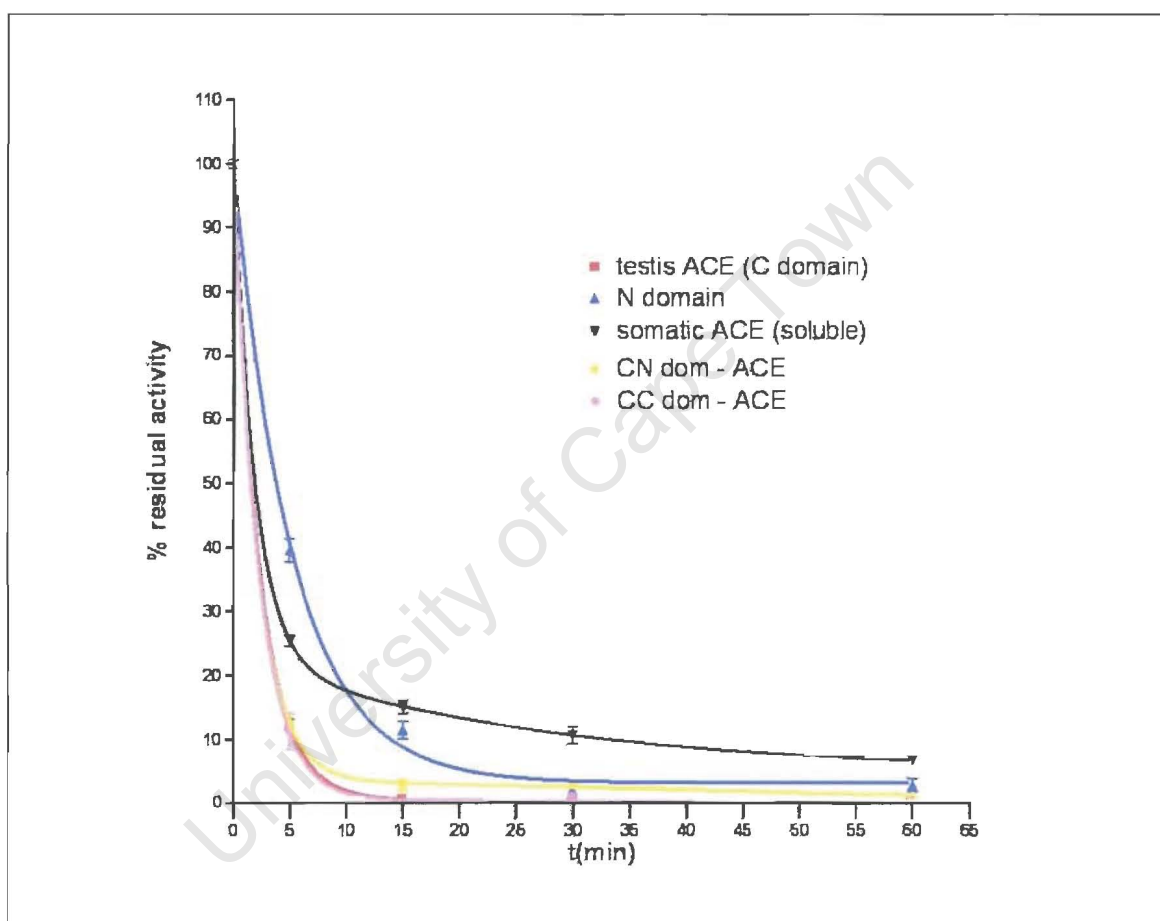
Lisinopril is a widely-used commercially available ACE inhibitor that is slightly more specific for the C domain of ACE as compared with the N domain (Wei *et al.*, 1992). Its binding affinity for sACE¹²¹¹, CNdom-ACE and CCdom-ACE was determined and K_i values calculated as 46 nM, 34 nM and 43 nM respectively. Given the relatively low C domain-selectivity of lisinopril, no major differences were found in its binding to sACE¹²¹¹, CNdom-ACE and CCdom-ACE. This was taken to suggest that a domain swop-over or the tandem duplication of domains did not influence the binding of inhibitor.

3.3.4. Thermal stabilities

In order to determine the effect of domain swop-overs, domain duplications and regional tACE-N domain substitutions on the thermal stability of the protein constructs, their residual activities were determined using the FRET peptide AbzYRK(Dnp)P (specific for both C- and N domains)(Araujo *et al.*, 2000) following thermal denaturation at 55°C. The data are shown in Figures 3.8. and 3.9.

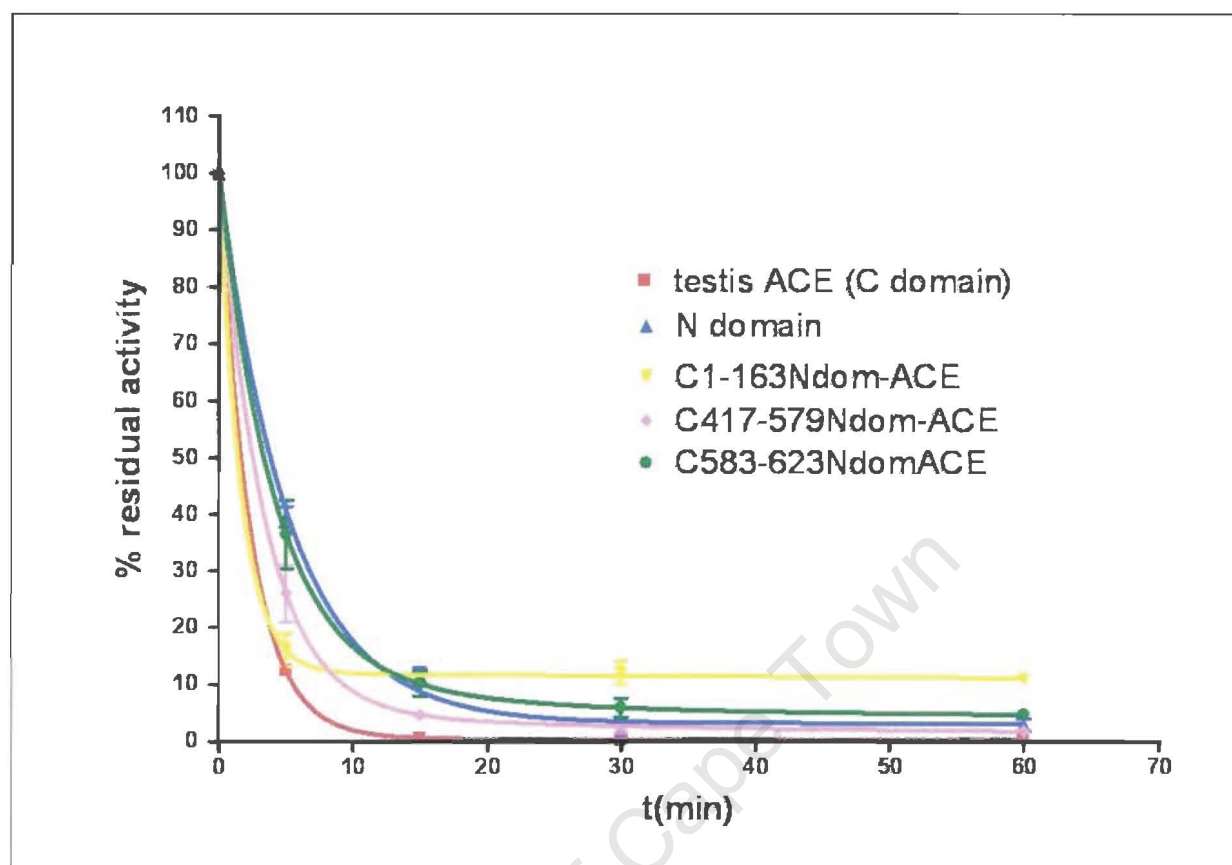
tACE displayed a significantly lower thermal stability as compared with the N domain. Following a 5 minute inactivation at 55°C, the N domain retained 39.6% of its activity while tACE exhibited only 12.4% residual activity. This greater thermal stability of the N domain as compared with tACE was evident for all subsequent inactivation periods. After 5 minutes, sACE¹²¹¹ retained 25.5% of its activity, which was intermediate between the N domain and tACE. However, following longer inactivations at 55°C, sACE¹²¹¹ retained more residual activity as compared with both tACE and the N domain. This data suggested that the enhanced thermal stability of sACE¹²¹¹ was derived from the presence of two domains and possibly a stabilising interaction between them (Figure 3.8.). This was investigated further by determining the thermal stabilities of CNdom-ACE, in which the relative orientations of the two domains were “swopped” and CCdom-ACE where two C domains occur in tandem.

CCdom-ACE displayed a similar thermal denaturation profile to that of tACE with a rapid loss of residual activity after 5 minutes. CNdom-ACE, while having a markedly lower thermal stability than sACE¹²¹¹ after 5 minutes, retained more of its activity following subsequent inactivation periods as compared with CCdomACE and tACE (Figure 3.8.).



	0 min	5 min	15 min	30 min	60 min
<i>Testis ACE</i>	100%	12.4±1.4	0.6±0.05	0.2±0.09	0.4±0.3
<i>N domain</i>	100%	39.6±3.1	11.5±2.4	1.9±0.6	2.8±1.9
<i>sACE¹²¹¹</i>	100%	25.5±1.5	15.1±1.8	10.6±2.3	6.6±0.6
<i>CNdom-ACE</i>	100%	12.6±2.1	2.8±1.5	3.0±1.1	1.0±0.4
<i>CCdom-ACE</i>	100%	11.1±5.0	0.3±0.1	1.0±0.5	0.3±0.3

Figure 3.8. Thermal denaturation profiles of tACE, N domain, sACE¹²¹¹, CNdom-ACE and CCdom-ACE showing percentage residual enzyme activity with AbzYRK(Dnp)P following inactivation at 55°C for 0-60 minutes. Assays were conducted in triplicate. Data shown are the mean values ± SEM.



	0 min	5 min	15 min	30 min	60 min
<i>Testis ACE</i>	100%	12.4±1.4	0.6±0.05	0.2±0.09	0.4±0.3
<i>N domain</i>	100%	39.6±3.1	11.5±2.4	1.9±0.6	2.8±1.9
<i>C₁₋₁₆₃ Ndom-ACE</i>	100%	16.1±5.2	11.4±0.4	13.7±3.3	10.9±0.4
<i>C₄₁₇₋₅₇₉ Ndom-ACE</i>	100%	26.0±8.7	4.6±1.6	2.7±1.6	1.8±0.9
<i>C₅₈₃₋₆₂₃ Ndom-ACE</i>	100%	36.5±10.5	10.1±3.8	5.9±2.9	4.7±1.2

Figure 3.9. Thermal denaturation profiles of tACE, N domain and tACE-N domain chimeras showing percentage residual enzyme activity with AbzYRK(Dnp)P following inactivation at 55°C for 0-60 minutes. Assays were conducted in triplicate. Data shown are the mean values ± SEM.

This data suggested that the thermal stability of sACE¹²¹¹ arose from unique stabilising interactions between the N- and C domains and that these interactions are dependent upon the relative NH₂ and COOH-terminal orientations of the two domains. These stabilising interactions are reduced when the orientations of the domains are changed and do not take place when two identical C domains are present in tandem.

Chimeric proteins, C₄₁₇₋₅₇₉Ndom-ACE and C₅₈₃₋₆₂₃Ndom-ACE demonstrated thermal stabilities in a similar range to that of the N domain (Figure 3.9.) indicating that regional substitutions of tACE with these N domain sequences conferred N domain-like thermal properties. Similarly, the thermal stability of C₁₋₁₆₃ Ndom-ACE was more N domain-like and demonstrated a marked stability over longer inactivation periods (Figure 3.9.)

3.4. Discussion

The N- and C domains of sACE are thought to have arisen from a gene duplication event (Soubrier *et al.*, 1988). A subsequent evolutionary sequence divergence of ~40% has resulted in the development of distinctive features with respect to structure, function and stability. The k_{cat} for the hydrolysis of RAS substrates Ang I and BK by sACE is not significantly different from that determined for tACE, suggesting that the C domain (in both sACE and tACE) is the principal angiotensin-converting domain (Ehlers and Riordan, 1991). This may suggest an element of N domain redundancy with respect to physiological blood pressure regulation, electrolyte and fluid homeostasis. This hypothesis is reinforced by the ability of the N domain to hydrolyse non-RAS substrates such as the hemoregulatory peptide AcSDKP and LHRH (Ehlers and Riordan, 1991; Rousseau *et al.*, 1995). Thus, the two domains may in fact have diverse physiological roles (reviewed in Acharya *et al.*, 2003). In probing these physiological roles, functional characterization of the N- and C domains as well as their interactions within the context of full length somatic ACE has been enhanced by the recent elucidation of the structures of tACE (C domain) (Natesh *et al.*, 2003), *Drosophila* AnCE (Kim *et al.*, 2003) and ACE2 (Towler *et al.*, 2004). However, it is still unclear exactly which residues, regions or combinations thereof contribute to the unique characteristics of each domain. In order to discern the regions within tACE (and C domain) that distinguish it from the N domain, regions of tACE were substituted with corresponding N domain sequence, generating four chimeric proteins. Furthermore, the interactions between the N- and C domains in sACE were investigated in two constructs, one in which the relative orientations of the two domains were “swapped” and one in which two C domains were duplicated in tandem. The chimeric proteins and swop-over constructs were characterized with respect to their structure, catalytic properties and thermal stability as compared with wild-type tACE, the N domain and a soluble somatic ACE construct, sACE¹²¹¹.

In C₁₋₁₆₃Ndom-ACE, the N-terminal region 1-163 of tACE was substituted for corresponding N domain sequence. Marcic *et al.*, (2000) carried out work on a similar construct and reported that it was able to hydrolyse the N domain-specific substrate Ang₁₋₇ more efficiently than wild-type sACE (Deddish *et al.*, 1998; Marcic *et al.*, 2000).

This substrate ordinarily inhibits the C domain, indicating that despite being distal to the active site, residues 1-163 of the C domain play an important role in determining its substrate-specificity. These residues form part of a lid-like structure comprising helices $\alpha 1$, $\alpha 2$, and $\alpha 3$ (Figure 3.6.). This structure covers the central groove formed by the two sub-domains of tACE and might restrict access of large polypeptides to the active site (Acharya *et al.*, 2003). There are distinct differences in the charge and hydrophobicity of this region in the N- and C domains, which may explain why an N domain substitution here resulted in the acquisition of N domain-like catalytic properties by C₁₋₁₆₃Ndom-ACE.

tACE/C domain is less stable as compared with both sACE and the N domain (Velletri *et al.*, 1985, Sturrock *et al.*, 1997, Marcic *et al.*, 2000, Voronov *et al.*, 2002; Balyasnikova *et al.*, 2003). C₁₋₁₆₃Ndom-ACE demonstrated a marked increase in thermal stability as compared with tACE. Thus, an N domain substitution in the region 1-163 of tACE also conferred more N domain-like thermal characteristics. This was supported by secondary structure comparisons, which indicated that the substituted N domain region had a substantially higher proline and helix content as well as additional *N*-linked glycosylation sites, which may have conferred greater resistance to thermal denaturation. This explanation is supported by work carried out by Voronov *et al.*, (2002) where a similar N domain region (29-133) was identified as being thermally more stable than its corresponding C domain sequence (633-734).

Previous studies have reported C₁₆₄₋₄₁₆Ndom-ACE to be inactive in cell lysates and absent from the culture medium of CHO cells (Woodman, PhD thesis, 2003). This suggested that the chimeric protein underwent defective intracellular processing. In the present study, homology modelling revealed several alterations in the structure, which may have contributed to the inactivity of this protein. The introduction of an *N*-linked glycosylation site in helix $\alpha 11$ at the N terminus of the C-terminal sub-domain, may have contributed to the disruption in the overall integrity of the structure. Changes in the S₁' subsite residues may have altered the charge and hydrophobicity of the S₁' pocket resulting in its occlusion. The C-domain/tACE is more chloride-dependent than the N domain (Jaspard *et al.*, 1993).

The mechanism of substrate-dependent chloride activation could involve structural changes, which affect the activity of the enzyme. The deletion of the chloride ligand R¹⁸⁶ in C₁₆₄₋₄₁₆Ndom-ACE may have altered its chloride dependence as well as its overall structure, contributing to its inactivation. Williams *et al.*, (1996) replaced a 60 residue region H³⁵³-I⁴¹³ of tACE with corresponding N domain sequence to generate an enzymatically active chimera with C domain substrate specificity. Since a substitution of D¹⁶⁴-V⁴¹⁶ in C₁₆₄₋₄₁₆Ndom-ACE inactivated the protein, the region D¹⁶⁴-C³⁵² may be critical for the activity and intracellular processing of tACE.

C₄₁₇₋₅₇₉Ndom-ACE demonstrated a significant reduction in the rate of substrate hydrolysis with a shift in substrate-specificity to that of the N domain. Homology modelling revealed the replacement of tACE S₁ subsite residues and the protrusion of N⁴⁹⁴ into the S₁ pocket potentially favouring the hydrolysis of more N domain-specific substrates (Tzakos *et al.*, 2003). While C₄₁₇₋₅₇₉Ndom-ACE displayed lower residual activity than the N domain following thermal denaturation at 55°C for 5 minutes, subsequent denaturation periods indicated that it was thermally more stable than tACE under identical conditions. Thus, a regional substitution between residues 417-579 with corresponding N domain sequence not only resulted in the acquisition of more N domain-like catalytic properties but also more N domain-like thermal stability.

The substituted region of C₅₈₃₋₆₂₃Ndom-ACE did not include key active site residues while the two tACE N-linked glycosylation sites at N⁵⁸⁶ and N⁶²⁰ that were lost are surface localised and do not play a crucial role in maintaining the catalytic activity of the enzyme (Gordon *et al.*, 2003). Moreover, secondary structure prediction did not identify major differences in proline or helix content suggesting that the altered substrate specificity of C₅₈₃₋₆₂₃Ndom-ACE as well as its marked increase in thermal stability was likely the result of other tertiary structural features.

Although both domains are catalytically active in isolation (Wei *et al.*, 1991), they do not function independently in full-length sACE (Ehlers and Riordan, 1991; Binevski *et al.*, 2003; Andujar-Sanchez *et al.*, 2004).

Several studies have reported strong negative cooperativity for sACE, such that the k_{cat} for Z-FHL, Ang I, and Ang₁₋₇ was approximately the mean of the k_{cat} values determined for the individual domains (Binevski *et al.*, 2003; Rice *et al.*, 2004). A recent *in vivo* study in the mouse indicated that Ang I hydrolysis was completely abolished by selective inhibition of either the N domain or the C domain (Georgiadis *et al.*, 2003), suggesting some form of domain interaction or cooperativity. Moreover, this domain interaction extends to the binding of inhibitors. Active site titration with 1 mol/mol of the tight-binding inhibitor lisinopril, was sufficient to abolish catalytic activity in both sACE and tACE (Ehlers and Riordan, 1991; Andujar-Sanchez *et al.*, 2004).

Although the crystal structure of full-length sACE is not yet available, its structure may be inferred from the structures of the individual domains (Natesh *et al.*, 2003 and Corradi *et al.*, in press) as well as that of the interdomain bridge region linking them. This 14-residue region is susceptible to proteolysis (Sturrock *et al.*, 1997) and may enable considerable freedom of movement of the two domains relative to each other. This flexibility may facilitate close intermolecular contact between the N- and C domains during the binding and hydrolysis of peptide substrates, thus enabling inter-domain cooperativity. This is reinforced by the greater observed immunogenicity of the N domain which may arise as a result of the folding of this more extensively glycosylated, solvent accessible domain over the C domain (Danilov *et al.*, 1994).

The increased resistance of sACE¹²¹¹ to thermal denaturation suggests furthermore that a stabilising interaction occurs between the N- and C domains. Previous studies have demonstrated that thermal inactivation of the C domain (Melting point = 55°C) within bovine sACE does not influence the properties of the active N domain (Melting point = 70.5°C) suggesting that the two domains fold independently within the globular protein (Voronov *et al.*, 2002). Thus, thermal inactivation of CNdom-ACE would be expected to maintain the activity of an independently folded N domain irrespective of its position relative to the C domain. However, in the present study, the thermal stability of CNdom-ACE was significantly lower than that of both sACE¹²¹¹ and the N domain.

In CNdom-ACE where P₆₀₄-P₇₃₉ of sACE is fused to D₁₄₂-S₇₀₁ of the N domain, the N domain of this construct includes a short sequence of C domain at its N-terminus, which constitutes a major part of region 1-163 (Figure 3.2 and 3.3.). Based upon results obtained with the chimeric protein C₁₋₁₆₃Ndom-ACE, this region has been implicated in playing an important role in conferring the N domain its unique thermal stability and when substituted into the C domain, conferring N domain-like thermal properties. Therefore, substitution of this N domain region in CNdom-ACE with thermally less stable C domain sequence may provide an explanation for the observed reduced thermal stability of an independently-folding “N domain” in this construct. The reduced stability of native CNdom-ACE is supported by Woodman *et al.*, (2005) who demonstrated that interdomain bridge proteolysis occurred to a limited extent in this construct as compared with the absence of such proteolysis in more stable sACE. In contrast to sACE and CNdom-ACE, two C domains in tandem (CCdom-ACE) demonstrated positive cooperativity and were as susceptible to thermal denaturation as an individual C domain (tACE). This suggested that the two C domains are in sufficiently close contact to cooperate in a positive manner but that a thermally-stabilising interaction, as may be the case between the N- and C domains of sACE¹²¹¹, is absent. In CCdom-ACE, a putative sheddase recognition motif present on the N-terminal C domain may direct proteolysis of the interdomain bridge by the ACE sheddase while the chimeric protein is still membrane-bound (Woodman *et al.*, 2005). The greater susceptibility of this construct to proteolysis within its interdomain bridge region may also be due to reduced thermally-stabilising interactions between the two heat-labile C domains.

In delineating the regions that are important in domain-specific catalytic activity, regions of tACE were replaced with corresponding N domain sequence. The active chimeras allowed further characterisation of the differences between the two domains of sACE. In three of the chimeras, substitutions resulted in the acquisition of N domain-like catalytic and thermodynamic properties. However, substituted N domain sequence including residues D¹⁶⁴ to V⁴¹⁶ was unable to maintain the native conformation of tACE for processing and substrate hydrolysis to occur. This suggests that this region within tACE contains critical structural elements for the protein.

In sACE, a flexible inter-domain bridge may permit a close thermally-stabilising interaction between the more stable N domain and the C domain resulting in negative inter-domain cooperativity with respect to peptide substrate hydrolysis. Thus, in addition to the potentially divergent physiological roles of the two domains within sACE, the N domain may have a function in regulating the catalytic properties of the C domain and consequently its hydrolysis of key substrates in the RAS.

CHAPTER 4

Inhibitor selectivity of the N- and C domains of sACE

4.1. Introduction

ACE inhibitors, including lisinopril, keto-ACE and phosphinic peptides have been reviewed in **Chapter 1**. The crystal structures of tACE (Natesh *et al.*, 2003) in complex with various inhibitors (Kim *et al.*, 2003 and Natesh *et al.*, 2004) as well as the crystal structure of the N domain (Corradi *et al.*, in press) and its homology model (Tzakos *et al.*, 2003) have highlighted key differences underlying the inhibitor selectivities of the N- and C domains. Furthermore, these structures have proven a useful tool in exploring the molecular basis for the interactions of lisinopril and keto-ACE with the enzyme substrate-binding pockets.

Since the C domain of sACE is primarily responsible for blood pressure regulation and since inappropriate N domain inhibition may be the cause of adverse drug events (reviewed in **Chapter 1**), the development of more C domain-selective ACE inhibitors is considered an important therapeutic goal. Lisinopril, is a potent ACE inhibitor with a K_i in the nanomolar range. Its inhibitory potency is derived largely from its zinc-binding carboxylate as well as interactions between its P_1' lysyl amine and the S_1' pocket, its P_1 benzyl ring and the S_1 pocket and its hydrophobic prolyl ring and S_2' subsite residues (Figure 4.1.). However, lisinopril is less C domain-specific than keto-ACE (Deddish *et al.*, 1998; Tzakos and Gerothanassis, 2005). Keto-ACE, potent in the lower micromolar range, has a 40-50 fold greater specificity for the C domain as compared with the N domain (Almquist *et al.*, 1980; Deddish *et al.*, 1998). This has been attributed to the presence of a bulky P_1 group and P_2 benzyl ring (Acharya, 2003).

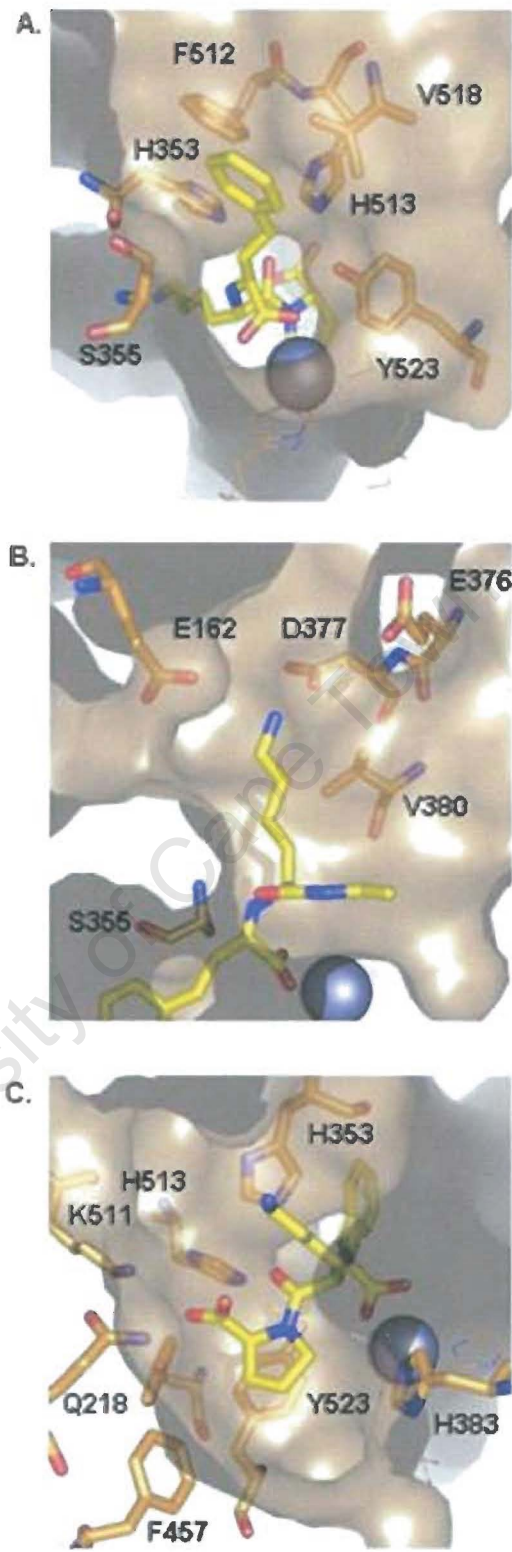


Figure 4.1. Interactions of lisinopril (yellow) with the S₁ (A), S₁' (B) and S₂' (C) subsites of tACE. The catalytic zinc atom is shown in purple.

Recently, Georgiadis *et al.*, (2004) described the synthesis of a highly potent (nM) phosphinic ACE inhibitor, RXPA380 which is ~3000-fold more C domain-selective. RXPA380 incorporates a bulky tryptophan moiety at the P₂' position, reinforcing the importance of a deep S₂' pocket as a determinant of C domain-selectivity. Taking this feature into account together with the relative contributions of the unique functionalities of lisinopril and keto-ACE and with the aim of improving their C domain-selectivity, a series of novel lisinopril and keto-ACE derivatives have been designed which incorporate additional modifications at the P₁', P₂ and P₂' positions. In the present study, the *in vitro* inhibitory activities of these derivatives were assessed, potential candidate compounds for further development have been identified and the underlying basis for the differences in inhibitor selectivity between the C- and N domains has been investigated.

4.2. Materials and Methods

Keto-ACE and lisinopril derivatives were synthesised by Dr. Aloysius T. Nchinda in our group and included:

1. Keto-ACE with a Boc-group replacing phenylalanine at the P₂ position: Compound **2** (Table 4.1)
2. Keto-ACE incorporating a phenylalanine group at the P₁' position: Compounds **3-4** (Table 4.1). Compounds **3** and **4** are diastereoisomers.
3. Keto-ACE incorporating a phenylalanine group at the P₂' position: Compounds **5-6** (Table 4.1). In compound **6**, a Boc-group replaces the phenylalanine group at the P₂ position of compound **5**.
4. Keto-ACE incorporating a tryptophan moiety at the P₂' position: Compounds **7-8** (Table 4.1). In compound **8** a Boc-group at the P₂ position replaces the phenylalanine group of compound **7**.
5. Keto-ACE incorporating a phenylalanine group at the P₁' and P₂' positions: Compounds **9-10** (Table 4.1). Compounds **9** and **10** are diastereoisomers.
6. Keto-ACE incorporating a phenylalanine group at the P₁' position and a tryptophan moiety at the P₂' position: Compounds **11-12** (Table 4.2). Compounds **11** and **12** are diastereoisomers.
7. Lisinopril incorporating a tryptophan moiety at the P₂' position: Compounds **13-14** (Table 4.2). Compounds **13** and **14** are diastereoisomers.

(Appendix 2.3.).

4.2.1. Enzyme expression and purification

Human tACEΔ36NJ is a soluble form of tACE lacking the 36-residue *O*-glycosylated region and truncated to exclude the transmembrane region and cytosolic tail (Yu *et al.*, 1997). A soluble human N domain construct L¹-D⁶²⁹ is similarly truncated at D629 (Balyasnikova *et al.*, 2003). The expression and purification of tACEΔ36NJ and N domain L¹-D⁶²⁹ has been described in **Chapter 2**.

4.2.2. ACE inhibition assay

ACE inhibitory activities were determined as previously described (Almquist *et al.*, 1980) with some modifications (Appendix 2.3.). 40 nM enzyme (tACE Δ 36NJ and N domain L¹-D⁶²⁹) was pre-incubated with 0-500 nM lisinopril or 0-500 μ M compound (1-14) at ambient temperature for 60 minutes. Residual enzyme activity was determined from 3.0 μ l enzyme-inhibitor solution using 30 μ l of Z-FHL at two substrate concentrations (0.1 mM and 1.0 mM Z-FHL). Assays were conducted as described previously in **Chapter 2**. Fluorescence was measured at λ_{Ex} =360 nm and λ_{Em} =486 nm on a Cary Eclipse fluorometric plate reader (Varian). All assays were conducted in triplicate. Inhibition curves were plotted and K_i values were determined from Dixon plots of $1/V$ vs $[I]$. IC_{50} values with 1.0 mM Z-FHL were determined from sigmoidal dose response curves (GraphPad Prism 4.01). IC_{50} values were determined for all the compounds. K_i values were determined for those compounds showing significant domain-selectivity based on IC_{50} values. Where K_i values were calculated, N/C selectivity was based on K_i , where only IC_{50} values were calculated, N/C selectivity was based on IC_{50} (Appendix 2.3).

4.2.3. Molecular docking

Molecular docking experiments were carried out using INSIGHT II (Accelrys Inc., Version 98.0). The starting structure was that of testis ACE in complex with a known inhibitor lisinopril (Natesh *et al.*, 2003) as well as the homology modelled structure of the N domain (Tzakos *et al.*, 2003). The Consistent Valence Force Field and the Extensible Systematic Force Field (metal adapted) were used in all energy minimizations and dynamic runs. Conjugate gradient minimization was used after the initial 1000 steps, followed by 3000 cycles of molecular dynamics and 3000 cycles of energy minimization in an NTV (constant volume) ensemble, at a temperature of 300 K. All calculations were carried out in a dielectric constant of 1.00 and a cut-off distance of 9.5 Å. The structures of the compounds were generated with standard bond lengths and angles using the builder tool of INSIGHT II (Accelrys Inc.) and then minimized.

The initial position of the compounds in the active sites of tACE and the N domain was determined by superimposing the important pharmacophoric groups on the corresponding atoms of lisinopril in the tACE-lisinopril complex. After removal of the reference inhibitor (lisinopril), the structures of the ACE-inhibitor complexes were refined by running energy minimizations and molecular dynamics.

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4.3. Results

In elucidating the functional differences between the N- and C domains of ACE with respect to inhibitor selectivity, the inhibitory activities of several novel keto-ACE and lisinopril derivatives towards tACE and the N domain were determined. The structures of these compounds as well as their K_i and IC_{50} values are shown in Tables 4.1 and 4.2.

4.3.1. ACE inhibitory activities

Keto-ACE and its derivatives all showed K_i values in the micromolar range as compared with the nanomolar K_i value of the commercial ACE inhibitor, lisinopril (Tables 4.1 and 4.2). However, keto-ACE (**1**) was found to be more C-selective (about 25-fold) as compared with lisinopril (about 2.6-fold). Although the ACE inhibitory activities of P_1' phenethyl keto-ACE derivatives were modest as compared with lisinopril, compound **3** was about 10-fold more potent for the C domain ($IC_{50}=1.1\ \mu\text{M}$) as compared with keto-ACE (**1**) ($IC_{50}=10.9\ \mu\text{M}$) (Table 4.1), indicating that the incorporation of the phenethyl group at the P_1' position conferred increased potency towards the C domain. However, the P_1' phenethyl group did not influence the relative N/C selectivity based on comparative IC_{50} values (Compound **3** =7.5-fold and keto-ACE (**1**) =5.3-fold (Table 4.1)).

Table 4.1. Structures and ACE inhibitory activities of compounds 1-10

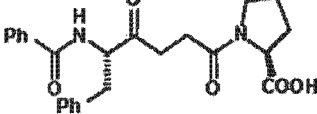
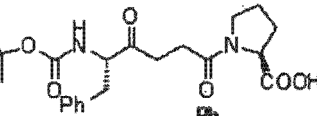
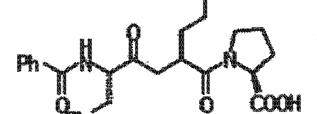
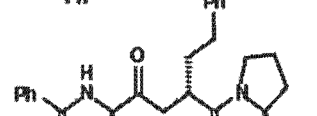
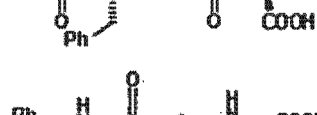
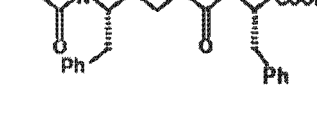
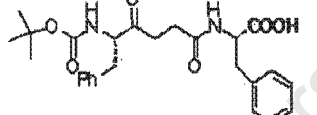
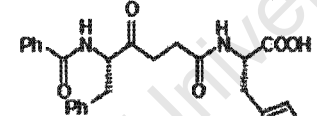
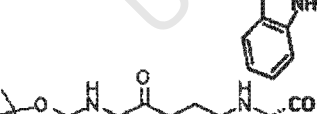
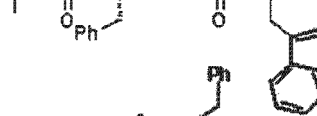
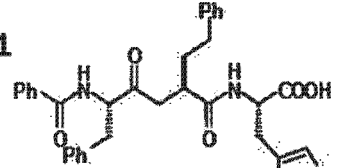
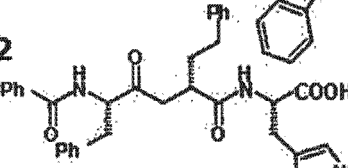
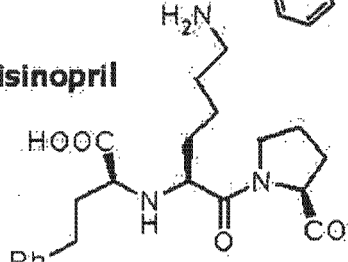
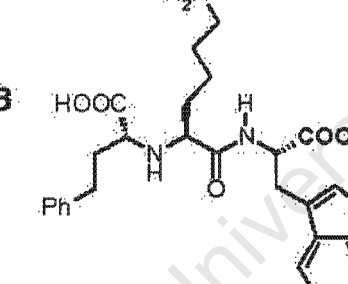
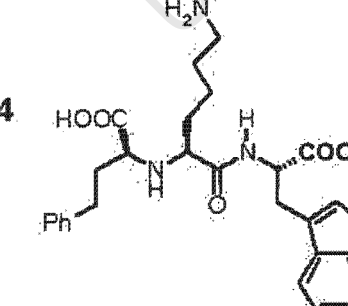
		<i>C</i> domain K_i	<i>C</i> domain IC_{50}	<i>N</i> domain K_i	<i>N</i> domain IC_{50}	Selectivity <i>N/C</i>
1		1.8 μM	10.9 μM	45.3 μM	57.4 μM	25.0
2		143.5 μM	113.6 μM	33.6 μM	5.4 μM	0.2
3			1.1 μM		8.2 μM	7.5
4			> 500 μM		232.8 μM	< 0.5
5		23.7 μM	26.2 μM	84.3 μM	74.4 μM	3.6
6		312.9 μM	247.8 μM	43.0 μM	108.7 μM	0.1
7		0.8 μM	11.3 μM	195.7 μM	47.4 μM	245
8		381.3 μM	282.7 μM	13.3 μM	195.5 μM	0.04
9			19.8 μM		35.6 μM	1.8
10			208.4 μM		196.5 μM	0.94

Table 4.2. Structures and ACE inhibitory activities of compounds 11-14

		<i>C</i> domain K_i	<i>C</i> domain IC_{50}	<i>N</i> domain K_i	<i>N</i> domain IC_{50}	Selectivity N/C
11			7.1 μ M		10.9 μ M	1.5
12			242.4 μ M		43.9 μ M	0.2
Lisinopril		51.0 nM	28.3 nM	131.5 nM	25.8 nM	2.6
13		26.3 μ M	34.4 μ M	> 500 μ M	> 500 μ M	> 19
14		7.0 μ M	13.2 μ M	> 500 μ M	> 500 μ M	> 72

The diastereoisomers of compound **3** (Compound **4**), compound **9** (Compound **10**) and compound **11** (Compound **12**) with an *S*- configuration at the chiral centre were less potent towards the C domain (Tables 4.1-4.2) than their respective counterparts with an *R*-configuration. This indicated that there was a preference for *R*-stereochemistry at the P₁' group and that inversion to an *S* - configuration resulted in a decrease in inhibitory potency. Incorporation of a Phe at the P₂' of keto-ACE (compound **5**)(Table 4.1) resulted in a reduction in C domain selectivity as compared with the parent compound. When a Phe functionality was included at the P₁' position in addition to the P₂' Phe (compound **9**), C domain-selectivity was reduced even further.

The C domain-selectivity of keto-ACE **1** (25-fold) was significantly enhanced by the incorporation of a tryptophan moiety at the P₂' position (Compound **7**=245-fold)(Table 4.1). However, further incorporation of a P₁' Phe (Compound **11**) abrogated this selectivity (Table 4.2).

Both compounds **5** and **7** which retained a benzoyl group at the P₂ position, showed greater potency toward the C domain as compared with their more N-selective counterparts **6** and **8** which possessed a Boc group at the P₂ position (Table 4.1).

Compounds **13** and **14** were two diastereoisomers of a lisinopril derivative incorporating a Trp at the P₂' position. Their ACE inhibitory activities are shown in Table 4.2. Although they displayed *K_i* values in the micromolar range, the introduction of a tryptophan moiety at the P₂' position resulted in a marked increase in C domain-selectivity (>19- >72 fold) as compared with lisinopril (2.6-fold). Neither compound inhibited the N domain up to a concentration of 500 μM (Table 4.2). Compound **14**, with an *S*-configuration at the stereogenic centre bearing the zinc-binding carboxylate group, was more C-selective than its *R*-diastereoisomer (Compound **13**).

4.4. Discussion

An expanding body of structural data derived from the co-crystallisation of tACE with various inhibitors as well from the crystal structure and homology model of the N domain, have highlighted key differences underlying the inhibitor selectivities of the two domains of sACE (Natesh *et al.*, 2003; Acharya *et al.*, 2003; Tzakos *et al.*, 2003; Sturrock *et al.*, 2004; Corradi *et al.*, in press).

The design of novel keto-ACE and lisinopril derivatives has exploited unique features in the parent compounds that confer inhibitory potency and C domain-selectivity. The incorporation of various functionalities could potentially enhance these properties. The aim of the present work was to determine the effect of these functionalities on the *in vitro* biological activities of novel keto-ACE and lisinopril derivatives to aid in the identification of candidate C domain-selective ACE inhibitors. Furthermore, the underlying basis for the different inhibitor selectivities of the N- and C domains was investigated.

The rationale behind the incorporation of P₁' Phe functionality in keto-ACE, was based upon the presence of a deep S₁' pocket in the C domain structure (Natesh *et al.*, 2003). In lisinopril, which is more potent, yet less C domain-selective than keto-ACE, a P₁' lysyl amine interacts with E¹⁶² and D³⁷⁷ of the tACE S₁' subsite (Figure 4.1). In the N domain, these residues are replaced by D¹⁴⁰ and Q³⁵⁵ respectively, accounting for the increased C domain-selectivity of this inhibitor.

The stereochemistry of the P₁' substituent is very important in determining inhibitory potency. In lisinopril, P₁' substituents with *S*-stereochemistry have been shown to possess greater potency than their *R*- counterparts (Patchett *et al.*, 1980). The effect of the incorporation of a hydrophobic phenyl group at the P₁' position on the inhibitory potency and domain-selectivity of keto-ACE was investigated. As is the case with lisinopril, the stereochemistry of keto-ACE derivatives incorporating P₁' Phe functionalities was important in determining their inhibitory potencies.

However, unlike lisinopril, the *R*-configuration of keto-ACE phenylethyl derivatives **3**; **9** and **11** was associated with significantly higher inhibitory potency as compared with an *S*-configuration at the P₁' position in compounds **4**, **10** and **12**. Molecular docking of these compounds into the active site pockets of tACE and the N domain homology model has revealed two stereochemistry-dependent binding modes. In the more potent *R*-configuration, the phenyl ring of the P₁' phenylethyl moiety adopts a pseudo-parallel conformation permitting a better fit into the S₁' pocket. This fit is hindered by the assumption of a pseudo-perpendicular conformation of the P₁' phenylethyl moiety in the *S*-enantiomers **4**, **10** and **12**. Interestingly, compound **3** with P₁' phenylalanine, demonstrated a greater inhibitory potency for both N- and C domains while maintaining the C domain selectivity of keto-ACE. This data suggested that the P₁' functionality may be more important in influencing potency rather than domain-selectivity.

Inhibitory activity profiles have reinforced the role of key active site residues in the S₂ and S₂' pockets as determinants of C domain selectivity (Georgiadis *et al.*, 2004; Tzakos and Gerothanassis, 2005). The C domain of ACE has a preference for bulky aromatic groups at the P₂ position, while the S₂ pocket of the N domain more readily accommodates bulky aliphatic groups. The greater C domain selectivity of keto-ACE has been attributed to the presence of a P₂ benzyl ring (Acharya, 2003). Here, the benzyl ring is orientated for a stacking interaction with the aromatic side chain of F³⁹¹ in the C domain S₂ subsite. In the N domain, F³⁹¹ is replaced by Y³⁶⁹, resulting in an unfavourable contact with the P₂ benzyl ring (Tzakos and Gerothanassis, 2005). Replacement of this functionality with a *Boc* moiety in compound **2** abrogated its C domain- selectivity suggesting that the *Boc* moiety may be better tolerated by the N domain S₂ subsite which has a preference for both bulky aliphatic and acidic groups in the P₂ position. The greater N domain-selectivity of RXP407 arises from the unusual incorporation of an aspartate side-chain in the P₂ position (Dive *et al.*, 1999) which interacts with Arg³⁸¹ of the N domain S₂ subsite. In the C domain, this residue is replaced by Glu⁹⁷⁹ (Acharya *et al.*, 2003; Dive *et al.*, 2004).

The F³⁹¹/Y³⁶⁹ alteration in the S₂ subsite also influences the domain-selectivity of RXP407 where the P₂ aspartate functionality forms a hydrogen bond with the side chain of Y³⁶⁹ in the N domain (Tzakos and Gerothanassis, 2005).

Furthermore, the C domain also demonstrates a preference for bulky and polar residues at the P₂' position which may interact with its S₂' pocket (Bala *et al.*, 2002; Acharya *et al.*, 2003; Georgiadis *et al.*, 2004; Bersanetti *et al.*, 2004). Significant interactions occur between lisinopril and this relatively large pocket. The P₂' hydrophobic prolyl ring fits into this pocket, interacting with S₂' subsite residues such as Q²⁸¹, K⁵¹¹ and Y⁵²⁰ (Tzakos and Gerothanassis, 2005). These interactions increase the inhibitory potency of lisinopril by at least 25-fold and they are facilitated by the bent conformation assumed by the bound lisinopril molecule which places the free carboxylate in a favorable orientation for interacting through hydrogen bonding with the Lys⁵¹¹ and Tyr⁵²⁰ side chains of ACE (Georgiadis *et al.*, 2004).

A large P₂' residue is an essential functionality for C domain-selectivity (Dive *et al.*, 2004). The highly C domain-selective phosphinic ACE inhibitor, RXPA380 incorporates a bulky tryptophan moiety at the P₂' position. (Georgiadis *et al.*, 2004). This functionality interacts with bulky, hydrophobic residues Val⁹⁵⁵ (tACE numbering: Val³⁷⁹) and Val⁹⁵⁶ (tACE numbering: Val³⁸⁰) in the C domain S₂' subsite. These residues are replaced by smaller, polar residues Ser³⁵⁷ and Thr³⁵⁸ in the N domain, disrupting favourable interactions between the P₂' tryptophan and the N domain S₂' subsite (Georgiadis *et al.*, 2004). Both lisinopril and keto-ACE lack bulky P₂' functionalities. In the present study, the introduction of a bulky P₂' tryptophan moiety significantly enhanced the C domain selectivity of compound **7** (a keto-ACE derivative) and compound **14** (a lisinopril derivative) as compared with their parent compounds. The S₂' subsite of the C domain readily accommodates these P₂' tryptophan moieties where the aromatic ring of the tryptophan makes contact with Thr²⁸², Val³⁷⁹, Val³⁸⁰ and Asp⁴⁵³ in the C domain increasing the overall hydrophobicity of the molecule (Figure 4.2 and 4.3).

In the N domain, Val³⁷⁹ and Val³⁸⁰ are replaced by polar residues Ser³⁵⁷ and Thr³⁵⁸ while Asp⁴⁵³ in the C domain is replaced by a less polar Glu⁴³¹ in the N domain.

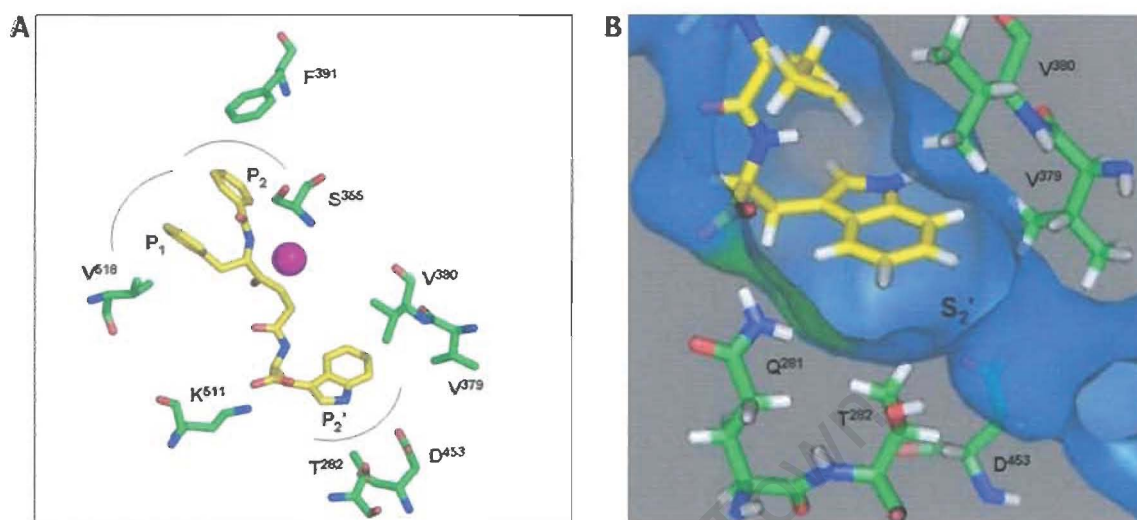


Figure 4.2. Interaction of compound 7 P₁, P₂ and P₂' functionalities with residues of the tACE active site (A). Binding of the P₂' tryptophan moiety of compound 7 to the S₂' pocket of tACE (B).

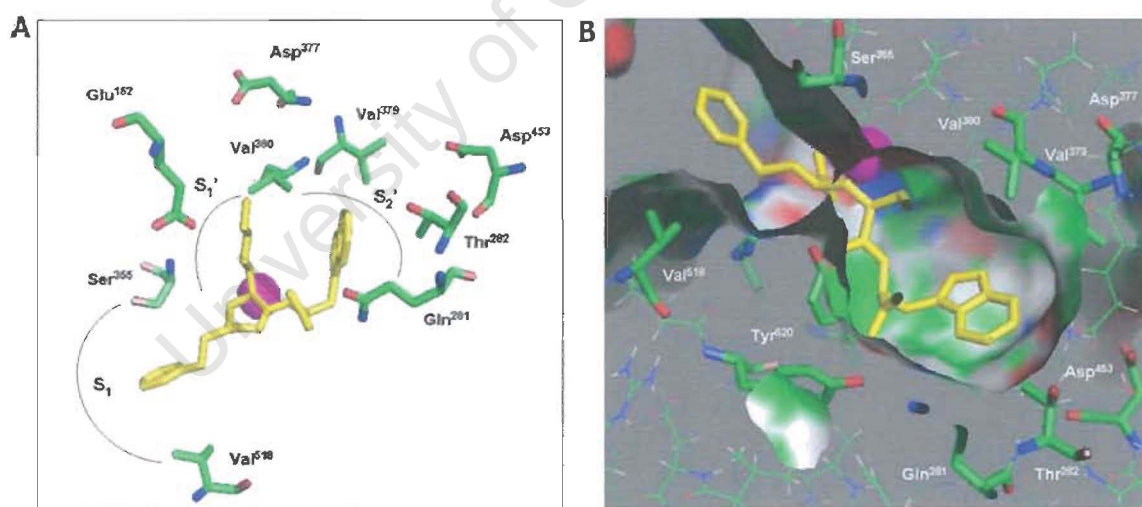


Figure 4.3. Interaction of compound 13 residues of the S₁, S₁' and S₂' subsites of tACE (A). A surface representation of the S₂' substrate-binding pocket of tACE, accommodating the P₂' tryptophan moiety of compound 13 (B).

Glu⁴³¹ would increase the distance from the side chain, decreasing the size of the binding pocket. Thus, compounds **7** and **14** form more stable complexes with the C domain, exhibiting greater C-selectivity as compared with their parent compounds. Like lisinopril, the *S*-enantiomer (compound **13**) was more potent than the *R*-enantiomer (compound **14**).

The unique inhibitor selectivities of the N- and C domains are primarily the result of subtle differences in the residue composition and subsite architecture of their active sites which are only evident from the 3D structure of the two domains. The present study has highlighted the role of the S₂ and S₂' pockets as structural determinants of domain-specific inhibitor selectivity while the S₁' pocket appears to play a role in inhibitory potency. The novel keto-ACE and lisinopril derivatives that have been designed to exploit these differences may serve as potential compounds in the development of C domain-selective ACE inhibitors.

Conclusion

The central position of ACE in the renin-angiotensin and kallikrein-kinin systems, its role as a critical regulator of cardiovascular homeostasis and its consequent importance as a therapeutic target have underscored the need for detailed investigation into the structure-function relationships of this enzyme. While significant advances have been made in this regard, greatly aided by the recent structural elucidation of ACE, several aspects were explored further in the present work. These included the role of *N*-linked glycosylation in ACE processing and activity, the contribution of specific regions and residues of the N- and C domains to substrate-specificity, inhibitor selectivity and thermal stability as well as the nature of the interaction between the two domains within the somatic enzyme. In all studies, a truncated form of tACE (tACE Δ 36NJ), lacking the 36-residue N-terminal region, TM region and cytosolic tail was used since it is identical to the C domain of sACE in terms of its catalytic function.

The N- and C domains displayed differences in their processing- and functional requirements for *N*-linked glycosylation with the N domain demonstrating a different site-specific glycosylation requirement as compared with tACE. The likely reasons for these observations may include the fact that the N domain, occurring usually only as the N-terminal region of a larger sACE molecule may have a different requirement for glycosylation in effecting correct folding and processing of sACE to the cell surface.

Differences in the substrate-specificity and thermal stability of the N- and C domains were reinforced by the acquisition of N domain-like catalytic and thermal properties upon substitution of C domain regions with corresponding N domain sequence. The N-terminal region of the N domain, including residues 1-163 was identified as an important determinant of substrate-specificity and thermal stability. Furthermore, substrate-selectivity was also dependent upon specific residues occupying the S_1 and S_1' substrate-binding pockets.

Within the full-length somatic enzyme, the N- and C domains demonstrated evidence of negative cooperativity with respect to substrate-hydrolysis suggesting thermally-stabilising intermolecular interactions irrespective of the relative orientation of the two domains. When two C domains were duplicated in tandem, intermolecular interactions enabled positive cooperativity with respect to substrate-hydrolysis but did not increase the thermal stability of the construct as compared with the individual C domains.

The rational, structure-based design of highly C domain selective inhibitors is considered to be an appropriate strategy in overcoming the drawbacks of current generation ACE inhibitors where inappropriate suppression of N domain bradykinin hydrolysis amongst others is thought to be a major contributing factor to adverse drug events. In this regard, the present study has identified novel lisinopril and keto-ACE analogues which have exploited the ability of the C domain to accommodate a bulky, hydrophobic P₂' tryptophan functionality. These analogues may thus serve as potential compounds in the development of C domain-selective ACE inhibitors.

Appendix 1: Supplementary Figures

Human sACE

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1      MGAASGRGPF GLLLPLPLLL LLPPQPALAL DPGLQPGNFS ADEAGAQLFA QSYNSSAEQV
61     LFQSVAAAWA HDTNITAENA RRQEEAALLS QEFAEAWGQK AKELYEPIWQ NFTPDPQLRRI
121    IGAVRTLGA NLPLAKRQQY NALLSNMSRI YSTAKVCLPN KTATCWSLDP DLTNILASSR
181    SYAMLLFAWE GWHNAAGIPL KPLYEDFTAL SNEAYKQDGF TDTGAYWRSW YNSPTFEDDL
241    EHLYQQLEPL YLNLHAFVRR ALHRRYGDY INLRGPIPAH LLGDMWAQSW ENIYDMVVPF
301    PDKPNLDVTS TMLQQGWNAT HMFRVAEEFF TSLELSPMPF EFWEGSMLEK PADGREVVCH
361    ASAWDFYNRK DFRIKQCTRV TMDQLSTVHH EMGHIQYYLQ YKDLPVSLRR GANPGFHEAI
421    GDVLALSIST PEHLHKIGLL DRVTNDTESD INYLLKMALE KIAFLPFGYL VDQWRWGVFS
481    GRTPPSRYNF DWYLRTRYQ GICPPVTRNE THFDAGAKFH VPNTVPYIRY FVSFVLQFQF
541    HEALCKEAGY EGPLHQCDIY RSTKAGAKLR KVLQAGSSRP WQEVKDKMVG LDALDAQPLL
601    KYFQPVQTWL QEQQNQNGEV LGWPEYQWHP PLPDNYPEGI DLVTDEAEAS KFVEEYDRTS
661    QVVWNEYAEA NWNNTNTITT ETSKILLQKN MQIANHTLKY GTQARKFDVN QLQNTTIKRI
721    IKKVQDLERA ALPAQEELEY NKILLDMETT YSVATVCHPN GSCLQLEPDL TNVMTASRKY
781    EDLLWAWEGW RDKAGRAILQ FYPKYVELIN QAARLNGYVD AGDSWRSMYE TPSLEQDLER
841    LFQELQPLYL NLHAYVRRAL HRHYGAQHIN LEGPIPAHLL GNMWAQTWSN IYDLVVPFPS
901    APSMDTTEAM LKQGWTPRRM FKEADDFFTS LGLLPVPPEF WNKSMLEKPT DGREVVCHAS
961    AWDFYNGKDF RIKQCTTVNL EDLVVAHHEM GHIQYFMQYK DLPVALREGA NPGFHEAIGD
1021   VLALSISTPK HLHSLNLLSS EGGSDEHDIN FLKMKALDKI AFIPFSYLVD QWRWRVFDGS
1081   ITKENYNQEW WSLRLKYQGL CPPVPTQGD FDPGAKFHIP SSVPIRYFV SFIIQFQFHE
1141   ALCQAAGHTG PLHKCDIYQS KEAGQRLATA MKLGFSRPWP EAMQLITGQP NMSASAMLSY
1201   FKPLLDWLRT ENELHGEKLG WPQYNWTPNS ARSEGPLPDS GRVSFLGLDL DAQQARVGVQW
1261   LLLFLGIALL VATLGLSQRL FSIRHRSRHR HSHGPQFGSE VELRHS

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Human sACE N domain

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1      MGAASGRGPF GLLLPLPLLL LLPPQPALAL DPGLQPGNFS ADEAGAQLFA QSYNSSAEQV
61     LFQSVAAAWA HDTNITAENA RRQEEAALLS QEFAEAWGQK AKELYEPIWQ NFTPDPQLRRI
121    IGAVRTLGA NLPLAKRQQY NALLSNMSRI YSTAKVCLPN KTATCWSLDP DLTNILASSR
181    SYAMLLFAWE GWHNAAGIPL KPLYEDFTAL SNEAYKQDGF THTGAYWRSW YNSPTFEDDL
241    EHLYQQLEPL YLNLHAFVRR ALHRRYGDY INLRGPIPAH LLGDMWAQSW ENIYDMVVPF
301    PDKPNLDVTS TMLQQGWNAT HMFRVAEEFF TSLELSPMPF EFWEGSMLEK PADGREVVCH
361    ASAWDFYNRK DFRIKQCTRV TMDQLSTVHH EMGHIQYYLQ YKDLPVSLRR GANPGFHEAI
421    GDVLALSIST PEHLHKIGLL DRVTNDTESD INYLLKMALE KIAFLPFGYL VDQWRWGVFS
481    GRTPPSRYNF DWYLRTRYQ GICPPVTRNE THFDAGAKFH VPNTVPYIRY FVSFVLQFQF
541    HEALCKEAGY EGPLHQCDIY RSTKAGAKLR KVLQAGSSRP WQEVKDKMVG LDALDAQPLL
601    KYFQPVQTWL QEQQNQNGEV LGWPEYQWHP PLPDNYPEGI DLVTDEA

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Human tACE

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1      MGQGWATAGL PSLFLLLCY GHPLLVPSEQ ASQQVTVTHG TSSQATTSSQ TTTHQATAHQ
61     TSAQSPNLVT DEAEASKFVE EYDRTSQVVW NEYAEANWNY NTNITTETSK ILLQKNMQIA
121    NHTLKYGTQA RKFDVNQLQN TTIKRIKKV QDLERAALPA QELEEYNKIL LDMETTSVA
181    TVCHPNGSCL QLEPDLTNVM ATSRKYEDLL WAWEGWRDKA GRAILQFYPK YVELINQAAR
241    LNGYVDAGDS WRSMYETPSL EQDLERLFQE LQPLYLNLHA YVRRALHRHY GAQHINLEGP
301    IPAHLGNNMW AQTWSNIYDL VVPFAPSAPM DTTEAMLKQG WTPRRMFKEA DDFFTSLGLL
361    PVPPEFWNKS MLEKPTDGRE VVCHASAWDF YNGKDFRIKQ CTTVNLEDLV VAHHEMGIHQ
421    YFMQYKDLPV ALREGANPGF HEAIGDVLAL SVSTPKHLHS LNLSSSEGS DEHDINFLMK
481    MALDKIAFIP FSYLVDQWRW RVFDGSITKE NYNQEWWSLR LKYQGLCPPV PRTQGDFFDPG
541    AKFHIPSSVP YIRYFVSFII QFQFHEALCQ AAGHTGPLHK CDIYQSKEAG QRLATAMKLG
601    FSRPWPEAMQ LITGQPNMSA SAMLSYFKPL LDWLRTENEL HGEKLGWPQY NWTNPNSARSE
661    GPLPDSGRVS FLGLDLDAQQ ARVGQWLLLF LGIALLVATL GLSQRFLSIR HRSRHRHSHG
721    PQFGSEVELR HS

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Figure 1.1. Primary sequences of human sACE, N domain and tACE.

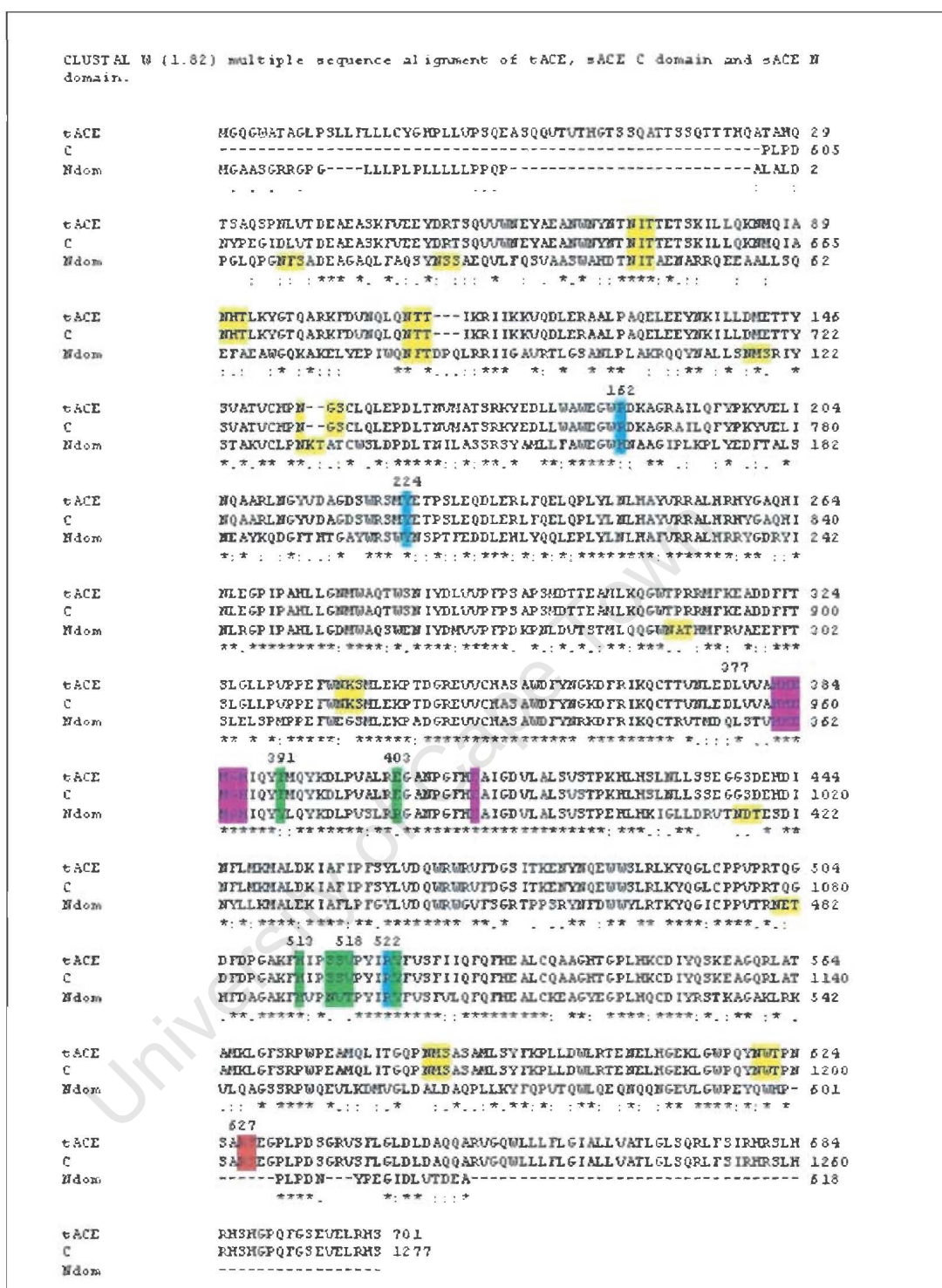


Figure 1.2. Sequence alignment of tACE, C domain and N domain showing N-linked glycosylation sequons (yellow); chloride-binding residues (pale blue); zinc-coordinating motif (purple); other subsites and catalytic residues (light green) and secretase cleavage site (red). Numbers above certain key residues are according to tACE numbering.

Appendix 2: Materials and Methods

2.1. Synthetic tripeptide substrates: Hip-His-Leu and Z-Phe-His-Leu

2.1.1. Buffers and solutions

2.1.1.1. Potassium Phosphate buffer, pH 8.3 containing 300 mM NaCl.

A 5x stock was prepared: 0.5 M KH_2PO_4 ; 0.5 M K_2HPO_4 , pH 8.3 and 1.5 M NaCl.

68.5 g KH_2PO_4 ($M_w=136.99$); 87.1 g K_2HPO_4 ($M_w=174.18$) and 87.66 g NaCl ($M_w=58.44$) in 1L of distilled water. Adjusted pH to 8.3 with NaOH.

Working solution: 1 x buffer.

2.1.1.2. 3N HCl

32% HCl=10N

150 ml 32% HCl made up to 500 ml with distilled water and autoclaved.

2.1.1.3. 1M NaOH

20 g NaOH ($M_w=40$) made up to 500 ml with distilled water and autoclaved.

2.1.1.4. 0.28 N NaOH

140 ml 1.0 M NaOH in 500 ml distilled water and autoclaved.

2.1.1.5. 0.025M NaOH

12.5 ml 1M NaOH made up to 500 ml with distilled water and autoclaved.

2.1.1.6. *o*-phthaldialdehyde (150 mM)

20 mg *o*-phthaldialdehyde ($M_w=134.1$)(Sigma) in 1.0 ml methanol.

2.1.1.7. 5.7 mM Hippuryl-His-Leu

48.5 mg N-Hippuryl-His-Leu tetrahydrate ($M_w = 501.5$) (Sigma) dissolved in 4.165 ml heated 0.025 M NaOH. Then added:
4.0 ml 5 x Potassium Phosphate buffer, pH 8.3 (without NaCl).
2.0 ml 3M NaCl.
9.835 ml sterile distilled water.

2.1.1.8. 20 mM Z-Phe-His-Leu stock:

110 mg Z-Phe-His-Leu-OH (Bachem) ($M_w = 549.63$) dissolved in 1.0 ml 0.28 N NaOH. Then added:
9.0 ml sterile distilled water in a drop-wise fashion to prevent precipitation.

Aliquoted into 1.0 ml Eppendorf tubes and stored aliquots at -20°C until use.

2.1.1.9. 1.0 mM Z-Phe-His-Leu working solution:

4.0 ml 5 x Potassium Phosphate buffer, pH 8.3 containing 150 mM NaCl (2.1.1.1).
15.0 ml sterile distilled water
20.0 μl 10 mM ZnSO_4 and then added:
1.0 ml 20 mM Z-Phe-His-Leu aliquot.

2.1.1.10. Z-Phe-His-Leu substrate concentration series (for kinetic studies)**8.0 mM stock solution:**

12.0 ml 5 x Potassium Phosphate buffer, pH 8.3 containing 150 mM NaCl (2.1.1.1).
20.0 μl 10 mM ZnSO_4 and then added:
8.0 ml 20 mM Z-Phe-His-Leu stock

8.0 mM solution diluted out in 1 x buffer to generate a range of concentrations: 0-2.5 mM.

2.1.2. His-Leu standard curve**2.1.2.1. His-Leu (HL) standards**

Stock solution of His-Leu ($M_w = 268.3$) (Sigma):

10 mg in 10 ml sterile distilled water = 3.7 nmoles/ μl His-Leu.
Standards: Prepared in 5.0 ml 1 x Potassium Phosphate buffer, pH 8.3 without added NaCl.
nmole/ μl HL: 0; 0.037; 0.074; 0.148; 0.296; 0.592.

35 μ l contains: 0; 1.3; 2.59; 5.18; 10.36 and 20.72 nmoles His-Leu.

35 μ l standard per well of a 96-well fluorometric plate

To this was added:

180 μ l 0.28 N NaOH and mixed

12.0 μ l 150 mM o-phthalaldehyde and mixed

26.0 μ l 3N HCL and mixed

Fluorescence measured at $\lambda_{Ex}=360$ and $\lambda_{Em}=486$ nm on a Cary Eclipse fluorometric plate reader (Varian).

A standard curve of fluorescent units vs nmoles HL was plotted using GraphPad Prism 4.01. Curve was linear to 20.72 nmoles HL. Slope was determined via linear regression analysis.

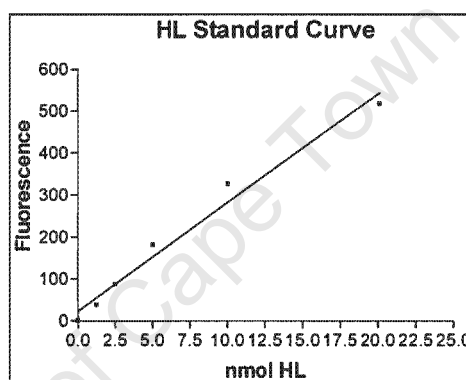


Figure 2.1. A His-Leu standard curve

2.1.2.2. Enzyme activity in culture media/cell lysates

Using 5.7 mM HHL or 1.0 mM Z-FHL.

Enzyme activity (nmol/min) = Fluorescent units/HL standard curve slope/incubation time.

1nmol/min = 1 mU.

Expressed in mU/ml by dividing out volume of assayed medium or cell lysate.

2.1.2.3. k_{cat} , K_m and k_{cat}/K_m values:

From a non-linear regression analysis of a Michaelis-Menten plot (GraphPad Prism 4.01):

K_m in mM.

V_{max} (nmoles/min) = χ fluorescent units/HL standard curve slope/incubation period.

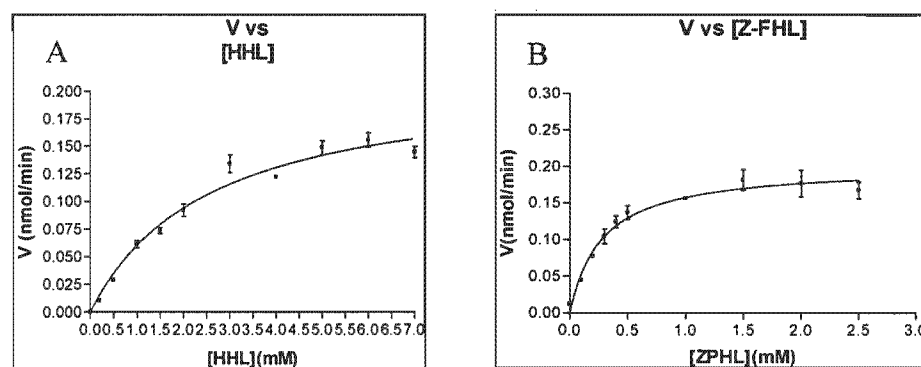


Figure 2.2. Michaelis-Menten plots for the hydrolysis of HHL (A) and Z-FHL(B).

$$k_{\text{cat}} (\text{min}^{-1}) = V_{\text{max}} / \text{nmoles added enzyme}$$

$$k_{\text{cat}} (\text{sec}^{-1}) = k_{\text{cat}} (\text{min}^{-1}) / 60$$

$$k_{\text{cat}} / K_m = k_{\text{cat}} (\text{sec}^{-1}) / K_m$$

2.2. Fluorogenic peptide substrates

2.2.1. 2 x Reaction buffer

0.2 M Tris-HCl, pH 7.0

100 mM NaCl

20 μM ZnCl_2

In 250.0 ml distilled water:

7.88 g Tris-HCl ($M_w=157.56$)

25.0 ml of 1 M NaCl

50.0 μl of 0.1 M ZnCl_2

adjusted pH to 7.0 and autoclaved.

Diluted 50% to 1 x Reaction buffer in sterile distilled water.

2.2.2. Preparation of fluorogenic peptide substrates

These were supplied as solids and diluted in a suitable volume of dimethyl sulfoxide (DMSO). Based upon an $E_{365}=17300\text{M}^{-1}\text{cm}^{-1}$, the A_{365} of stock solutions were determined and concentrations calculated using the Beer-Lambert equation: $A_{365}=E_{365} \cdot c \cdot l$; where c =concentration (M) and l =pathlength (cm). Concentrations were typically 1000-3000 μM . Dilutions to 200 μM and 20 μM working solutions were made in 1 x Reaction buffer.

2.2.3. Fluorogenic peptide assays

Assays were conducted in a total volume of 2.5 ml and included 1.25 ml 2 x Reaction buffer, sterile distilled water, enzyme and substrate from a 20 or 200 μM working solution. Reactions were prepared in 10 mm open top fluorescence cells (Varian) with mixing via magnetic stir-stirrers. Reactions were pre-heated to 37°C prior to enzyme addition. Fluorescence was monitored continuously at 37°C, $\lambda_{\text{Ex}}=320$, $\lambda_{\text{Em}}=420$ nm using a Cary Eclipse fluorometric cuvette reader (Varian). Initially, calibration curves for each substrate were generated via total hydrolysis of 0-1.0 μM (0-2500 pmoles) substrate. Baseline fluorescence (background fluorescence prior to enzyme addition) was subtracted from peak plateau fluorescence (where fluorescence no longer increases) to generate a ΔF value for each substrate concentration.

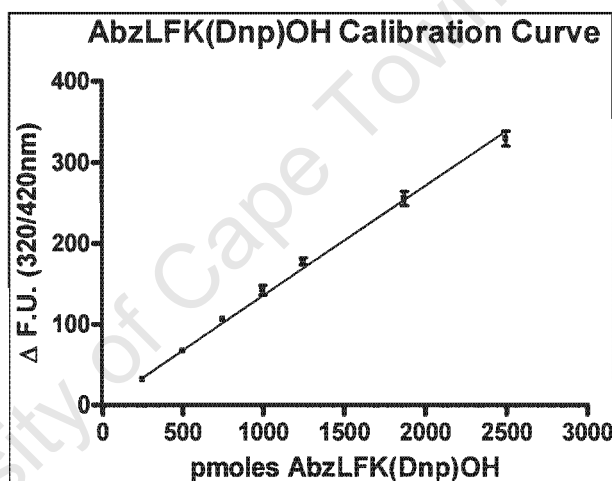


Figure 2.3. AbzLFK(Dnp)-OH calibration curve

Calibration curve slopes were determined via linear regression analysis (GraphPad Prism 4.01).

Kinetic assays were conducted in a similar manner over a 0-8.0 μM substrate concentration range. Initial rate conditions at 37°C were determined by incubating the lowest substrate concentration (0.5 μM) with varying amounts of enzyme and monitored over time. % substrate hydrolysis was calculated for each condition and using the calibration curve. $\Delta F = \chi$ pmoles substrate from curve.

% hydrolysis = pmoles of hydrolysed substrate/ total pmoles substrate present in reaction x 100.

Once initial rate conditions were established, where % hydrolysis < 10% for the lowest substrate concentration, identical conditions were employed for all subsequent reactions and substrate concentrations. ΔF values were determined for each substrate concentration.

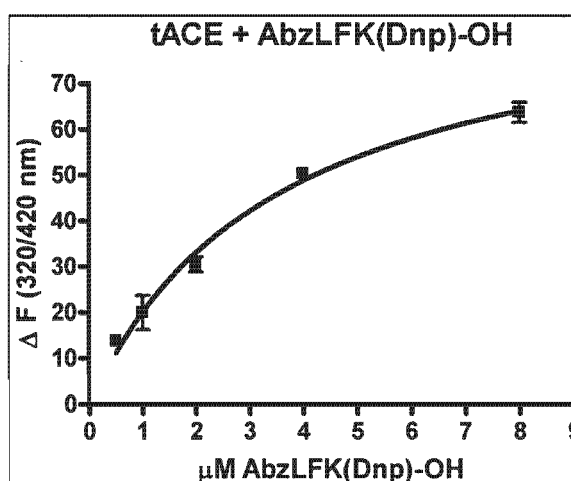


Figure 2.4. Michaelis-Menten plot for the hydrolysis of AbzLFK(Dnp)-OH

Kinetic constants, $V_{\max}$ and K_m were determined via non-linear regression analysis of Michaelis-Menten plots (GraphPad Prism 4.01). k_{cat} and k_{cat}/K_m constants were determined as described previously for HHL and Z-FHL.

2.3. Inhibition assays

2.3.1. Preparation of inhibitor stock solutions

Lisinopril Dihydrate (Zeneca Pharmaceuticals, U.K.) and its analogues, compounds **13** and **14** were dissolved in sterile distilled water. Keto-ACE and its derivatives, compounds **2-12** were dissolved in DMSO. The molecular weights of the compounds were as follows:

Compound	M_w (g.mol^{-1})	Compound	M_w (g.mol^{-1})
Lisinopril	442	8	507
2	423	9	576
3	526	10	576
4	526	11	615
5	472	12	615
6	468	13	494
7	511	14	494

2.3.2. Determination of inhibition constants IC_{50} and K_i

IC_{50} values were calculated from sigmoidal dose response curves and K_i values via linear regression analysis of Dixon plots (GraphPad Prism 4.01).

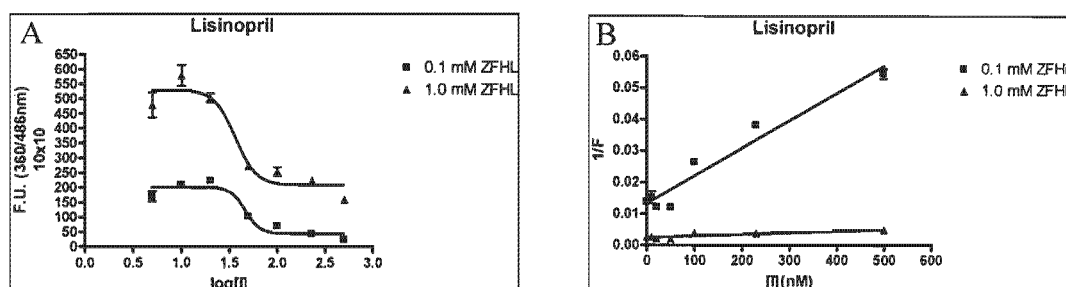


Figure 2.5. Sigmoidal dose response curve (A) and Dixon plot (B) showing tACE inhibition by lisinopril.

For the determination of K_i :

From linear regression of two straight lines, K_i = negative χ coordinate corresponding to the point of intersection of the straight lines, where:

$m\chi + c = m'\chi + c'$ (m =gradient; χ =abscissal coordinate; c =y-intercept). $-\chi = K_i$.

2.4. DNA cloning

2.4.1. Restriction digestion

1.0 μ g DNA was digested with 10 U of restriction enzyme/s in 1 x reaction buffer at 37°C for 60 minutes, in a waterbath. Reactions (20.0 μ l) were stopped with 2.0 μ l chilled 6 x loading dye on ice. 20.0 μ l of each reaction was subjected to agarose gel electrophoresis.

2.4.2. Agarose gel electrophoresis

2.4.2.1. 0.5 M EDTA

14.6 g EDTA (M_w = 292.24) in 100 ml distilled water. Adjusted pH to 8.0 and autoclaved.

2.4.2.2. Tris borate EDTA (TBE) buffer

In 1L sterile distilled water:

108 g Tris (M_w = 121.1)
 55 g Boric acid (M_w = 61.83)
 40 ml 0.5 M EDTA, pH 8.0
 Autoclaved.

100 ml 10xTBE + 900 ml distilled water. To this is added:
60 µL 5mg/ml ethidium bromide to a final concentration of 0.3 µg/ml.

2.4.2.3. 6 x loading dye

0.025g Bromophenol Blue
0.025 g Xylene Cyanol
6.0 ml 50 % glycerol
4.0 ml sterile distilled water.
Aliquoted into sterile 1.0 ml Eppendorf tubes.

2.4.2.4. HindIII-EcoRI λ DNA molecular weight marker

40.0 µl λ DNA (Promega)
5.0 µl *EcoRI* (10 U/µl)
5.0 µl *Hind III* (10 U/µl)
16.0 µl 10 x buffer B (Promega)
94.0 µl nuclease-free water (Promega)
Incubated at 37°C for 2 hours and stopped with 16.0 µl 6 x loading dye.

2.4.2.5. 1% agarose gel

0.4 g agarose (low melting temperature molecular grade)/40.0 ml 1xTBE (containing 0.3 µg/ml ethidium bromide).
DNA samples were electrophoresed at 60-80V with 1 x TBE as a running buffer and visualised using a ChemiGenius Bio Imaging System (Syngene).

2.4.3. Fragment excision and purification

DNA bands were excised from agarose gels under U.V. transillumination using sterile scalpel blades. DNA was purified using the Wizard SV Gel kit (Promega), as per manufacturer's instructions. Purified DNA, resuspended in nuclease-free water, was subjected to agarose gel electrophoresis and yields (ng/µl) calculated.

2.4.4. Ligation

Quantities of insert required for ligation into vector were calculated as follows for an insert/vector ratio of 1:1; 3:1 and 10:1.

$$\frac{\text{ng vector} \times \text{kb insert}}{\text{kb vector}} \times \text{I/V} = \text{ng insert}$$

I=insert
V=vector

2.4.4.1. Ligation reactions

In a 20.0 µl reaction:

χ µl DNA insert
χ µl vector
2.0 µl 10 x reaction buffer (Promega)
1.0 (3U/µl) T4 DNA Ligase (Promega)

Ligation reactions were carried out at 4°C or 25°C.

2.4.5. Transformation of competent *E.coli*

2.4.5.1. Luria broth

In 1L distilled water : 10 g bactotryptone
5 g yeast extract
10 g NaCl.

Autoclaved in 1L flasks, 500 ml flasks and 5 ml bottles.

Ampicillin (100 mg/ml) was added to a final concentration of 100 µg/ml prior to use where necessary.

2.4.5.2. Luria agar

In 500 ml luria broth: 6 g agar.

Cooled to 50°C, added ampicillin (100 mg/ml) to a final concentration of 100 µg/ml and poured into Petri dishes.

2.4.5.3. Generation of competent *E.coli* cells

1M RbCl₂

6.05 g RbCl₂ (M_w =120.94) in 50.0 ml sterile distilled water.

750 mM CaCl₂

5.51 g CaCl₂.2H₂O (M_w= 147.02) in 50.0 ml sterile distilled water.

100 mM MOPS, pH 7.0

1.05 g MOPS (3-[N-Morpholino]propanesulfonic acid]) (M_w=209.3) in 50.0 ml sterile distilled water. Adjusted pH to 7.0.

50 ml Transformation Buffer 1 (TFB1)

5.0 ml 1M RbCl₂

0.495 g MnCl₂·4H₂O (M_w=197.9)

0.147 g KOAc (M_w=98.15)

0.67 ml 750 mM CaCl₂

15.0 ml 50 % glycerol

pH adjusted carefully to 5.8 with glacial acetic acid. Filter sterilised using a 0.2 micron filter.

50.0 ml Transformation Buffer 2 (TFB2)

5.0 ml 100 mM MOPS, pH 7.0

0.5 ml 1M RbCl₂

5.0 ml 750 mM CaCl₂

15.0 ml 50% glycerol

Filter sterilised using a 0.2 micron filter.

A single colony was inoculated into 5.0 ml Luria broth and incubated for 16 hours at 37°C in a shaking incubator at 200 rpm. This starter culture was inoculated into 100 ml Luria broth and incubated at 37°C, 200 rpm until an O.D._{550nm} of 0.35 was reached.

The culture was transferred into two sterile 50.0 ml centrifuge tubes and chilled on ice for 15 minutes followed by centrifugation at 2500 rpm for 5 minutes at 4°C. The supernatant was decanted and pellet resuspended in 21.0 ml cold **TFB1** by gentle vortexing. The suspensions were pooled and incubated for 90 minutes on ice following which they were centrifuged at 2500 rpm for 5 minutes at 4°C. The supernatant was decanted and the pellet resuspended in 3.5 ml cold **TFB2**. Suspensions were aliquoted into 1.0 ml Eppendorf tubes, flash-frozen in liquid nitrogen and stored at -70°C until use.

2.4.5.4. Transformation of competent *E.coli*

Sterile Falcon 2059 tubes were pre-chilled on ice following which 100 µl of competent *E.coli* cells was added. <10 µl DNA was added to the cells and they were mixed by gentle flicking. Cells were incubated on ice for 30 minutes following which they were heat shocked at exactly 42°C for 45 seconds. Cells were placed on ice for 2 minutes and 900 µl pre-warmed (37°C) Luria broth was added. Tubes were incubated at 37°C for 1-2 hours, transferred to a 1.0 ml sterile Eppendorf tube and centrifuged for 1 minute. Supernatant to 100 µl was removed and cells resuspended gently by flicking of the tube. 100 µl suspension was added to an agar plate and spread. Plates were incubated for 16-24 hours at 37°C and colonies picked.

2.4.6. Isolation of plasmid DNA

For screening purposes (Mini-DNA)

Picked colonies were inoculated into 5.0 ml Luria broth containing 100 µg/ml ampicillin and incubated at 37°C for 16 hours in a shaking incubator at 200 rpm. Cultures were transferred to sterile 1.0 ml Eppendorf tubes and centrifuged for 2 minutes at high speed. Supernatants were decanted and pellets resuspended in 70 µl **smart buffer**:

10 mM Tris, pH 8.0
1 mM EDTA
15% sucrose
100 µg/ml BSA
200 µg/ml RNase A
2 mg/ml lysozyme.

Cells were incubated with buffer in a 37°C waterbath for 30 minutes. Enzymes were inactivated by boiling of tubes for 90 seconds. Tubes were placed on ice for 10 minutes and then centrifuged for 5 minutes in a microfuge at high speed. The DNA (typically 10.0 µl) was ready for agarose gel electrophoresis and restriction digests.

Isolation of plasmid DNA for subcloning, sequencing and transfections (Midi-DNA)

5.0 ml of remaining culture was inoculated into 50.0 ml Luria broth containing 100 µg/ml ampicillin and incubated for 16-24 hours at 37°C in a shaking incubator at 200 rpm. DNA was isolated and purified using a QIAGEN Plasmid Midi Kit and as per manufacturer's instructions. Yields were typically 1.0 µg/µl.

2.5. Site-directed mutagenesis of N domain *N*-linked glycosylation sites

2.5.1. Primers

Site-directed mutagenesis primers were designed in which codons encoding Asn-residues were mutated to those encoding Gln-residues at N domain *N*-linked glycosylation sites 9, 25, 45, 82, 131, 289, 416 and 480. The primers were 25-45 bases in length, with a T_m greater than or equal to 78°C, a minimum GC content of 40% with one or more G or C at both 5' and 3' ends. Restriction sites, engineered via silent mutation, were introduced into the primer sequences, enabling screening of positive clones following transformation. Primers were designed using WatCut (Michael Palmer, University of Waterloo, Canada. <http://watcut.uwaterloo.ca/watcut>) and synthesised by MWG Biotech and Metabion, GmbH

2.5.2.Dpn I method

Primers, complimentary to opposite strands of the vector, are extended using a high fidelity *Pfu* polymerase. Primer incorporation generates mutated plasmid with staggered nicks. Dpn I, specific for methylated DNA is used to digest methylated parental DNA template. Nicked vector, incorporating the desired mutations is transformed into competent *E.coli*.

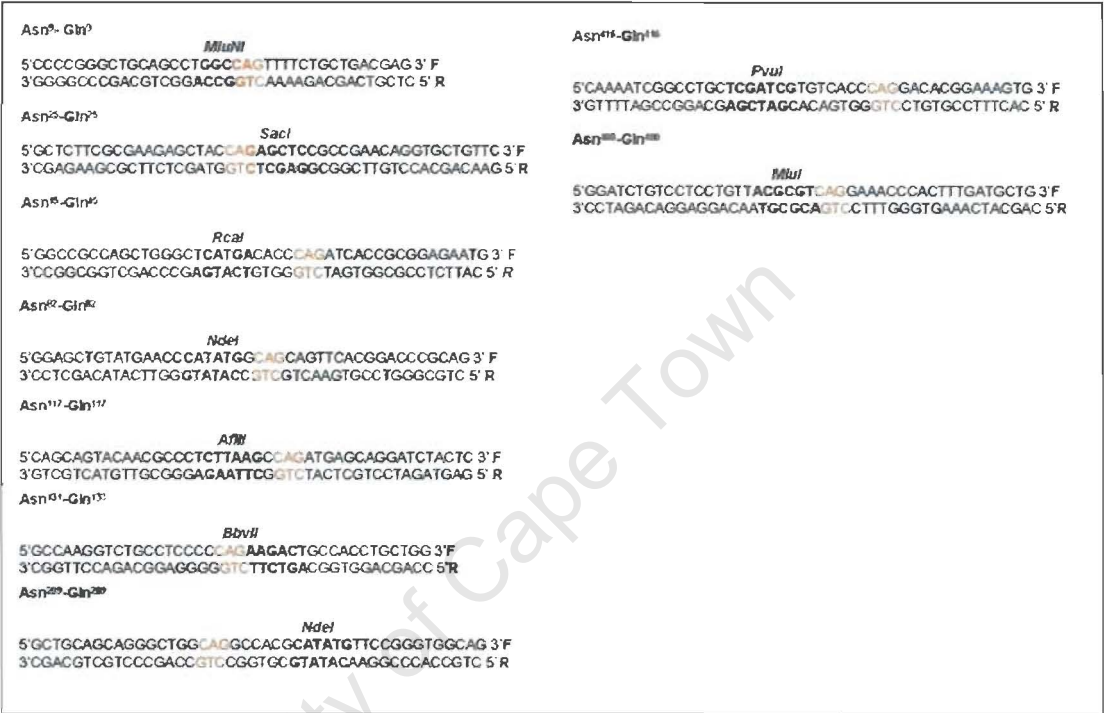


Figure 2.6. Site-directed mutagenesis primers. Codons highlighted in yellow encode mutant Gln. Bases in bold are restriction enzyme recognition sequences. Restriction enzymes are indicated in italics above each primer pair.

2.6. Tissue culture

2.6.1. Media and buffers

2.6.1.1 10% Foetal Calf Serum (FCS) medium

- 50 ml contains:
- 21.5 ml Dulbecco’s Modified Eagles Medium (DMEM)(Gibco-BRL)
- 21.5 ml HAMS-F12 (Gibco-BRL)
- 5.0 ml of FCS heat inactivated at 56°C for 30 minutes (Gibco-BRL)
- 1.0 ml 1M HEPES, pH 7.5
- Filter sterilised using a 0.2 micron filter.

2.6.1.2. 30% Foetal Calf Serum (FCS) medium

- 10 ml contains:
- 3.15 ml Dulbecco’s Modified Eagles Medium (DMEM)(Gibco BRL)
- 3.15 ml HAMS-F12 (Gibco-BRL)
- 3.33 ml FCS heat inactivated at 56°C for 30 minutes (Gibco-BRL)
- 200.0 µl 1M HEPES, pH 7.5

2.6.1.3. 1 M HEPES, pH 7.5

23.83 g HEPES ($M_w = 238.31$) in 100.0 ml distilled water.
Adjusted pH to 7.5 and autoclaved.

2.7. SDS-Polyacrylamide gel electrophoresis**2.7.1. Buffers and solutions****2.7.1.1. Sample buffer**

0.0625 M Tris-HCl, pH 6.8
2% SDS
10% glycerol
5% mercaptoethanol
0.001% bromophenol blue

2.7.1.2. Acrylamide

40% Acrylamide
1.1% Bis-acrylamide

2.7.1.3. Stacking gel buffer

0.375 M Tris-HCl, pH 6.8
0.3% SDS

2.7.1.4. Running gel buffer

1.125 M Tris-HCl, pH 8.8
0.3 % SDS

2.7.1.5. 10 x Running buffer

0.25M Tris-HCl, pH 8.3
1.92 M Glycine
1% SDS

2.7.1.6. Staining solution

0.25% Coomassie brilliant blue
50% methanol
10% acetic acid.

2.7.1.7. Destaining solution

25% ethanol
10% acetic acid.

2.8. Western blotting

2.8.1. Buffers and solutions

2.8.1.1. Transfer buffer

In 1L:
3.025 g Tris
14.4 g glycine
200.0 ml methanol

2.8.1.2. Blocking buffer

In 1L:
5% skim milk
0.1% Tween-20
0.2 M NaCl
0.05 M Tris-HCl, pH 7.4.

2.8.1.3. Wash buffer

Same composition as blocking buffer except with no skim milk added.

2.8.2. Western blotting method

Transfer was allowed to take place at 100V, 300 mA for 60 minutes. The blot was removed and blocked for 60 minutes in blocking buffer following which the blot was incubated for 90 minutes with rabbit anti-human ACE C-domain polyclonal antibody (1:1000) diluted in blocking buffer. The blot was washed several times in wash buffer and then incubated for 60 minutes with goat anti-rabbit horseradish peroxidase-conjugated IgG (1:1000) diluted in wash buffer. Blots were detected using the LumiGLO Reserve chemiluminescent substrate (KPL) as per manufacturer's instructions. Blots were visualised on a ChemiGeniusQ Bio Imaging System (Syngene).

2.9. Lisinopril affinity chromatography

2.9.1. Buffers and solutions

2.9.1.1. Washing buffer

0.5 M NaCl; 20 mM HEPES, pH 7.5

In 1L distilled water:

250 ml 2M NaCl

20 ml 1M HEPES, pH 7.5

2.9.1.2. Elution buffer

50 mM Borate, pH 9.5

500 mM stock solution: 15.46 g boric acid in 500 ml distilled water

50 mM working solution with pH adjusted to 9.5

2.9.2. Methodology

Pooled bulk culture medium (~500 ml) was run over a lisinopril-sepharose column at a flow rate of 0.5ml/minute. The column was washed overnight with 600-800 ml washing buffer. Protein was eluted with 50 mM Borate pH 9.5 and 2.0 ml fractions collected. Protein fractions were monitored at $\lambda=280\text{nm}$. 10.0 μl of peak fractions were diluted to 1.0 ml with distilled water and 10.0-15.0 μl assayed for enzyme activity using HHL and as follows:

In a chilled 1.0 ml Eppendorf tube was added 10.0-15.0 μl diluted enzyme. 120.0 μl of chilled 5.7 mM HHL was added to the sample/s on ice and tubes mixed by inversion. Substrate incubations were undertaken in a 37°C waterbath for 15 minutes and reactions were stopped by adding 725.0 μl 0.28N NaOH. To the mixed reactions was added 50.0 μl 150 mM o-phthaldialdehyde. Tubes were mixed by inversion and incubated at room temperature for 10 minutes. Reactions were stopped by adding 100.0 μl 3N HCL and mixed. 250.0 μl of the stopped reaction was transferred per well of a 96-well fluorometric plate and fluorescence measured at $\lambda_{\text{Ex}}=360/\lambda_{\text{Em}}=486\text{nm}$ on a Cary Eclipse fluorometric plate reader (Varian). As a control, a BZT (blank zero time) reaction was performed. Here, 0.28N NaOH was added to the sample prior to incubation at 37°C.

The BZT value was subtracted from the fluorescence value of the sample and enzyme activity (expressed in mU/ml) was calculated as described previously using a His-Leu standard curve.

The peak fractions were combined and dialysed against 3 changes of 5 mM HEPES, pH 7.5 at 4°C for 16 hours. Dialysed samples were assayed for enzyme activity using HHL as described previously.

Samples were concentrated using Ultrafree-0.5 centrifugal filters with a Biomax-30K NMWL membrane and as per manufacturer's instructions. Protein concentration of concentrated samples was determined according to the Bradford method.

Concentrated protein samples were subjected to SDS-PAGE. 10.0 μ l sample was mixed with 10.0 μ l 2 x sample buffer and boiled. 10.0 μ l was loaded and run on a 3% stacking, 10% running gel at 25mA. Gels were stained, destained and visualised.

2.10. Bradford Assay

Protein sample was made up to 800 μ l with sterile distilled water and then 200 μ l Bradford reagent (Biorad) was added and incubated at ambient temperature for 5 minutes. Absorbance measured at 595 nm and protein concentration calculated from a an IgG standard curve. A concentration range of 0; 1; 2; 3; 4; 5; 10 and 20 μ g/ml was generated from each stock solution in distilled water. To 800.0 μ l of each standard was added 200.0 μ l Bradford reagent (Biorad). Following a 5 minute incubation, the A_{595} of each standard concentration was measured and a standard curve was plotted.

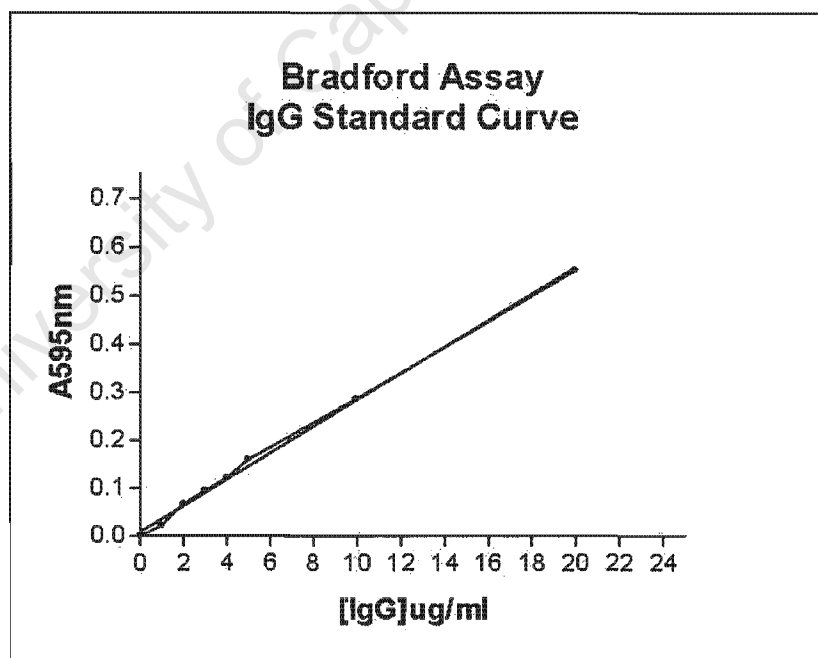


Figure 2.7. An IgG standard curve. Curve slope was determined by linear regression analysis.

Appendix 3: Abbreviations

Abz: ortho-amino benzoic acid
ACE: angiotensin I-converting enzyme
ACE 2: angiotensin I-converting enzyme 2
AcSDKP: *N*-acetyl-Ser-Asp-Lys-Pro
AD: Alzheimer's Disease
AMP: adenosine monophosphate
Ang I: angiotensin I
Ang II: angiotensin II
Ang: angiotensin
AT-1: angiotensin type I receptor
AT-2: angiotensin type II receptor
AT-4: angiotensin type IV receptor
B₁: type I kinin receptor
B₂: type II kinin receptor
BchE: butylcholinesterase
BK: bradykinin
BPP: bradykinin-potentiating peptide
BSA: bovine serum albumin
BZT: blank zero time
cAMP: cyclic AMP
CHO: Chinese hamster ovary
CKII: casein kinase II
COX-2: cyclooxygenase II
D: deletion polymorphism
DMSO: dimethylsulfoxide
dNTP: deoxynucleoside triphosphate
EDDnp: 2,4-dinitrophenyl ethylene diamine
EI: enzyme-inhibitor complex
ER: endoplasmic reticulum
FAPGG: furylacryloyl-phenylalanyl-glycyl-glycine
FCS: foetal calf serum
FRET: fluorescence resonance energy transfer
GlcNAc: N-acetylglucosamine
GPI: glycosylphosphatidylinositol
GS: glutamine synthetase
HHL: hippuryl-*L*-histidyl-*L*-leucine
HMWK: high molecular weight kininogen
I: insertion polymorphism
IC₅₀: inhibitor concentration reducing V_{max} by 50%.
JNK: c-Jun N-terminal kinase
K(Dnp): 2,4-dinitrophenyl-lysine
 k_{cat}/K_m : specificity constant
 k_{cat} : turnover number
 K_i : inhibition constant
KKS: Kallikrein-Kinin System
 K_m : Michaelis-Menten constant
LHRH: luteinizing hormone releasing hormone
LMWK: low molecular weight kininogen

MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MSX: methionine sulfoxamine
NBDNJ: *N*-butyl deoxynojirimycin
NO: nitric oxide
OD: optical density
OST: oligosaccharyltransferase complex
P: substrate/inhibitor functionality
PAGE: polyacrylamide gel electrophoresis
PG: prostaglandin
PGE₂: prostaglandin E₂
pI: isoelectric point
PKC: protein kinase C
PMA: phorbol myristate acetate
PMSF: phenylmethylsulfonylfluoride
RAS: Renin-Angiotensin System
RMSD: root mean squared deviation
S: enzyme subsite
sACE: somatic angiotensin I-converting enzyme
SARS: severe acute respiratory syndrome
SDS: sodium dodecyl sulphate
SEM: standard error of the mean
SP: substance P
tACE: testis angiotensin I-converting enzyme
V_{max}: maximal velocity
Z-FHL: z-phenylalanyl-*L*-histidyl-*L*-leucine

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