Exploring the structure and function of zinc metallopeptidases: old enzymes and new discoveries

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Abstract
Neprilysin [or neutral endopeptidase (NEP)] and angiotensin-converting enzyme (ACE) are zinc metallopeptidases involved in the extracellular metabolism of biologically active peptides. Recent genomic advances have led to the identification of novel homologues of each of these ectoenzymes and new physiological and pathological roles are emerging for them. The structures of each of these peptidases have recently been solved providing insight into their distinct catalytic sites. In addition to its originally identified role in neuropeptide metabolism in the nervous system, NEP is implicated in regulation of the cardiovascular system and is protective in prostate and certain other cancers. Hence the cellular concentration of NEP is critical to tissue homeostasis. Most recently, NEP has been shown to exert neuroprotective actions, principally through its ability to catabolize the neurotoxic Alzheimer’s amyloid peptide. The only known homologue of ACE, termed ACE2, is critical to cardiovascular function, but its physiological substrates and precise metabolic roles remain to be elucidated. Other members of these growing metallopeptidase families await further characterization and possible exploitation as therapeutic targets.

Introduction
Proteolysis plays vital roles in cellular processes from fertilization through development to death. The human genome encodes well over three hundred distinct proteases, many of whose physiological roles are yet to be identified and which may provide potential therapeutic targets in the treatment of human disease. This article will focus on two distinct zinc metallopeptidase families: the neprilysin [or neutral endopeptidase (NEP)] family and the angiotensin-converting enzyme (ACE) family. Members of each of these families have served as important drug targets, particularly in cardiovascular disease and, more recently, have provided insight into mechanisms involved in the processing of the amyloid precursor protein (APP) and its products in Alzheimer’s disease. The recent discovery of ACE2 in our laboratory [1], as a result of genomics approaches to the identification of zinc metalloproteinases, together with its critical role in cardiac development and function [2,3], emphasize the validity of this strategy for identification of novel therapeutic targets.

The NEP family
NEP, or neprilysin as it is now known, was originally identified as a major antigen of renal membranes over 30 years ago and, at that time, was implicated in the metabolism of insulin. However, subsequently there has been little support for its role in the physiological inactivation of insulin, especially since another zinc metallopeptidase insulin-degrading enzyme (IDE; insulysin) appears to fulfil that function. Furthermore, NEP degrades only the insulin B chain in vitro and not the intact insulin dimer. Whereas the highest concentrations of NEP are in the renal microvillar membrane, the first clues to its physiological roles came from studies on the metabolism of neuropeptides (especially the enkephalins and substance P) in the central nervous system where NEP is several orders of magnitude less abundant [4,5]. It is now accepted that NEP functions to turn off neuropeptide signals at the synapse in an analogous fashion to the hydrolysis of acetylcholine by acetylcholinesterase at cholinergic synapses (see e.g. [6] for review). Figure 1 illustrates how NEP, a type II integral membrane protein of synaptic and axonal membranes, is topologically and anatomically poised to fulfil this role. Evidence in vitro demonstrating that synaptic membranes efficiently degraded enkephalins and substance P and that NEP was the primary enzyme responsible for these events [7], combined with data obtained in vivo in rodents using potent and selective NEP inhibitors, such as phosphoramidon and thiorphan [8], established such key roles for NEP. Subsequently, renal NEP was shown to be the principal enzyme inactivating the vasodilator, atrial natriuretic peptide [9], which has led to much investment in the development of NEP inhibitors, either alone or in combination with ACE inhibitors (‘vasopeptidase inhibitors’), as drugs in the treatment of hypertension, congestive heart failure and renal...
NEP exists as an ectoenzyme with its catalytic domain facing the extracellular space. NEP is located on axonal membranes and on both pre- and post-synaptic membranes where it can inactivate released neuropeptides such as the enkephalins (Enk) after their interaction with their respective peptide receptors.

The topology and location of the zinc metallopeptidase, NEP, at a peptidergic synapse

NEP, at a peptidergic synapse

The human genome is now known to contain at least seven NEP-related enzymes (summarized in [14]), of which the best characterized is endothelin-converting enzyme-1 (ECE-1), which catalyses the final step in the biosynthesis of the potent vasoconstrictor peptide, endothelin-1. Several of the NEP-like enzymes are, as yet, orphan peptidases with no recognized peptide substrates. Novel strategies are urgently needed to allow the identification of physiologically relevant substrates for such newly identified proteases.

The ACE family

ACE was originally identified almost 50 years ago as a ‘hypertensin-converting enzyme’ [15] and its primary substrate was identified as angiotensin I, which it converts into the vasoconstrictor angiotensin II. In parallel, it inactivates the vasodilator bradykinin. Hence inhibition of ACE has a powerful effect in reduction of blood pressure and the enzyme has therefore been a major cardiovascular target for many years. The catalytic activity of ACE is primarily as a ‘peptidyl dipeptidase’, removing dipeptides from the C-terminus of a susceptible peptide substrate. In the hydrolysis of some peptides (e.g. substance P, luliberin), ACE can act as an endopeptidase, although with much lower catalytic efficiency [16]. Mammalian ACE exists as two distinct forms, arising from the use of alternative promoters. The simplest form is germinal or testicular ACE, which is essential for male fertility and which carries a single zinc-binding and catalytic domain. Elsewhere in the body, the somatic form of ACE is duplicated and carries two active sites. The elusive X-ray structures of both single-domain mammalian testicular ACE [17] and of Drosophila ACE [18] have recently been solved by independent groups and both reveal that ACE most closely resembles a neurotensin-degrading zinc endopeptidase known as neurolysin rather than NEP, or carboxypeptidase A, on whose structure the design of ACE inhibitors were originally based [19].

While seeking novel expressed sequence tags encoding zinc metallopeptidases, we recently identified and cloned the first human homologue of ACE, which we originally termed ACEH, but which now is more commonly referred to as ACE2 [1]. There are, of course, considerable similarities with ACE. ACE2 is also a type I integral membrane peptidase showing 40% identity and 61% similarity with ACE and conserving the critical active-site residues. It contains a single catalytic domain like testicular ACE and it is most abundantly expressed in kidney, heart and lung [1,2]. However, there are also striking and unexpected differences yet to be explained on a structural basis. ACE2 functions exclusively as a carboxypeptidase, hydrolysing either aromatic or basic residues from the C-terminus and preferring a prolyl residue in the P1 position [16,20]. It hydrolyses both angiotensin I and angiotensin II, but not bradykinin, and ACE2 is insensitive to all ACE inhibitors tested to date. Figure 2 illustrates the substrate specificity of ACE2. The cytosolic, transmembrane and juxtamembrane regions of ACE2 bear no similarity to ACE, but resemble a membrane protein of the renal collecting duct, collectrin, that is believed to be involved in the physiological response to renal injury [21]. Collectrin has no
Figure 2 | Substrate specificity of ACE2

ACE2 acts as a carboxypeptidase on a number of biologically active peptides, some examples of which are shown. A strong preference exists for either a hydrophobic or a basic residue at the C-terminus (arrows) and for a prolyl residue in the penultimate (P1) position [1,2,20].

![Substrate specificity of ACE2](image)

peptidase catalytic domain, suggesting that ACE2 evolved as a natural chimera of ACE and collectrin genes, and that the cytosolic domain of ACE2 may have roles quite distinct from the carboxypeptidase activity of the extracellular catalytic domain. Clues to the roles of ACE2 have come from the development of mice deficient in the ACE2 gene [3].

These mice have severe cardiac contractility defects, increased plasma angiotensin II levels and an up-regulation of cardiac hypoxia-related genes, implicating ACE2 as an essential regulator of heart function. The ace2 gene itself may be a candidate gene for hypertension [3]. Intriguingly, a double knockout in mice of both the ACE and ACE2 genes is able to rescue the cardiac defect seen with the ACE2-deficient mice [3].

ACE2 mRNA and protein levels are substantially reduced in the kidney in diabetic rats, suggesting that the enzyme may have a role in the development of diabetic complications [22].

ACE2 is the only known mammalian carboxypeptidase that contains the classical His-Glu-Xaa-Xaa-His zinc-binding motif in its active site. However, in this respect it resembles a group of bacterial carboxypeptidases typified by that from Pyrococcus furiosus, whose structure has recently been solved [23]. Close similarities were noted between the structures of P. furiosus carboxypeptidase and of neurolysin although they have little or no sequence similarity, suggesting convergent structural evolution. As ACE also resembles neurolysin, it is likely that all these enzymes fall into a distinct structural family of zinc metallopeptidases which are quite distinct from the NEP family. Now that the structures of ACE, neurolysin and P. furiosus carboxypeptidase are available, it should facilitate comparative modelling of the active site of ACE2 and aid in the design of novel inhibitors to investigate further the physiological roles of the enzyme. The first classes of ACE2 inhibitors have recently been described in the literature [24,25].

One feature that both ACE [26] and ACE2 ([2] and D.W. Lambert, N.M. Hooper and A.J. Turner, unpublished work) share is the ability to be shed from the plasma membrane by cleavage within the juxtamembrane region releasing the bulk of the protein, including the intact catalytic domain into the extracellular medium. This process is common to a growing number of membrane proteins of diverse characteristics (see e.g. [27] for review), an event which is generally receptor-regulated and sensitive to inhibition by a group of hydroxamate metalloproteinase inhibitors such as batimastat. This has led to the identification of members of the ADAMs (a disintegrin and metalloproteinase) family of zinc proteinases, typified by tumour necrosis factor-α converting enzyme (‘TACE’; ADAM17) as candidate shedding enzymes [28].

From ACE to Alzheimer’s disease

We originally described the process of membrane protein shedding when we observed that incubation of renal microvillar membranes in a buffered solution led to the release of ACE, but not other microvillar peptidases such as NEP, into the 100 000 × g supernatant fraction, a process that was inhibited by metal-chelating agents, implicating the action of a metalloproteinase [26]. We referred to this shedding activity as a ‘membrane protein secretase’ [27] and have subsequently characterized the activity in some detail. As it turns out, the shedding of ACE has served as a prototype of many other such shedding events. One that we have focused on in some detail is the non-amyloidogenic (‘α-secretase’) processing of the APP [29–31], an event which is protective against the production of amyloid β-peptide and hence Alzheimer’s disease. This event, which involves the shedding of the ectodomain of APP to produce a polypeptide that has neuritogenic and neurotrophic properties, is mediated by one or more members of the ADAMs family, of which ADAM10 is the favoured candidate [28]. The shedding process is generally stimulated by activation of specific signalling pathways, e.g. via muscarine activation [32]. Activation of
such pathways, for example by the use of muscarinic agonists, has been explored as a possible therapeutic strategy in the treatment of Alzheimer’s disease, although with mixed results to date [33].

The amyloidogenic pathway of APP processing (Figure 3), which leads to production of the neurotoxic amyloid β-peptide of 40 or 42 amino acids in length, involves the sequential action of two aspartic proteinases, β-secretase [34] and γ-secretase, the latter involving a membrane-bound protein complex comprising the Alzheimer’s disease-related gene product, presenilin, which itself may be the catalytic core of the complex [35]. This amyloidogenic pathway may occur preferentially within lipid-raft domains within the membrane [36]. The accumulation of amyloid β-peptide, according to the ‘amyloid cascade hypothesis’ put forward by Hardy and Higgins just over a decade ago [37], is the primary event leading to neuronal cell death and dementia in Alzheimer’s disease (Figure 3). For much of the subsequent time, amyloid formation has been considered as an irreversible process and hence preventing its deposition or fibrillar aggregation have been seen as the logical therapeutic targets. However, more recent studies [38,39] have shown unequivocally that enzymic mechanisms exist to facilitate the removal of amyloid β-peptide and are therefore neuroprotective (Figure 3). Our story comes full circle since the zinc metallopeptidases NEP [38,39], its homologue ECE-1 [40,41], IDE [42] and possibly ACE [43], have been identified as potential regulators of amyloid β-peptide levels in the brain (reviewed in [12,44]). In particular, in mice deficient in NEP or ECE-1, amyloid deposits are seen to deposit at significant levels in the brain [41,45]. Furthermore, injection of amyloid β-peptide into the brains of rodents significantly enhances the concentrations of NEP mRNA and protein, suggesting the operation of a regulatory feedback mechanism to protect neurons from toxic damage [46]. NEP levels appear to be reduced in high-plaque-bearing areas of human brain in Alzheimer’s disease and in cerebral amyloid angiopathy [47,48], but no association has been detected to date between polymorphisms in the NEP gene and Alzheimer’s disease [49,50]. Up-regulation of one or more of the amyloid-degrading enzymes might provide effective protection against the neurodegeneration that occurs in Alzheimer’s disease.

Conclusions
The NEP and ACE gene families, which play key roles in the processing of biologically active peptides, are providing novel insights into human biology and disease. In particular, in Alzheimer’s disease, such zinc metallopeptidases operate to protect the organism from the neurotoxic effects of APP processing. The physiological roles of several recent recruits to the NEP family as well as the only known ACE homologue in humans, ACE2, still remain to be elucidated and could provide new avenues for the treatment of some of the major human diseases of the aging populations of the Western world: cardiovascular disease, cancer and dementia.

Note added in proof (received 23 April 2003)
A study examining the phenotype of IDE knock-out mouse [51] has confirmed that IDE has a physiological role both in the degradation of insulin and amyloid beta-peptide in vivo. Further confirmatory evidence that NEP is critical to amyloid removal comes from the demonstration that lentiviral gene transfer of NEP in transgenic mice significantly reduces amyloid pathology [52].

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