CMLS, Cell. Mol. Life Sci. 61 (2004) 2704–2713
1420-682X/04/212704-10
DOI 10.1007/s00018-004-4240-7

CMLS Cellular and Molecular Life Sciences

Angiotensin-converting enzyme-2: a molecular and cellular perspective

F. J. Warner [a, b, *], A. I. Smith [b], N. M. Hooper [a] and A. J. Turner [a]

[a] School of Biochemistry and Microbiology, University of Leeds, Leeds LS2 9JT (United Kingdom)
[b] Baker Heart Research Institute, St Kilda Rd Central, P.O. 6492, Melbourne, Victoria 8008 (Australia),
Fax: +61 (0)3 85321100, e-mail: fiona.warner@med.monash.edu.au

Abstract. Angiotensin-converting enzyme-2 (ACE2) is the first human homologue of ACE to be described. ACE2 is a type I integral membrane protein which functions as a carboxypeptidase, cleaving a single hydrophobic/basic residue from the C-terminus of its substrates. ACE2 efficiently hydrolyses the potent vasoconstrictor angiotensin II to angiotensin (1–7). It is a consequence of this action that ACE2 participates in the renin-angiotensin system. However, ACE2 also hydrolyses dynorphin A (1–13), apelin-13 and des-Arg^9 bradykinin. The role of ACE2 in these peptide systems has yet to be revealed. A physiological role for ACE2 has been implicated in hypertension, cardiac function, heart function and diabetes, and as a receptor of the severe acute respiratory syndrome coronavirus. This paper reviews the biochemistry of ACE2 and discusses key findings such as the elucidation of crystal structures for ACE2 and testicular ACE and the development of ACE2 inhibitors that have now provided a basis for future research on this enzyme.

Key words. Angiotensin converting enzyme; ACE2; carboxypeptidase; peptidase; renin-angiotensin system; structure.

Introduction

Over 60 years have passed since the renin-angiotensin system (RAS) was initially described [1]. Since that time, the RAS has become one of the best-characterized hormonal systems, reflecting its central role in the control of blood pressure. Within the RAS, angiotensin II (Ang II) is considered the primary physiological product, and the basic pathway leading to Ang II formation has remained unchanged in theory. The RAS cascade is inaugurated with the hydrolysis of the precursor angiotensinogen to angiotensin I (Ang I) by the aspartic protease, renin. Ang I is, in turn, converted to Ang II principally by the zinc metallopeptidase angiotensin-converting enzyme (ACE), although in the heart the serine protease chymase also appears to play a role (fig. 1) [2]. Throughout the past decade, the formation and function of other angiotensin fragments, such as Ang (1–7), Ang (1–9), Ang III [Ang (2–8)] and Ang IV [Ang (3–8)] have been investigated. In particular, the role of Ang (1–7) has been of greatest impact, since this fragment mediates opposing effects to Ang II, thus providing a novel channel of regulation within the RAS [3] (fig. 1). Ang (1–7) acts through its own specific G-protein-coupled receptor, Mas [4].

ACE has been identified as a fundamental regulator of the RAS in humans and is an important target in regulation of blood pressure homeostasis. Classical ACE inhibitors, such as captopril and lisinopril, have proven to be effective anti-hypertensive therapeutics [5]. In 2000, a human homologue of ACE was discovered independently by two groups using differing genomic-based strategies and was referred to as ACEH [6] or ACE2 [7]. The ability of ACE2 to produce Ang (1–7) via two alternative pathways, in concert with ACE (fig. 1), has led to its increasing therapeutic relevance and interest. Most recently, ACE2 has been implicated in cardiac dysfunction, hypertension [8, 9], heart failure and ventricular remodelling [10, 11], diabetes [12], Ang (1–7) regulation during pregnancy [13] and as a functional receptor to the coronavirus that cause severe acute respiratory syndrome (SARS) [14]. The foundation for future research into the biochemistry and physiological role of this enzyme has been provided both by the development of specific ACE2 in-

* Corresponding author.

hibitors [15, 16] and the elucidation of crystal structures for ACE2 [17], testicular ACE (tACE) [18] and its *Drosophila* orthologue, AnCE [19].

Structure of the ACE2 gene

Orthologues of human ACE2 have been identified in both the rat and mouse genomes and display ~86 and 82% identity, respectively, to the nucleotide sequence of human ACE2. The human ACE2 gene consists of 18 exons, with the first 12 exons of ACE2 being of similar size to the first 11 exons of the ACE gene [6, 7] (fig. 2). In ACE2 there is a union of exons 5 and 6, which jointly correspond to exon 5 of ACE (fig. 2); the zinc-binding motif (HEXXH) of ACE2 is located within exon 9, compared to exon 8 of the ACE gene. A significant difference between ACE2 and ACE is that only a single ACE2 protein species appears to be formed. The mammalian ACE gene gives rise to two isoforms, somatic ACE (sACE) with two catalytic domains (N- and C-domain) and tACE, which has only a single catalytic domain and resembles the C-domain of sACE (fig. 2). The initiation sites of the transcripts for sACE and tACE occur in different regions of the ACE gene and are under the control of alternate, tissue-specific promoters [20]. The regulatory elements for the ACE2 gene have thus far not been described.

The ACE2 gene maps to the X chromosome in humans (Xp22) [6,7], rodents (Xq32) [8] and mice (XF5) [21]. In three different rat models of either spontaneous or salt-induced hypertension, the rat gene was mapped to a defined quantitative trait locus (QTL) on the X chromosome. In these hypertensive rat models, a reduction in ACE2 gene and protein expression was observed, suggesting that ACE2 is a strong candidate for a hypertensive QTL on the X chromosome [8]. Furthermore, two single-nucleotide polymorphisms have been identified in the ACE2 locus and were associated with a number of cardiovascular endpoints in Caucasian subjects [22].

The strong association of the ACE2 gene with hypertension is further strengthened by the tissue-specific expression of the ACE2 messenger RNA (mRNA) at high levels as a single 4.3-kb transcript in kidney and heart [6, 7]. Immunohistochemical studies of heart sections have localised ACE2 to the endothelium of ventricular intramyocardial vessels and occasionally over the smooth muscle and adventitia of larger blood vessels. Surprisingly, no difference in immunoreactive staining was observed between sections of non-failing and failing hearts, although ACE2 was originally cloned from a human heart failure complementary DNA (cDNA) library [7], and ACE2 activity is increased in human heart ventricles of idiopathic dilated cardiomyopathy patients [10]. In the kidney, ACE2 was observed on endothelium of intrarenal arteries

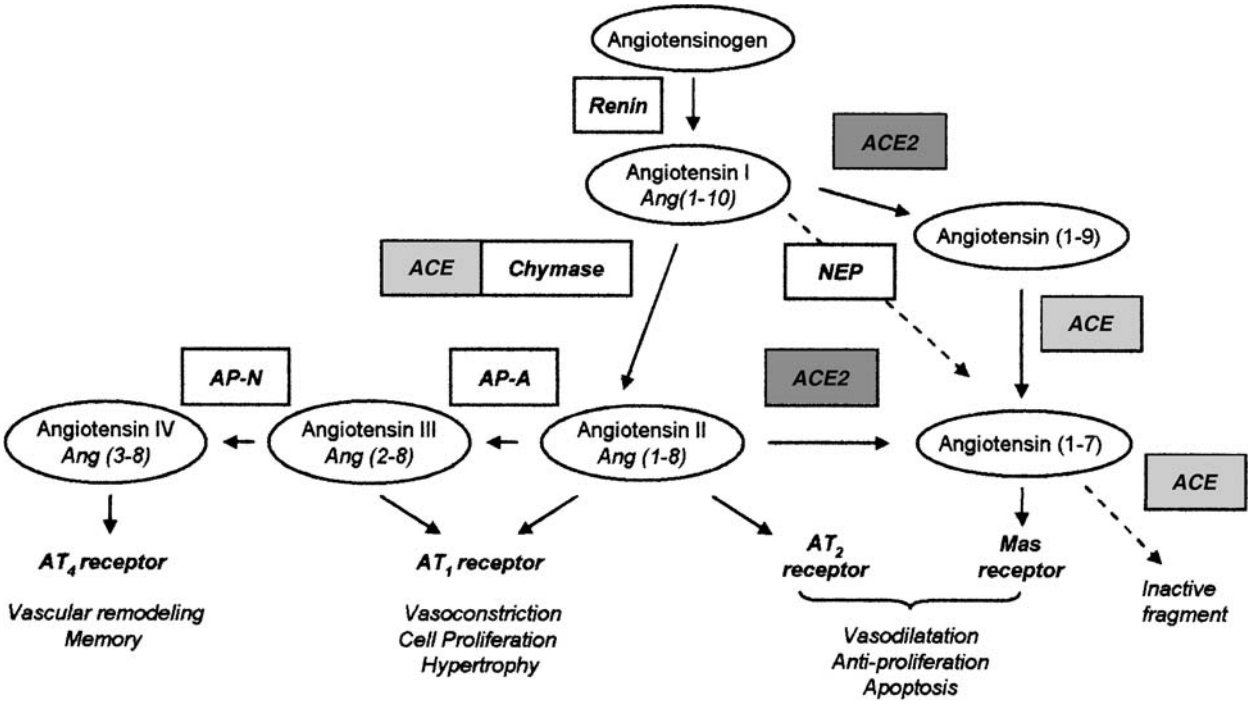

Figure 1. Schematic diagram of the renin-angiotensin system (RAS). In the classical RAS, angiotensin-converting enzyme (ACE) catalyses the production of angiotensin (Ang) II from Ang I. Ang II is a biologically active vasoconstrictor that interacts with angiotensin G-protein-coupled receptors AT1 and AT2. Ang (1–7), a metabolite of Ang I and Ang II, has opposing effects to Ang II which are mediated via interaction with the Mas [Ang (1–7)] receptor. ACE2 converts Ang II to Ang (1–7) with 370-fold greater catalytic efficiency than its conversion of Ang I to Ang (1–9). NEP, neprilysin; AP-N, aminopeptidase N; AP-A, aminopeptidase A.

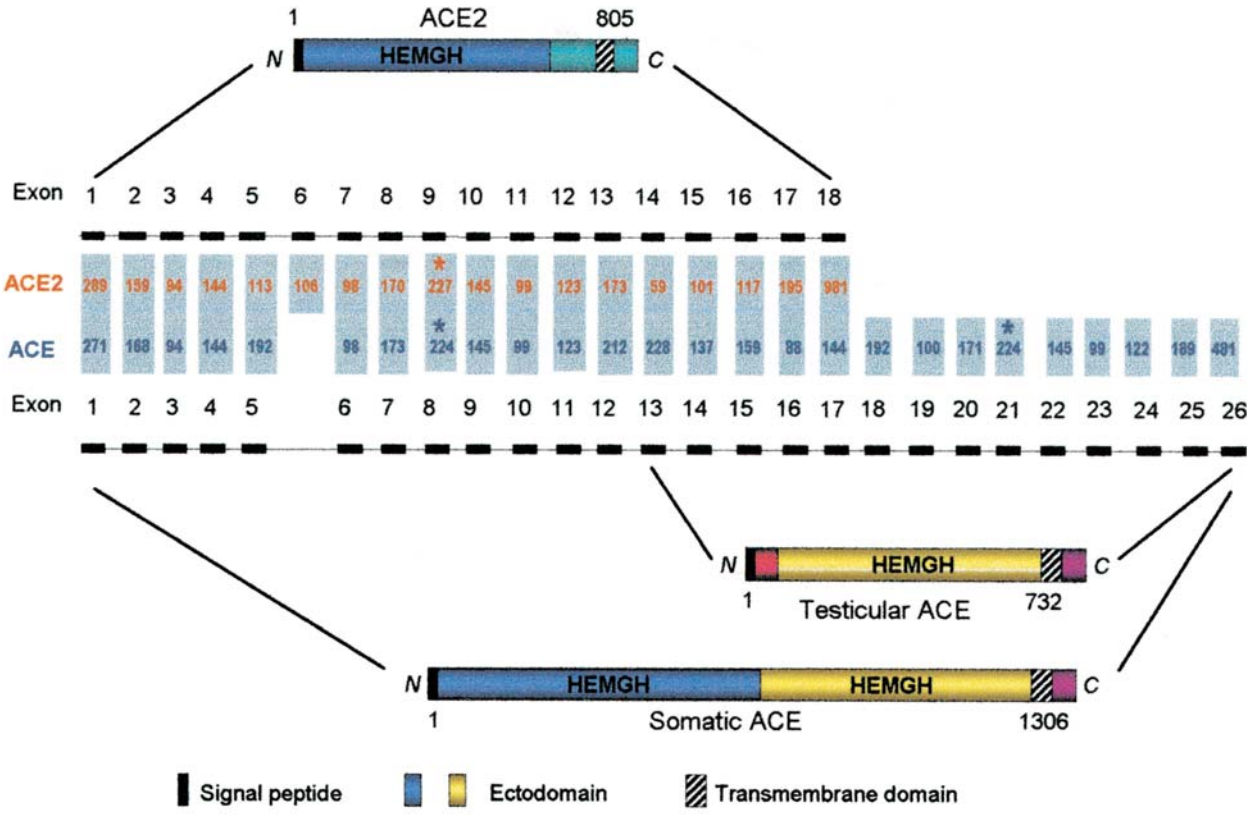

Figure 2. Exon alignment of ACE2 and ACE genes. Many of the exons of ACE2 are of similar size to the exons of ACE, although in ACE2 there is fusion of the equivalent exons 5 and 6 of ACE. Exons 13–18 of ACE2 bear no similarity to those of ACE and encode the unique juxtamembrane, transmembrane and cytosolic domains of the ACE2 protein. Somatic ACE mRNA is transcribed from exons 1 to 26, excluding exon 13. Testicular ACE mRNA (tACE) is transcribed from exons 13 to 26. Exon 13 encodes for the unique 67 amino acids at the beginning of the N-terminal region of tACE, whereas downstream exons encode the sequence common to both isoenzymes. * Denotes the zinc-binding HEXXH motif. The numbers shown in red (ACE2) and blue (ACE) represent the size (in nucleotides) of each exon.

and the epithelia of proximal tubules [7]. As with other zinc metalloproteases, such as tACE [23] and neprilysin [24], ACE2 is also highly expressed in testes [6, 7], although ACE2 knockout male mice appear fertile [8]. Recently, a more extensive assessment of ACE2 expression using quantitative real-time polymerase chain reaction (PCR) and tissue immunohistochemistry has revealed an even higher level of ACE2 gene and protein expression in all regions of the gastrointestinal (G-I) tract, in addition to its high expression in the heart, kidney and testes [25, 26].

The ACE2 protein

The open reading frame of human ACE2 encodes an 805-amino-acid polypeptide [6]. Hydropathy analysis of the ACE2 protein sequence reveals two hydrophobic regions, a potential 18-amino-acid signal peptide at the N-terminus and a 22-amino-acid hydrophobic region near the C-terminus. Like ACE, ACE2 is likely to be a type I integral membrane protein, anchored to the cell membrane via the hydrophobic region located towards the C-terminus of the protein. The N-terminal region of ACE2, which contains the active site, faces the extracellular space. ACE2 has been localised to the cell surface in stably transfected Chinese hamster ovary (CHO) cells by both immunofluorescence and cell surface biotinylation studies (F. J. Warner and Turner A. J. 2003, unpublished data). Thus, ACE2, like ACE, is an ectoenzyme, poised at the cell surface to hydrolyse circulating peptides.

Glycosylation

Human ACE2 has six potential N-glycosylation sites, as indicated by the presence of the Asn-X-Ser/Thr motif (at positions Asn^{53}, Asn^{90}, Asn^{103}, Asn^{322}, Asn^{432}, Asn^{546}) in its primary structure [6, 17]. Complete deglycosylation of denatured soluble ACE2 with *N*-glycosidase F, which hydrolyses all classes of Asn-linked glycans, results in a shift in the migration of the polypeptide from 120 to ~85 kDa, the predicted M_r for unglycosylated ACE2 [6]. Comparatively, sACE contains 17 N-glycosylation sites, of which 10 are located in the N-domain, the remaining 7 being found in both the C-domain of sACE and in tACE. In addition to N-linked glycans, tACE is also extensively

O-glycosylated within the first 36 amino acids of the N-terminal sequence, although these O-linked sugars are not essential for expression or activity [27]. O-Linked glycosylation has not been reported for sACE or ACE2.

Whether the presence of all sugar moieties associated with ACE2 glycosylation is necessary for correct targeting and activity remains to be established. The contribution of five of the potential glycosylation sites for expression, processing and activity in human tACE and rabbit ACE has been investigated [28–30] in an effort to produce a minimally glycosylated form of tACE for crystallisation studies [18]. Glycosylation at either the first or third sites (from the N-terminus) is sufficient to produce a catalytically active form of human tACE [30]. Similarly, the first and second sites are essential for rabbit tACE [28]. Of the seven potential glycosylation sites of ACE2, the first putative site, Asn^{53}, in humans is found to be conserved in rat ACE2, as well as in human, mouse and rabbit tACE, and *Drosophila* Ance and Acer, suggesting that this site is essential for maintaining activity. Overall, these studies of sACE and tACE [28–32] imply that carbohydrate processing has an essential role in the expression, maturation and proteolytic activity of membrane-bound and soluble ACE by influencing polypeptide folding and intracellular targeting and transport. Similar studies are required to reveal which of the potential glycosylation sites are important for the processing of ACE2.

Possible mechanisms of anchorage and solubilisation

Both sACE and tACE are solubilised from the membrane surface through the action of ACE secretase [33], the precise identity of which is still unknown. Even so, it is thought to be a member of the adamalysin (ADAM) family of zinc metalloproteases [34]. Comparative inhibitor studies suggest that ACE secretase is related, although distinct from, the well-characterised, tumour necrosis factor-α convertase (TACE, ADAM 17) [35–37].

ACE2 also exists in both a membrane-bound and a soluble form [6–8]. In CHO cells stably expressing ACE2, an active form of ACE2 is secreted from cells into the medium [6]. These studies suggest that the soluble form of ACE2 is derived from the membrane-bound form via a post-translational proteolytic cleavage event similar to that occurring with ACE. The cleavage site for human tACE and sACE has been mapped to the Arg^{1203}/Ser^{1204} bond (sACE numbering), 24 amino acids proximal to the cell membrane, in vitro and in vivo [38, 39]. The exact sequences around the cleavage site or distance from the membrane do not appear to be direct determinants of secretase activity [40, 41], but may be dependent on the conformational state of the juxta-membrane stalk region [42]. The juxtamembrane regions of human ACE and ACE2 display minimal sequence homology. Nevertheless, at least two potential (Arg-Ser and Arg-Leu) secretase cleavage sites exist within the juxtamembrane stalk region of ACE2.

Whilst the formation of soluble ACE2 has been established in vitro [6, 7], a soluble form of ACE2 has not yet been detected in vivo in human body fluids, although ACE2 has been detected in urine collected from rats [M. C. Chappell, 2003, unpublished data]. Until now, the majority of studies in rat and mouse models [8, 9] have thus far relied on an indirect means of measuring ACE2 activity in plasma through changes in levels of either Ang II or its metabolite Ang (1–7), by radioimmunoassay [43]. Future investigation into the mechanisms involved in the solubilisation of ACE2 and whether this secretory event is regulated will aid our understanding of the physiological relevance of ACE2 in modulating Ang I, Ang II and its other potential substrates both locally and systemically.

Catalytic properties of ACE2

ACE2 is a zinc metalloprotease that belongs to the gluzincin clan (MA) of metalloproteases, of which thermolysin is the archetypal member. Catalysis by members of this family occurs via a mechanism whereby zinc facilitates nucleophilic attack by a water molecule on the scissile carbonyl bond of the substrate. Rather than form a covalent intermediate, metalloproteases utilise co-ordination of a water molecule to a metal ion to form a non-covalent intermediate. Three other ligands co-ordinate the zinc ion and are conserved in all metalloproteases of the MA family (fig. 3a). In ACE2, the first two zinc co-ordinating ligands, His^{374} and His^{378}, are found within the consensus sequence, HEXXH, whereas the third zinc ligand, Glu^{402}, lies 23 amino acids C-terminal to this motif. Corresponding residues are found in the two domains of ACE and tACE (fig. 3a) [18, 20]. In ACE, an EXXXD motif contains the third zinc ligand (E) followed by an aspartyl residue. In ACE2, Glu^{406} replaces this aspartyl residue, whose role in ACE is to position the first histidine zinc ligand (fig. 3a). Mutagenesis of Asp^{991} to Glu in the C-domain of sACE reduces, but does not eliminate, the activity of wild-type ACE [44]. The role of Glu^{406} in ACE2 remains to be explored.

Based upon sequence alignment, ACE2 more closely resembles the N-domain of sACE with ~60% similarity and 41% identity to the overall protein sequence of sACE [6]. Although the juxtamembrane, transmembrane and cytoplasmic domains of ACE2 do not resemble sACE, they do share 48% identity with a 222-amino acid transmembrane glycoprotein, collectrin, which is derived from renal collecting ducts [45] (fig. 2). ACE2 has been classified as member of the M2 family along with sACE, tACE and *Drosophila* Ance and Acer. However, unlike other

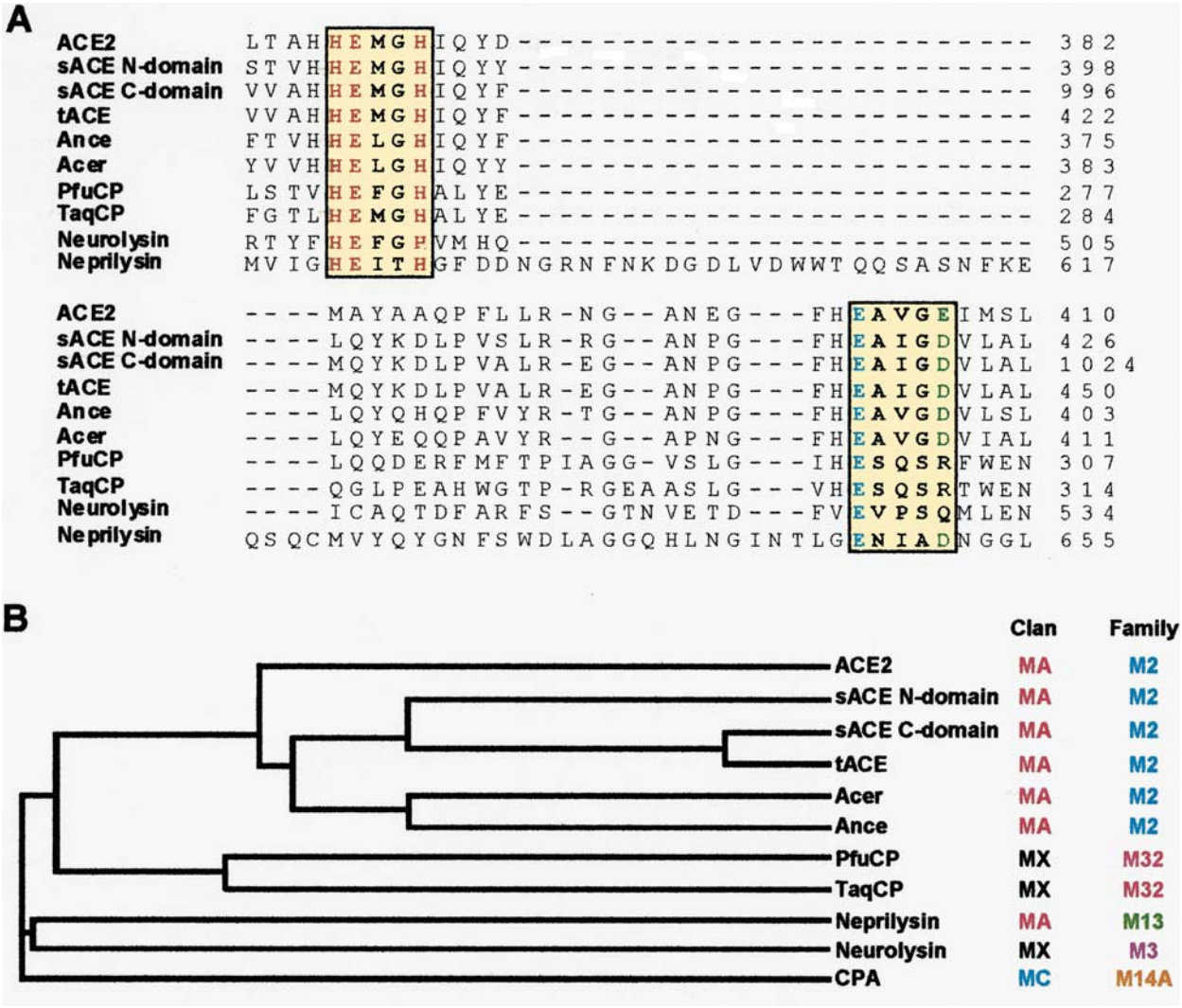

Figure 3. (*A*) Comparison of partial protein sequences for ACE2, ACE and other zinc metalloproteases. The amino acid sequences of human ACE2, testicular ACE (tACE), somatic ACE (sACE), neurolysin, neprilysin (NEP), carboxypeptidase A (CPA), *Pyrococcus furiosus* carboxypeptidase (PfuCP), *Thermus aquaticus* carboxypeptidase (TaqCP) and *Drosophila melanogaster* ACEs, Acer and Ance, were aligned by Clustal W. Boxed is the zinc-binding motif (red) and third zinc ligand (blue) consensus sequences. (*B*) A phylogenetic tree (cladogram) of ACE2 and other zinc metalloproteases of the M2, M3, M13 M14A and M32 families. ACE2 displays a highly similar tertiary structure to neurolysin and PfuCP, despite these proteins displaying little sequence homology and being very distantly related.

members of the M2 family, ACE2 is a carboxypeptidase rather than a peptidyl dipeptidase, being the first mammalian carboxypeptidase to be identified to contain the HEXXH motif rather than the typical carboxypeptidase A-like motif, $HXXE(X)_{123-132}H$. In this sense, ACE2 more closely resembles the bacterial carboxypeptidases of the M32 family, *Thermus aquaticus* carboxypeptidase (TaqCP) [46] and *Pyrococcus furiosus* carboxypeptidase (PfuCP) [47], which also utilize an HEXXH motif for zinc binding [48] (fig. 3a). The high sequence homology between ACE2 and the bacterial carboxypeptidases is, however, only confined to the region surrounding the catalytic binding domains. Consequently, ACE2 has been separated from the M32 family carboxypeptidases when portrayed schematically in a cladogram based upon protein sequence alignment (fig. 3b). However, despite there being a lack of sequence homology, close similarities have been noted between the tertiary structures of ACE2 [17] tACE, [18], PfuCP [47] and the neurotensin-degrading enzyme, neurolysin [49], suggesting convergent structural evolution of these metalloproteases.

Substrate specificity

The recent elucidation of crystal structures for human ACE2 [17] and a previously described comparative model of the ACE2 active site, based upon the crystal structure of tACE [18, 50], have identified key active site differences between ACE2 and tACE. Both studies indicate that the catalytic mechanism of ACE2 closely re-

sembles that of ACE. Differences in substrate specificity, however, suggest distinctive differences exist between the substrate binding subsites of ACE2 and tACE. ACE2 cleaves a single amino acid from the C-terminus of its substrates, whereas ACE functions as a peptidyl dipeptidase. The basis for this difference relates to the pocket (S2′ subsite) and binding of the substrate C-terminus. In ACE2, the S2′ subsite is smaller as a result of the R273 ⇒ Q substitution and is only able to accommodate one amino acid instead of a second terminal peptide bond, as compared with that of tACE [17, 50]. The inhibitory action of ACE inhibitors, captopril and lisinopril, is based, in part, upon their ability to mimic the dipeptidyl C-terminal binding of ACE substrates [51]. This feature may be one factor that contributes to the ineffectiveness of captopril and lisinopril to inhibit the proteolytic activity of ACE2 [6].

A diverse range of peptides have been screened as potential physiological substrates for ACE2 [50, 52]. Around 12 peptides have been identified that are hydrolysed to a significant extent by ACE2 (table 1). ACE2 cleaves both Ang I and Ang II, although ACE2 has an almost 400-fold higher catalytic efficiency for hydrolysis of Ang II [52]. Ang I also inhibits ACE2 hydrolysis of fluorogenic substrate Mca-APK(Dnp)-OH weakly (K_i = 2.2 μM) [14]. The majority of studies investigating a physiological role for ACE2 have concentrated on the ability of ACE2 to hydrolyse Ang II and its role in the RAS. However, ACE2 also hydrolyses the opioid peptide dynorphin A (1–13) and the hypotensive peptide apelin-13 with comparable kinetics to that of Ang II, as well as des-Arg^9 bradykinin, β-casomorphin and the growth hormone secretagogue ghrelin [52]. The ability of ACE2 to hydrolyse substrates other than Ang II suggests ACE2 may have a role in these other peptidergic systems.

Alignment of the substrates hydrolysed by ACE2 reveals a potential consensus sequence, P_3-X-P_1 ⇓ $X_{hydrophobic/basic}$, where the cleavage site occurs between the P_1 and a C-terminal hydrophobic/basic residue [50]. The vast majority of peptides efficiently cleaved by ACE2 possess a proline at the penultimate position (P_1) of the peptide and a phenylalanine at the C-terminus. However, a basic residue can be substituted at the C-terminus, as observed in neurotensin (1–8), neurotensin (1–11) and ghrelin (table 1) [50, 52]. The topology of the S1 subsite, in particular residue Y510, appears to be a determining factor in limiting the size of the residue found at the substrate P_1 position. Substrates with medium-sized amino acids such

Table 1. Substrates and inhibitors of human soluble ACE2 (adapted from [52]).

Substrate	Sequence P_3-X-P_1 ↓ $X_{hydrophobic/basic}$	Hydrolysis	Reference
Angiotensin I (1–10)	DRVYIHPFH ↓ L	P	6, 7, 50, 52
Angiotensin (1–9)	DRVYIHPFH	N	6, 7, 50, 52
Angiotensin II (1–8)	DRVYIHP ↓ F	C	6, 7, 50, 52
Angiotensin (1–7)	DRVYIHP	N	6, 50, 52
Angiotensin III	RVYIHP ↓ F	N.D.	
Angiotensin IV	VYIHP ↓ F	C	50
Bradykinin	RPPGFSPFR	N	6, 7, 50, 52
Bradykinin (1–7)	RPPGFSP	N	50, 52
des-Arg^9 bradykinin	RPPGFSP ↓ F	C	7, 50, 52
Lys-des-Arg^9 bradykinin	KRPPGFSP ↓ F	P	
Apelin-13	QRPRLSHKGPMP ↓ F	C	52
Apelin-36	…...QRPRLSHKGPMP ↓ F	C	52
β-Casomorphin	YPFVEP ↓ I	C	52
Dynorphin A 1–13	YGGFLRRIRPKL ↓ K	C	52
Neurotensin (1–13)	pELYENKPRRPYIL	N	50, 52
Neurotensin (1–11)	pELYENKPRRP ↓ Y	P	50
Neurotensin (1–8)	pELYENKP ↓ R	P	50, 52
Ghrelin	….ESKKPPAKLQP ↓ R	P	52
Inhibitors		**Inhibition parameters**	
Angiotensin I	DRVYIHPFHL	K_i = 2.2 μM	15
Pro-Phe	PF	IC_{50} ≈ 150 μM	50
DX600	RLPSCHSYDG-AC YYPWWKCTYPDPEGGG-NH_2	K_i = 2.8 nM IC_{50} = 10 nM	16
MLN-4760	(SS) 2-{(1)-carboxy-2-[3-(3,5-dichlorobenzyl)-3H-imidazol-4-yl] ethylamino}-4-methyl-pentanoic acid	IC_{50} = 0.44 nM	15

The sequence of each peptide is depicted using single-letter codes for each amino acid. An arrow indicates the site of hydrolysis. C, complete hydrolysis; P, partial hydrolysis; N, no hydrolysis all indicate the degree to which peptide substrates were hydrolysed. N.D., not determined.

as proline or leucine, but not bulky residues like tyrosine or phenylalanine, at the P_1 position are cleaved [17]. The presence of a proline at P_3, but not P_2, may also be a determinant for ACE2 activity [50]. For instance, both Ang I and dynorphin A (1–13) which contain a proline at the P_3 position are cleaved by ACE2, whereas a proline at the P_2 position, as observed in Ang (1–9) and bradykinin, prevents hydrolysis by ACE2. The exception to this is neurotensin (1–13), which despite having a proline in the P_3 position, is not a substrate for ACE2 (table 1). Further clarification of the rules governing ACE2 hydrolysis is required.

pH and anion activation

Current studies [50, 52, 53] have established the dependence of ACE2 on pH and monovalent anions for catalytic activity. Anion activation of Ang I hydrolysis by ACE was first recognised by Skeggs and co-workers in 1954 [54]. The dependence of ACE on chloride ions is both substrate- [55] and domain-specific [56]. Both ACE and ACE2 exhibit a preference for Cl^- over other monovalent ions, F^- and Br^- [52, 57]. The molecular mechanism for anion activation is yet to be fully understood, although it has been proposed that chloride ions may induce a slight change in the conformation of the active site that facilitates binding [58]. The crystal structure for lisinopril-complexed tACE has revealed two concealed chloride ions [18]. Four ligands, Arg^{489}, Arg^{186}, Trp^{485} and water bind the first chloride ion (Cl1). The ligands, Tyr^{224}, Arg^{522} and a water molecule bind the second chloride ion (Cl2), which is located closer to the co-ordinated zinc ion [18]. The C-domain ACE residue Arg^{1098} (equivalent to Arg^{522} in tACE) has been identified as a crucial residue for Cl^- activation of sACE activity [59]. Both Tyr^{244} and Arg^{522} are conserved in human ACE2 (Tyr^{207} and Arg^{514}), in addition to all known ACE sequences [59], implying that these conserved residues may also be involved in mediating the Cl^- effect observed for ACE2 and other related ACEs. However, the native crystal structure of the ACE2 ectodomain shows only a single bound chloride ion, instead of two as observed in the lisinopril-complexed tACE [17]. The location of this single chloride ion in the native ACE2 structure is almost identical to the position of the first chloride ion (Cl1) in the lisinopril-complexed tACE [17, 18]. Since no native crystal structure for tACE has been solved [18] and the resolution of the crystal structure of ACE2 complexed with MLN4760 (ACE2 inhibitor) was not high enough to differentiate water from chloride ions [17], it remains unknown if the second chloride ion (Cl2) in lisinopril-complexed tACE is unique to the inhibitor bound state. Future site-directed mutagenesis studies of the potential residues involved in chloride binding will give a greater understanding of the mechanisms underlying anion activation.

As for ACE, activation of ACE2 catalytic activity by Cl^- is substrate-dependent. At high concentrations of NaCl (1.0 M), ACE2 hydrolysis of Ang I [50] and fluorogenic substrates Mca-APK(Dnp)-OH [50, 52], AbzGFSPY(NO_2) and AbzSPY(NO_2) [53] markedly increases. In contrast, the presence of chloride ions for hydrolysis of Ang II by ACE2 is not essential. In fact, hydrolysis of Ang II is inhibited ninefold at 2.0 M NaCl [50].

The control of ACE2 activity through pH and anion activation presents the question of its physiological relevance. Both pH and chloride ion concentrations are, for the most part, relatively constant in blood plasma, to which ACE and ACE2 are theoretically exposed. Under physiological conditions, pH 7.4 and 100 mM [Cl^-], and based upon the data presented by Guy and co-workers [50], ACE2 would only exhibit ~15% of its maximal activity for Ang I, but be ~90% active towards Ang II. Hence, it may be that anion activation serves to regulate substrate specificity, particularly in organs such as the kidney where chloride ion concentrations fluctuate greatly.

Inhibitor design and studies

The first generation of selective inhibitors for ACE2 have been designed and synthesised (table 1) [15, 16]. A series of non-peptide compounds were constructed based upon the ACE2 substrate consensus P-X(1-3)-P_1-⇓-$X_{hydrophobic}$ and the requirement of a centrally located carboxylate to co-ordinate with the zinc ion [15]. This lead resulted in the synthesis of an inhibitor (MLN-4760, table 1) possessing sub-nanomolar affinity (IC_{50}, 50% inhibitor concentration 0.44 nM) for ACE2 and 220,000- and 22,000-fold less affinity for human tACE and bovine carboxypeptidase A, respectively [15].

Using an alternative strategy, potent ACE2 peptide inhibitors have been identified through screening of constrained peptide libraries [16]. The most potent inhibitor, DX600, a 26-amino acid N-terminal acetylated and C-terminal amidated peptide, displays a K_i of 2.8 nM using Ang I as a substrate. DX600 exhibited a combination of both competitive and non-competitive inhibition. Intravenous bolus injection of DX512 (K_i, 139 nM), an analogue of DX600, in conscious spontaneously hypertensive rats (SHR) produced a dose-dependent depressor response characterised by a decreased mean arterial pressure and accompanied by transient tachycardia [16]. These findings are in contrast to the hypothesis that ACE2 is linked to systemic arterial pressure [60]. This hypothesis is supported by studies in ACE2 knockout mice where ablation of ACE2 either did not have a direct effect on blood pressure or increased systolic blood pressure [8, 9]. Furthermore, ACE2 mRNA and protein expression levels are reduced in SHR compared with control Wistar Kyoto rats [8]. There are two possible expla-

nations for these conflicting results. First, Ang II may not be the sole physiological substrate involved in mediating the effects of ACE2 on blood pressure. Apelin-13 (k_{cat}/K_m, 2.0×10^6 M^{-1} s^{-1}) is cleaved by ACE2 with kinetics similar to that of Ang II (k_{cat}/K_m, 1.8×10^6 M^{-1} s^{-1}), and apelin-36 is also a substrate [52]. Both peptides also produce hypotensive systemic effects [61, 62] that may be accentuated in the absence of ACE2. Second, the inconsistencies observed among these studies may originate from the fundamental differences that exist between an animal which has never produced a protein and may develop compensatory mechanisms and an animal in which the same protein is inhibited after birth. Nevertheless, with the recent development of inhibitors these discrepancies can now be addressed, as well as promote new studies of ACE2 in normal physiology and in disease.

Conclusions and future perspectives

In a remarkably short space of time, much knowledge has accumulated on the structure, enzymology and physiological functions of ACE2, but much still remains to be elucidated. The counterbalancing roles of ACE and ACE2 in the RAS are gaining credence, but other metabolites, for example apelin, may be physiologically relevant substrates. ACE2 has been implicated in a number of disease conditions including hypertension, diabetes and cardiac function. Most recently, its identity as the SARS virus receptor opens new avenues for ACE2 research particularly in relation to the nature of the interactions between the viral spike glycoprotein and ACE2. More clinical studies are required to validate disease models, and for this purpose, generally available ACE2 inhibitors are of fundamental importance. The recent structural solutions of both ACE and ACE2 should facilitate further design of potent and selective ACE2 inhibitors. Finally, in this rapidly developing field, there may be yet further surprises that this new member of the ACE gene family has to provide.

Acknowledgements. The research described here was supported by the U.K. Medical Research Council and the British Heart Foundation (A.J.T., N.M.H.) and by the National Health and Medical Research Council (Australia) (A.I.S., F.J.W.). F.J.W. is a C. J. Martin Fellow (NHMRC, Australia).

1 Braun-Menendez E., Fasciolo J. C., Leloir L. F. and Munoz J. M. (1940) The substance causing renal hypertension. J. Physiol. **98:** 283–298
2 Dell'Italia L. J. and Husain A. (2002) Dissecting the role of chymase in angiotensin II formation and heart and blood vessel diseases. Curr. Opin. Cardiol. **17:** 374–379
3 Ferrario C. M., Chappell M. C., Tallant E. A., Brosnihan K. B. and Diz D. I. (1997) Counterregulatory actions of angiotensin-(1-7). Hypertension **30:** 535–541
4 Santos R. A. S., Simones e Silva, A. C., Maric C., Silva D. M. R., Machado R. P., de Buhr I. et al. (2003) Angiotensin (1-7) is an endogenous ligand for the G protein coupled receptor Mas. Proc. Natl. Acad. Sci. USA **100:** 8258–5263
5 Acharya K. R., Sturrock E. D., Riordan J. F. and Ehlers M. R. (2003) ACE revisited: a new target for structure-based drug design. Nat. Rev. Drug Discov. **2:** 891–902
6 Tipnis S. R., Hooper N. M., Hyde R., Karran E., Christie G. and Turner A. J. (2000) A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. J. Biol. Chem. **275:** 33238–33243
7 Donoghue M., Hsieh F., Baronas E., Godbout K., Gosselin M., Stagliano N. et al. (2000) A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ. Res. **87:** E1–E9
8 Crackower M. A., Sarao R., Oudit G. Y., Yagil C., Kozieradzki I., Scanga S. E. et al. (2002) Angiotensin-converting enzyme 2 is an essential regulator of heart function. Nature **417:** 822–828
9 Allred A. J., Donoghue M., Acton S. and Coffman T. M. (2002) Regulation of blood pressure by the angiotensin converting enzyme homologue ACE2. Am. J. Nephrol. **13:** 52A
10 Zisman L. S., Keller R. S., Weaver B., Quishan L., Speth R., Bristow M. R. et al. (2003) Increased angiotensin (1–7)-forming activity in failing human heart ventricles. Evidence for upregulation of the angiotensin-converting enzyme homologue ACE2. Circulation **108:** 1707–1712
11 Donoghue M., Wakimoto H., Maguire C. T., Acton S., Hales P., Stagliano N. et al. (2003) Heart block, ventricular tachycardia and sudden death in ACE2 transgenic mice with downregulated connexins. J. Mol. Cell. Cardiol. **35:** 1043–1053
12 Tikellis C., Johnston C. I., Forbes J. M., Burns W. C., Burrell L. M., Risvanis J. et al. (2003) Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. Hypertension **41:** 392–397
13 Brosnihan K. B., Neves L. A. A., Joyner J., Averill D. B., Chappell M. C., Sarao R. et al. (2003) Enhanced renal immunocytochemical expression of Ang (1-7) and ACE2 during pregnancy. Hypertension **42:** 749–753
14 Li W., Moore M. J., Vasilieva N., Sui J., Wong S. K., Berne M. A. et al. (2003b) Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature **426:** 450–454
15 Dales N. A., Gould A. E., Brown J. A., Calderwood E. F., Guan B., Minor C. A. et al. (2002) Substrate-based design of the first class of angiotensin-converting enzyme-related carboxypeptidase (ACE2) inhibitors. J. Am. Chem. Soc. **124:** 11852–1185
16 Huang L., Sexton D. J., Skogerson K., Devlin M., Smith R., Sanyal I. et al. (2003) Novel peptide inhibitors of angiotensin-converting enzyme 2. J. Biol. Chem. **278:** 15532–15540
17 Towler P., Staker B., Prasad S. G., Menon S., Tang J., Parsons T. et al. (2004) ACE2 structures reveal a large hinge-bending motion important for inhibitor binding and catalysis. J. Biol. Chem. **279:** 17996–18007
18 Natesh R., Schwager S. L., Sturrock E. D. and Acharya K. R. (2003) Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. Nature **421:** 551–554
19 Kim H. M., Shin D. R., Yoo O. J., Lee H. and Lee J.-O. (2003) Crystal structure of *Drosophila* angiotensin I-converting enzyme bound to captopril and lisinopril. FEBS Lett. **538:** 65–70
20 Soubrier F., Alhenc-Gelas F., Hubert C., Allegrini J., John M., Tregear G. et al. (1988) Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. Proc. Natl. Acad. Sci. USA **85:** 9386–9390
21 Komatsu T., Suzuki Y., Imai J., Sugano S., Hida M., Tanigami A. et al. (2002) Molecular cloning, mRNA expression and chromosomal localization of mouse angiotensin-converting enzyme-related carboxypeptidase (mACE2). DNA Sequence **13:** 217–220

22 Chen A. A., Barnes G., Foti A., Nolin E., Lewitzsky S., Metivier J. et al. (2002) Two single nucleotide polymorphisms in the ACE2 locus are associated with cardiovascular disease. Gen. Epidem. **23:** 272
23 Hubert C., Houot A. M., Corvol P. and Soubrier F. (1991) Structure of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene. J. Biol. Chem. **266:** 15377–15383
24 Erdös E. G. and Skidgel R. A. (1989) Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. FASEB J. **3:** 145–151
25 Harmer D., Gilbert M., Borman R. and Clark K. L. (2002) Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. FEBS Lett. **532:** 107–110
26 Hamming I., Timens W., Bulthuis M. L., Lely A. T., Navis G. J., van Goor H. (2004) Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J. Pathol. **203:** 631–637
27 Ehlers M. R., Chen Y. N. and Riordan J. F. (1992) The unique N-terminal sequence of testis angiotensin-converting enzyme is heavily O-glycosylated and unessential for activity or stability. Biochem. Biophys. Res. Commun. **183:** 199–205
28 Sadhukhan R. and Sen I. (1996) Different glycosylation requirements for the synthesis of enzymatically active angiotensin-converting enzyme in mammalian cells and yeast. J. Biol. Chem. **271:** 6429–6434
29 Yu X. C., Sturrock E. D., Wu Z., Biemann K., Ehlers M. R. W. and Riordan J. F. (1997) Identification of N-Linked glycosylation sites in human testis angiotensin-converting enzyme and expression of an active deglycosylated form. J. Biol. Chem. **272:** 3511–3519
30 Gordon K., Redelinghuys P., Schwager S. L. U., Ehlers M. R. W., Papageorgiou A. C., Natesh R. et al. (2003) Deglycosylation, processing and crystallisation of human testis angiotensin-converting enzyme. Biochem J. **371:** 437–442
31 Naim, H. Y. (1993) Human small intestinal angiotensin-converting enzyme: intracellular transport, secretion and glycosylation. Biochem. J. **296:** 607–615
32 Orth T., Voronov S., Binevski P., Saenger W. and Kost O. (1998) Glycosylation of bovine pulmonary angiotensin-converting enzyme modulates its catalytic properties. FEBS Lett. **431:** 255–258
33 Parvathy S., Oppong S. Y., Karran E. H., Turner A. J. and Hooper N. M. (1997) Angiotensin converting enzyme secretase is inhibited by metalloprotease inhibitors and requires its substrate to be inserted in a lipid bilayer. Biochem. J. **292:** 597–603
34 Black R. A. and White J. M. (1998) ADAMS: focus on the protease domain. Curr. Opin. Cell. Biol. **10:** 654–659
35 Parvathy S., Karran E. H., Turner A. J. and Hooper N. M. (1998). The secretases that cleave angiotensin enzyme and the amyloid precursor protein are distinct from tumour necrosis factor-α convertase. FEBS Lett. **431:** 63–65
36 Parkin E. T., Trew A., Christie G., Faller A., Mayer R., Turner A. J. et al. (2002) Structure-activity relationship of hydroxamate-based inhibitors on the secretases that cleave the amyloid precursor protein, angiotensin converting enzyme, CD23 and pro-tumor necrosis factor-alpha. Biochemistry **41:** 4972–4981
37 Allinson T. M. J., Parkin E. T., Turner A. J. and Hooper N. M. (2003) ADAMS family members as amyloid precursor protein α-secretases. J. Neurosci. Res. **74:** 342–352
38 Beldent V., Michaud A., Wei L., Chauvet M. T. and Corvol P. (1993) Proteolytic release of human angiotensin-converting enzyme. Localisation of the cleavage site. J. Biol. Chem. **268:** 26428–26434
39 Woodman Z. L., Oppong S. Y., Cook S., Hooper N. M., Schwager S. L. U., Brandt W. F. et al. (2000) Shedding of somatic angiotensin converting enzyme (ACE) is inefficient compared with testis ACE despite cleavage at identical stalk sites. Biochem. J. **347:** 711–718
40 Ehlers M. R., Schwager S. L., Scholle R. R., Manji G. A., Brandt W. F. and Riordan J. F. (1996) Proteolytic release of membrane-bound angiotensin-converting enzyme: role of the juxtamembrane stalk sequence. Biochemistry **35:** 9549–9559
41 Sadhukhan R., Sen G. C., Ramchandran R. and Sen I. (1998) The distal ectodomain of angiotensin-converting enzyme regulates its cleavage-secretion from the cell surface. Proc. Natl. Acad. Sci. USA **95:** 138–143
42 Eyries M., Michaud A., Deinum J., Agrapart M., Chomilier J., Kramers C. et al. (2001) Increased shedding of angiotensin-converting enzyme by a mutation identified in the stalk region. J. Biol. Chem. **276:** 5525–5532
43 Allred A. J., Chappell M. C., Ferrario C. M. and Diz D. I. (2000) Differential actions of renal ischemic injury on the intrarenal angiotensin system. Am. J. Physiol. **279:** F636–F645
44 Williams T. A., Corvol P. and Soubrier F. (1994) Identification of two active site residues in human angiotensin I-converting enzyme. J. Biol. Chem. **269:** 29430–29434
45 Zhang H., Wada J., Hida K., Tsuchiyama Y., Hiragushi K., Shikata K. et al. (2001) Collectrin, a collecting duct-specific transmembrane glycoprotein, is a novel homolog of ACE2 and is developmentally regulated in embryonic kidneys. J. Biol. Chem. **276:** 17132–17139
46 Lee S. H., Taguchi H., Yoshimura E., Minagawa E., Kaminogawa S., Ohta T. et al. (1996) The active site of carboxypeptidase Taq possesses the active-site motif His-Glu-X-X-His of zinc-dependent endopeptidases and aminopeptidases. Protein Eng. **9:** 467–469
47 Arndt J. W., Hao B., Ramakrishnan V., Cheng T., Chan S. I. and Chan M. K. (2002) Crystal structure of a novel carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Structure **10:** 215–224
48 Turner A. J. and Hooper N. M. (2002) The angiotensin-converting enzyme gene family: genomics and pharmacology. TiPS **23:** 177–183
49 Brown C. K., Madauss K., Lian W., Beck M. R., Tolbert W. D. and Rodgers D. W. (2001) Structure of neurolysin reveals a deep channel that limits substrate access. Proc. Natl. Acad. Sci. USA **98:** 3127–3132
50 Guy J. L., Jackson R. M., Acharya K. R., Sturrock E. D., Hooper N. M. and Turner A. J. (2003) Angiotensin-converting enzyme-2 (ACE2): comparative modelling of the active site, substrate specificity and chloride dependence. Biochemistry **42:** 13185–13192
51 Cushman D. W., Cheung H. S., Sabo E. F. and Ondetti M. A. (1977) Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. Biochemistry **16:** 5484–5491
52 Vickers C., Hales P., Kaushik V., Dick L., Gavin J., Tang J. et al. (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J. Biol. Chem. **277:** 14838–14843
53 Yan Z.-H., Ren K.-J., Wang Y., Chen S., Brock T. A. and Rege A. A. (2003) Development of intramolecularly quenched fluorescent peptides as substrates of angiotensin-converting 2. Anal. Chem. **312:** 141–147
54 Skeggs L. T. (1956) The preparation and function of the hypertensin-converting enzyme. J. Med. Med. **103:** 295–299
55 Shapiro R., Holmquist B. and Riordan J. F. (1983) Anion activation of angiotensin converting enzyme: dependence on nature of substrate. Biochemistry **22:** 3850–3857
56 Wei L., Alhenc-Gelas F., Corvol P. and Clauser E. (1991) The two homologous domains of the human angiotensin 1-converting enzyme are both catalytically active. J. Biol. Chem. **266:** 9002–9008

57 Bünning P. and Riordan J. F. (1983) Angiotensin-converting enzyme by monovalent anions. Biochemistry **22:** 110–116
58 Ehlers M. R. W. and Riordan J. F. (1990) Angiotensin-converting enzyme. Biochemistry and molecular biology. In: Hypertension Pathophysiology, Diagnosis and Management, pp. 1217–1231, Laragh J. H. and Brenner B. M. (eds), Raven Press, New York
59 Liu X., Fernandez M., Wouters M. A., Heyberger S. and Husain A. (2001) Arg-1098 is critical for the chloride dependence of human angiotensin-1 converting enzyme C-domain catalytic activity. J. Biol. Chem. **268:** 33518–33525
60 Yagil Y. and Yagil C. (2003) Hypothesis: ACE2 modulates blood pressure in the mammalian organism. Hypertension **41:** 871–873
61 Lee D. K., Cheng R., Nguyen T., Fan T., Kariyawasam A. P., Liu Y. et al. (2000) Characterisation of Apelin, the ligand for the AJP receptor. J. Neurochem. **74:** 34–41
62 Tatemoto K., Takayama K., Zou M. X., Kumaki I., Zhang W., Kumano K. et al. (2001) The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. Regul. Pept. **99:** 87–92