Journal of Proteomics & Bioinformatics - Open Access

Research Article JPB/Vol.1/December 2008

Novel Therapeutic Approaches to Treat Alzheimer's Disease and Memory Disorders

David B. Ascher[1], Gabriela A. N. Crespi[1], Hooi Ling Ng[1], Craig J. Morton[1], Michael W. Parker[1,2*] and Luke A. Miles[1,2]

[1]Biota Structural Biology Laboratory and Centre for Structural Neurobiology, St. Vincent's Institute of Medical Research, 9 Princes Street, Fitzroy, Victoria 3065, Australia

[2]Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, 30 Flemington Road, Parkville, Victoria 3010, Australia

*Corresponding author: Prof. Michael W. Parker, Biota Structural Biology Laboratory, St. Vincent's Institute of Medical Research, 9 Princes Street, Fitzroy, Victoria 3065, Australia

Tel: 0061 3 9288 2480; Fax: 0061 3 9416 2676; E-mail: mparker@svi.edu.au

Received August 09, 2008; Accepted October 14, 2008; Published December 05, 2008

Citation: David BA, Gabriela ANC, Hooi LN, Craig JM, Michael WP, et al. (2008) Novel Therapeutic Approaches to Treat Alzheimer's Disease and Memory Disorders. J Proteomics Bioinform 1: 464-476. doi:10.4172/jpb.1000054

Abstract

Cognitive decline most commonly associated with Alzheimer's dementia can also result from other conditions including cerebral ischemia or brain trauma. One quarter of people over the age of 65 are estimated to suffer some form of cognitive impairment underscoring the need for effective classes of cognitive-enhancing agents. In this mini-review we highlight recent work from our laboratory on the structural biology of Alzheimers disease and other memory disorders that was presented at the recent PRICPS – AOHUPO 2008 Conference held in Cairns, Australia. Our current work is focused on two proteins, amyloid precursor protein and insulin-regulated aminopeptidase, that are promising targets for the development of anti-Alzheimer's drugs and as cognitive enhancers. In both cases structures determined by X-ray crystallography are being used to discover promising lead compounds by structure-based drug design.

Keywords: Alzheimer's disease; Amyloid precursor protein; Drug design; Insulin-regulated aminopeptidase; X-ray crystallography

Abbreviations

Aβ: the peptide generated by cleavage of amyloid precursor protein; AD: Alzheimer's disease; Ang IV: Angiotensin IV; AOHUPO: 4th Asian-Oceania Human Proteome Organization; APA: Aminopeptidase A; APB: Aminopeptidase B; APN: Aminopeptidase N; APP: Amyloid Precursor Protein; ColAP: Cold Aminopeptidase; ERAP: Endoplasmic Reticulum Aminopeptidase; IRAP: Insulin-regulated Aminopeptidase; LTA4H: Leukotriene A4 hydrolase; PFA: Antibody that recognizes the Aβ peptide; PRICPS: 2nd Pacific Rim International Conference on Protein Science; TRDE: pyroglutamyl peptidase II; WO2: antibody that recognizes the Aβ peptide

Introduction

Approximately one quarter of people over the age of sixty five are estimated to suffer some form of cognitive impairment. The incidence of age-related neurological diseases is escalating primarily due to the increased life expectancy of the general population of many developed nations. One of the more prevalent and debilitating neurological disorders is Alzheimer's disease (AD).

AD is the leading cause of dementia among the elderly and is characterized by the presence of amyloid plaques,

Full Paper Accepted from the Joint 2nd Pacific Rim International Conference on Protein Science and 4th Asian-Oceania Human Proteome Organization, Cairns- Australia, 22-26 June 2008

extensive neuronal death and shrinkage of the brain. It is increasingly accepted that the neurotoxic Ab peptide is responsible for compromising neuronal functions and triggering cell death (Selkoe, 2002; Pereira et al., 2004). The peptide is derived from the cleavage of the amyloid precursor protein (APP) and is the main constituent of the amyloid plaques (Kang et al., 1987). Ab arises through the sequential cleavage by the b-site APP cleaving enzyme (BACE) in the ectodomain near the transmembrane domain and by the g-secretase protein complex within the membrane. This processing is thought to occur in endosomes or in an endosome-trans golgi network recycling pathway. APP is a Type-I transmembrane protein with a large extracellular portion, which has been structurally and functionally subdivided into several domains. We initiated a structural biology program focused on APP about ten years ago with aim of determining its structure as the basis for understanding its function and for structure-guided drug design. The program has been fruitful with numerous structures of the various APP domains now determined (Rossjohn et al., 1999; Barnham et al., 2003; Kong et al., 2005; Kong et al., 2007a; Kong et al., 2007b; Kong et al., 2008; Miles et al., 2008; Wun et al., 2008).

AD drug development strategies have focused on modulation of the Ab processing enzymes (β and γ secretases) and prevention of Aβ aggregation or oligomerization (Wolfe, 2002). Currently all drugs approved by the US Food and Drug Administration for AD address disease symptoms. Most belong to the class of cholinesterase inhibitors, which are of limited efficacy and only indicated for the treatment of mild-to-moderate forms of the disease (Birks, 2006). In spite of this, many drugs currently being developed to treat cognitive decline in AD are still targeting central cholinergic systems (www.alzforum.org/drg/drc). Development of memory-enhancing drugs is gaining momentum because of their increasingly widespread application in the treatment of other forms of memory disorders, including mild cognitive impairment, as well as that resulting from brain trauma and ischemic damage. Here we describe our recent studies on APP and on a protein receptor involved in memory.

Structural studies of an amyloid peptide-antibody complex

One promising stream of research to develop treatments or prophylactics for AD is anti-amyloid-β (Aβ immunotherapy) which aims to promote Aβ peptide clearance from the brain. In January 2002, however, the outlook for immunotherapies to AD took a pessimistic turn when the first clinical trials of active vaccination for AD were halted after a small subset of patients developed sterile meningoencephalitis. The synthetic Aβ peptide antigen from those trials, AN-1792, is thought to have elicited a severe T-cell mediated immune response inducing a higher incidence of meningoencephalitis. Despite this setback, it was subsequently demonstrated that vaccination with AN-1792 elicited a positive antibody response and that antibodies did not cross-react with APP as first feared. It was also shown that cognitive function was stabilised in over half of patients with high antibody titer (Weksler, 2004).

Six years later, Elan Corporation and Wyeth Pharmaceuticals, who initiated the AN-1792 trials, have begun a Phase 3 clinical program of bapineuzumab (AAB-001), for the treatment of patients with mild to moderate Alzheimer's disease. Bapineuzumab is a humanised monoclonal antibody targeting Aβ and is the first antibody based therapy to AD to enter Phase 3 clinical trials. It is hoped that by taking a passive immunotherapy approach, detrimental immune responses like those elicited by the AN-1792 immunogen can be avoided.

Results of the small Phase 2 clinical trial of Bapineuzumab on 240 patients showed significant slowing in the rate of mental decline and brain atrophy in some Alzheimer's patients receiving Bapineuzumab compared with placebo control patients. Unfortunately, in the Phase 2 trials twelve patients developed an accumulation of fluid on the brain known as vasogenic edema. Vasogenic edema was shown to occur at high doses and predominantly in patients carrying the E4 allele of apolipoprotein, a gene known to predispose a carrier to AD. With the incidence of vasogenic edema in mind, the Phase 3 trials initiated by Wyeth and Elan in 2007 were designed to treat APO E4 carriers and non-carriers separately (http://www.alzforum.org/new/detail.asp?id=1850). In addition to safety concerns raised in the Phase 2 trials, efficacy trends were weaker than expected. However, the much larger Phase 3 trials on Bapineuzumab will establish the true efficacy of this particular therapeutic in sufferers with mild to moderate forms of the disease.

A long term follow up study of the Phase 1 AN-1792 trial showed that immunisation of patients with pre-fibrillated Aβ (AN-1792) initiated a long term reduction in Aβ load and post mortem evidence of a plaque removal 5 years after the last injection. (Holmes et al., 2008). In addition, the two patients with almost complete elimination of plaques and lowest Aβ loads were shown to have had the highest antibody response during the Phase 1 trial. Unfortunately, there was no evidence that immunisation with AN-1792 had an impact on cognitive decline in the small cohort assessed in the follow up study. It may be that immunotherapies targeting

fibrillar Aβ (such as AN-1792) are not effective at reducing the concentration of soluble oligomeric forms of the peptide, and it has been suggested that disintegration of plaques may increase the concentration of these oligomers implicated in synaptic dysfunction and dementia in Alzheimer's disease (Patton et al., 2006).

Antibodies raised against Aβ that have been shown to reduce plaque burden and improve cognitive deficits in mouse models of AD are those raised against the N-terminal region of Aβ (McLaurin et al., 2002; Frenkel et al., 2003; Chauhan and Siegel, 2005). Following the AN-1792 trials, development of passive and active immunotherapies targeting Aβ has focused on this N-terminal region (~ residues 1 to 14), which contains the immunodominant B-cell epitope of Aβ but lacks the T-cell epitopes arguably responsible for abandonment of the AN-1792 trials. A number of antibodies specific to this region have been studied for their ability bind Aβ, inhibit fibrillogenisis and cytotoxicity, and reduce plaque burden *in vivo* (Solomon, 2004). In addition, several engineered immunogens based on this B-cell epitope have been proposed as vaccine candidates. Considering the intense interest in antibody based therapies targeting Aβ, it is surprising that only recently has structural data emerged characterising the molecular basis for antibody recognition of this important pathogen.

Crystal Structures

Concomitant structures of monoclonal antibody fragments (Fabs) with specificity for the immunodominant B-cell epitope of Aβ have been reported for three Fabs in unliganded states and in complex with Aβ peptides. Dealwis and co-workers (Gardberg et al., 2007) reported high resolution 3D structures of Fabs from the monoclonal antibodies PFA1 and PFA2 in complex with the $A\beta_{1-8}$ peptide (DAEFRHDS). We reported our own near-atomic resolution structures (Miles et al., 2008) for the antigen binding fragment of the WO2 antibody complexed to $A\beta_{1-16}$ and $A\beta_{1-28}$ peptides. Seven residues of Aβ (Ala 2 to Ser 8) have been modelled from electron density when complexed to PFA1, PFA2 or WO2. From our own work, extending the length of the peptide from 16 to 28 residues yielded equivalent structures with no electron density observed beyond this range, corresponding to peptide outside the restraints of the antigen binding site of WO2.

PFA1 and PFA2 have identical sequences across their light chains and indeed the WO2 light chain sequence is very similar (95% sequence identity) to the PFA light chains. Across the heavy chains PFA1 and PFA2 sequences share 96% sequence identity, whereas the WO2 heavy chain aligns to the PFA sequences with around 85% identity. The clearest point of difference is in the hypervariable CDR3 of the heavy chains, where the WO2 sequence is unique. A comparison of apo and liganded structures showed that the PFA antibodies change conformation by around 1 Å across the backbone of VH CDR3 upon binding Aβ, whereas WO2 CDRs remain virtually unchanged on binding Aβ. In this respect,

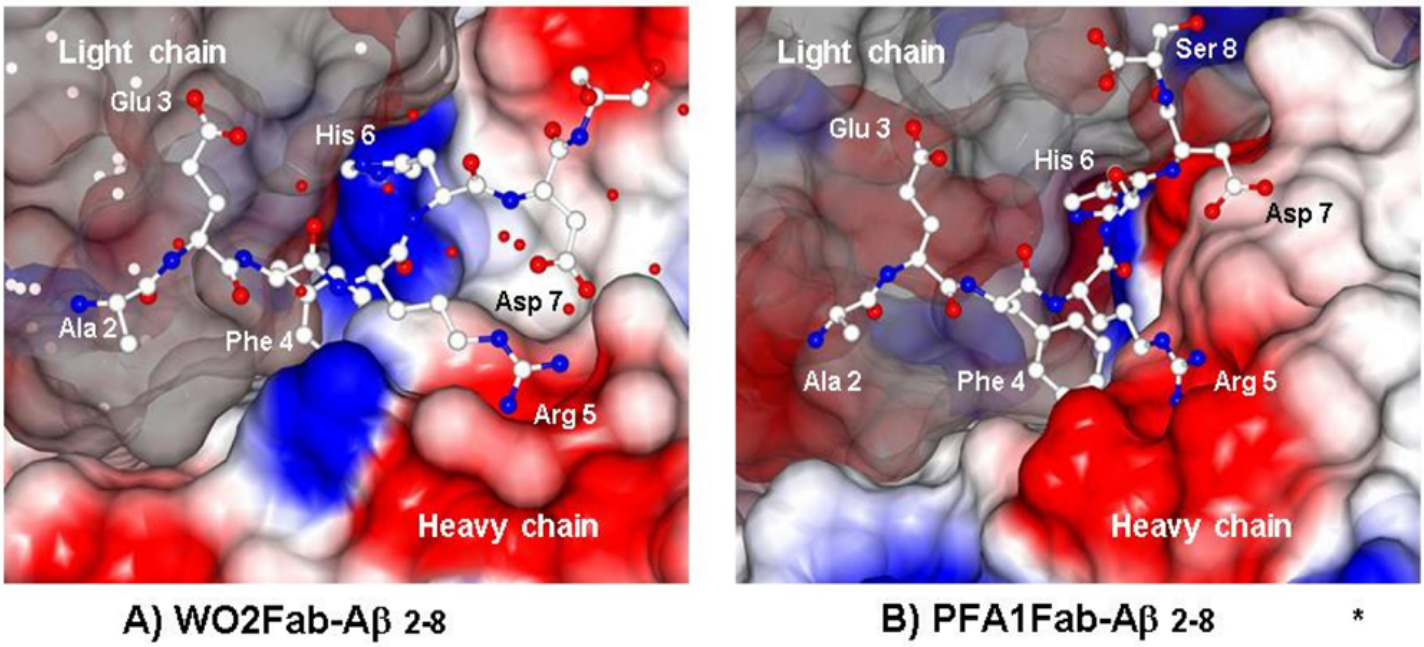

Figure 1: Electrostatic surface electrostatic representation of the WO2Fab antibody (A) and the PFA1 antibody (B) in complex with the Aβ peptide. In both representations the solid white is the heavy chain of the antibody and the translucent-dark region is the light chain. The surfaces are coloured by electrostatic potential with negative charge shown in red and, positive charge in blue. The Aβ peptide is shown as ball-and-stick and water molecules as small spheres.

WO2 represents a more rigid template for the design of engineered proteins that recognise the immunodominant B-cell epitope of Aβ.

Figure 1 shows Aβ in the binding cleft of the PFA1 and WO2 antibodies. A point of distinction between these structures is that in WO2 there is a large water filled cavity in the heavy chain beneath Asp 7 of Aβ. Unlike PFA1, WO2 makes no close contact or water mediated interaction with Asp 7 of Aβ. This may be a useful distinction between the two antibody binding sites considering the work of Zirah and coworkers who conducted an *in vitro* aging study of aspartates in $Aβ_{1-16}$ following a finding that an unusually high content of racemised and isomerised aspartates (common protein damage phenomena associated with ageing) were found in Aβ from amyloid deposits (Zirah et al., 2006). Zirah and coworkers showed that the major isomer generated in $Aβ_{1-16}$ that was aged *in vitro* was L-iso-Asp 7. Asp 7 is the only amino acid in the WO2 recognition epitope of Aβ not to make hydrogen bonds, salt bridges, or significant van der Waals contacts with WO2. Rather, it appears to be rigidified in the antibody by hydrogen bonds between its side chain and that of the adjacent Arg 5 of Aβ. Taken together with the fact that WO2 makes no direct contact with Asp 1 of Aβ, the WO2 Fv domain represents a promising lead for the development of an immunotherapy targeting the key region of Aβ, between the α- and β-secretase cleavage sites, with likely promiscuity for preserved and aged forms of the peptide.

Figure 2 shows schematic representations of the molecular basis for PFA1 and WO2 recognition of the Aβ immunodominant B-cell epitope. In the figure, interactions conserved between Aβ and the antibodies are highlighted in yellow, these occur predominantly at the light chain interface with Aβ. Again, the hydrogen bonding network that restrains Asp 7 of Aβ in the PFA antibody is absent in the WO2 mode of binding, and with Asp 7 unrestrained, the conformation of the C-terminus of this epitope is stabilised in WO2 by hydrophobic interaction between Ser 8 of Aβ and Tyr 100B (H) in CDR3 of WO2.

Despite these differences in binding, the conformation of Aβ when liganded to WO2 or PFA antibodies is remarkably similar across the core epitope EFRH (see superposition in Figure 3). This conformation should serve as a reliable template for the design of antigens for active immunotherapies targeting Aβ.

Structural studies of insulin-regulated aminopeptidase – a protein receptor involved in memory

A variety of brain functions are regulated by a disparate family of neuropeptides. An interesting example of these is one of the bioactive degradation products in the renin–angiotensin system, Angiotensin IV (Ang IV; Val-Tyr-Ile-His-Pro-Phe), a metabolite that possesses very different biological properties to its precursors. Intra-cranial administration of Ang IV or its analogue Nle1-Ang IV has been shown to enhance memory retrieval and retention in several animal models (Braszko et al., 1988; Wright et al., 1993; Wright et al., 1999; Braszko, 2004), while its precursors Ang I to Ang III have no effect. While able to facilitate learning and memory in normal rodents, Ang IV was also able to ameliorate the deficits in a range of rat models of amnesia, including scopolamine (Pederson et al., 1998) and mecalymine treatment (Olson et al., 2004), ischemic damage (Wright et al., 1996), chronic alcohol exposure (Wisniewski et al., 1993) and bilateral perforant pathway lesions (Wright et al., 1999). Ang IV is known to stimulate DNA synthesis and enhance thymidine incorporation and may be involved in several neurobiological actions including neural development and neuron survival (Hall et al., 1995).

It has been proposed that the AT4 receptor, the only specific binder of Ang IV in the brain, might be responsible for these effects (Wright and Harding, 1994). The AT4 receptor was subsequently identified as the transmembrane enzyme, insulin regulated aminopeptidase (IRAP), which occurs abundantly in vesicles associated with the insulin-sensitive glucose transporter GLUT4 in a wide variety of tissues (Albiston et al., 2003). Along with GLUT4, IRAP translocates to the plasma membrane in response to insulin and is rapidly internalised in the absence of this signal (Kanzaki, 2006; Huang and Czech, 2007). IRAP belongs to the M1 family of zinc metallopeptidases, which includes the aminopeptidases A, N and B (Rogi et al., 1996). This class of enzymes is characterized by two common structural features - a Zn^{2+}-binding motif $HEXXH(X)_{18}$-E and an exopeptidase motif GXMEN (Iturrioz et al., 2001). IRAP is a type II membrane-spanning protein with an extracellular catalytic site (Keller et al., 1995; Rogi et al., 1996). The extracellular domain of IRAP contains 18 potential N-linked glycosylation sites as well as a putative cleavage site (F^{154}/A^{155}) (Rogi et al., 1996) for release of the extracellular domain.

Following identification of IRAP as the AT4 receptor (Albiston et al., 2003), it was shown that Ang IV was a specific inhibitor of IRAP's aminopeptidase activity (Lew et al., 2003). This observation has led to the hypothesis that modulation of IRAP's aminopeptidase activity may be responsible for the cognitive effects seen with Ang IV, possi-

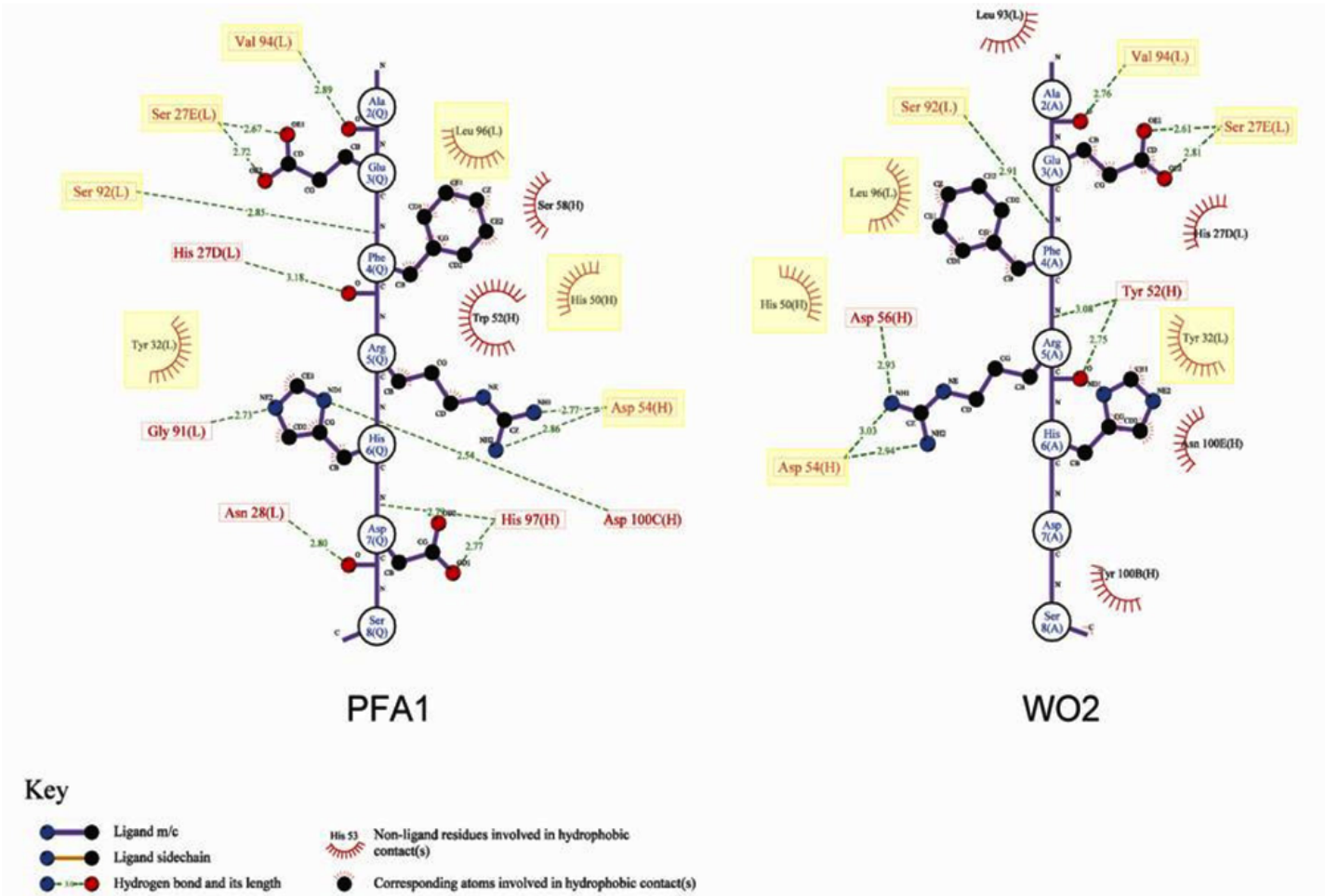

Figure 2: Schematic representations of the molecular basis for PFA1 and WO2 recognition of the Aβ immunodominant B-cell epitope. This figure was produced using LIGPLOT (Wallace et al., 1995).

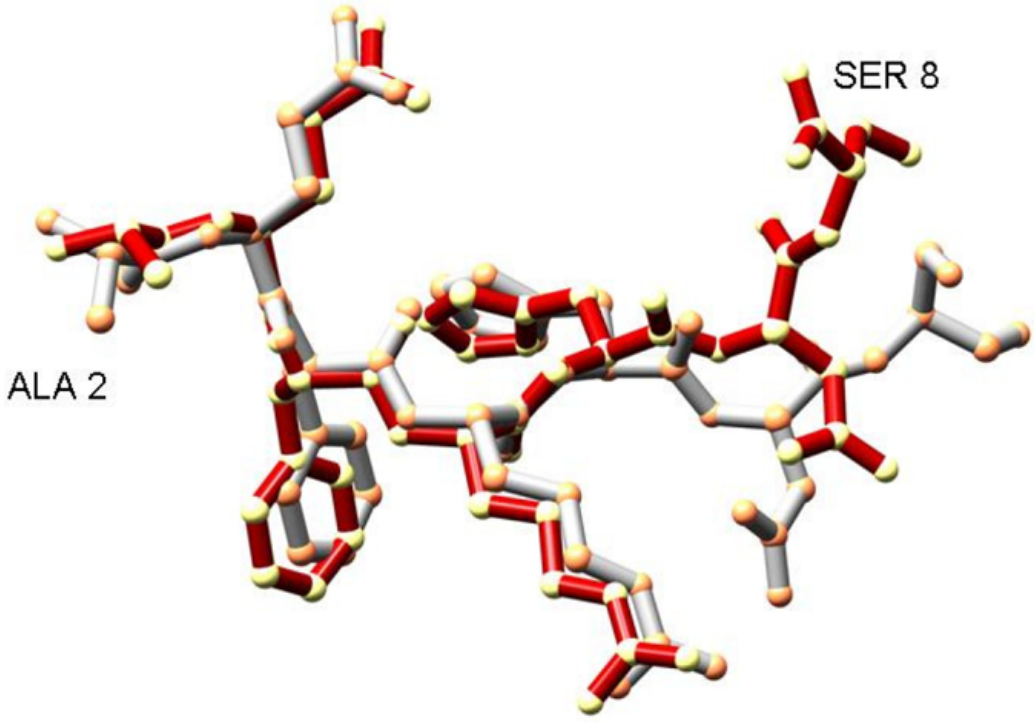

Figure 3: Superposition of the two Aβ 2-8 peptides when liganded to WO2 (red) and PFA (grey).

bly through prolonged circulation of neuropeptides that potentiate memory. This is supported by the observation that several *in vitro* substrates of IRAP, including vasopressin (de Wied et al., 1993; Engelmann et al., 1996), CCK-8 (Dauge and Lena, 1998; Fink et al., 1998; Huston et al., 1998; Sebret et al., 1999; Voits et al., 2001; Dauge et al., 2003), oxytocin (Tomizawa et al., 2003) and somatostatin (Schettini et al., 1988; DeNoble et al., 1989) have been shown to facilitate learning and memory in rodents in aversion conditioning and spatial learning tasks. Testing of this hypothesis was carried out through examining the effects of another known IRAP inhibitor, the peptide LVV-hemorphin-7, on memory and it was shown that this inhibitor had a very similar effect to Ang IV (Lee et al., 2004). While this does not unambiguously establish the actual mechanism and role of the inhibitors and IRAP in improving memory, it adds further support to the neuropeptide protection model. Other hypotheses for these effects that have been suggested include: (1) the regulation of GLUT4 translocation and therefore glucose uptake and neuronal activity, or (2) the direct activation of a signalling pathway. Evidence for the direct role of IRAP in the facilitation of this memory enhancement remains circumstantial.

Bioinformatics

Computational analysis of the IRAP amino acid sequence suggests that IRAP is comprised of several distinct regions (Fig. 4). Starting at the N-terminus, the cytoplasmic tail is predicted to be primarily disordered with some α-helical

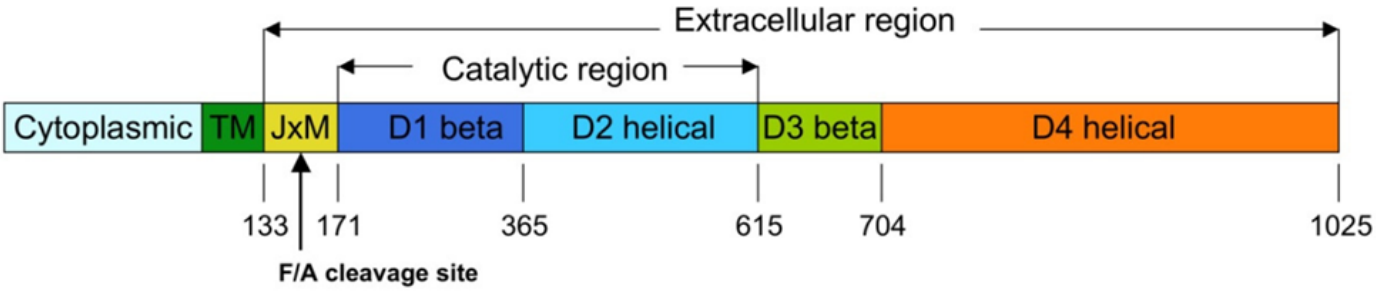

Figure 4: Diagrammatic representation of the domain arrangement of IRAP based on analysis of the IRAP amino acid sequence.

structure. Interestingly, IRAP is the only member of the M1 family that has a large cytoplasmic tail and it is believed that this tail may be involved in the trafficking of IRAP and GLUT4. This tail is followed by a typical single pass transmembrane region. Immediately proximal to the other side of the membrane is a β-sheet region, followed by an α-helical region that contains the conserved active site residues $HEXXH(X)_{18}$-E and GXMEN. A short β-sheet region then separates this from the helical final region whose function is unknown, although it has been proposed to act as a molecular chaperone (Rozenfeld et al., 2004).

Crystal Structures

To date no crystal structure of IRAP has been solved. Structures of several similar enzymes, however, have been determined including human leukotriene A4 hydrolase (LTA4H, 1HS6, 10% sequence identity to IRAP, (Thunnissen et al., 2001)), aminopeptidase N from *E. coli* (APN, 2DQ6, 11% sequence identity to IRAP, (Ito et al., 2006)) and *N. meningitides* (2GTQ, 12% sequence identity to IRAP, (Nocek et al., 2008)), *T. acidophilum* tricorn protease interacting factor F3 (Tricorn, 1Z5H, 17% sequence identity to IRAP, (Kyrieleis et al., 2005)) and cold aminopeptidase from *C. psychrerythraea* (ColAP, 3CIA, 10% sequence identity to IRAP, (Bauvois et al., 2008)).

LTA4H was the first M1 aminopeptidase family member for which a structure was solved (Fig. 5A, (Thunnissen et al., 2001)). LTA4H has a relatively short C-terminal region, similar to the more recent structure of cold aminopeptidase and the backbones of LTA4H and ColAP superimpose well with a root mean square deviation (r.m.s.d.) of 1.2 Å for 509 Cα atoms (Bauvois et al., 2008). They are both folded into three distinct domains (equivalent to D1, D2 and D4 of the proposed domain structure of IRAP, (Fig. 4)). D1 is composed of β-sheets which present a large surface to the solvent (Fig. 5). The catalytic domain (D2) is mainly composed of α-helices with the active site residues located on two helices. The active centre is located in a groove that consists of one β-strand and three α-helices in what has been described as a thermolysin-like catalytic domain (Thunnissen et al., 2001; Bauvois et al., 2008). D4 is α-helical and, of the three domains, has the lowest level of sequence conservation between LTA4H and ColAP (18% identity and 1.6 Å r.m.s.d. compared to 38% and 0.8 Å for

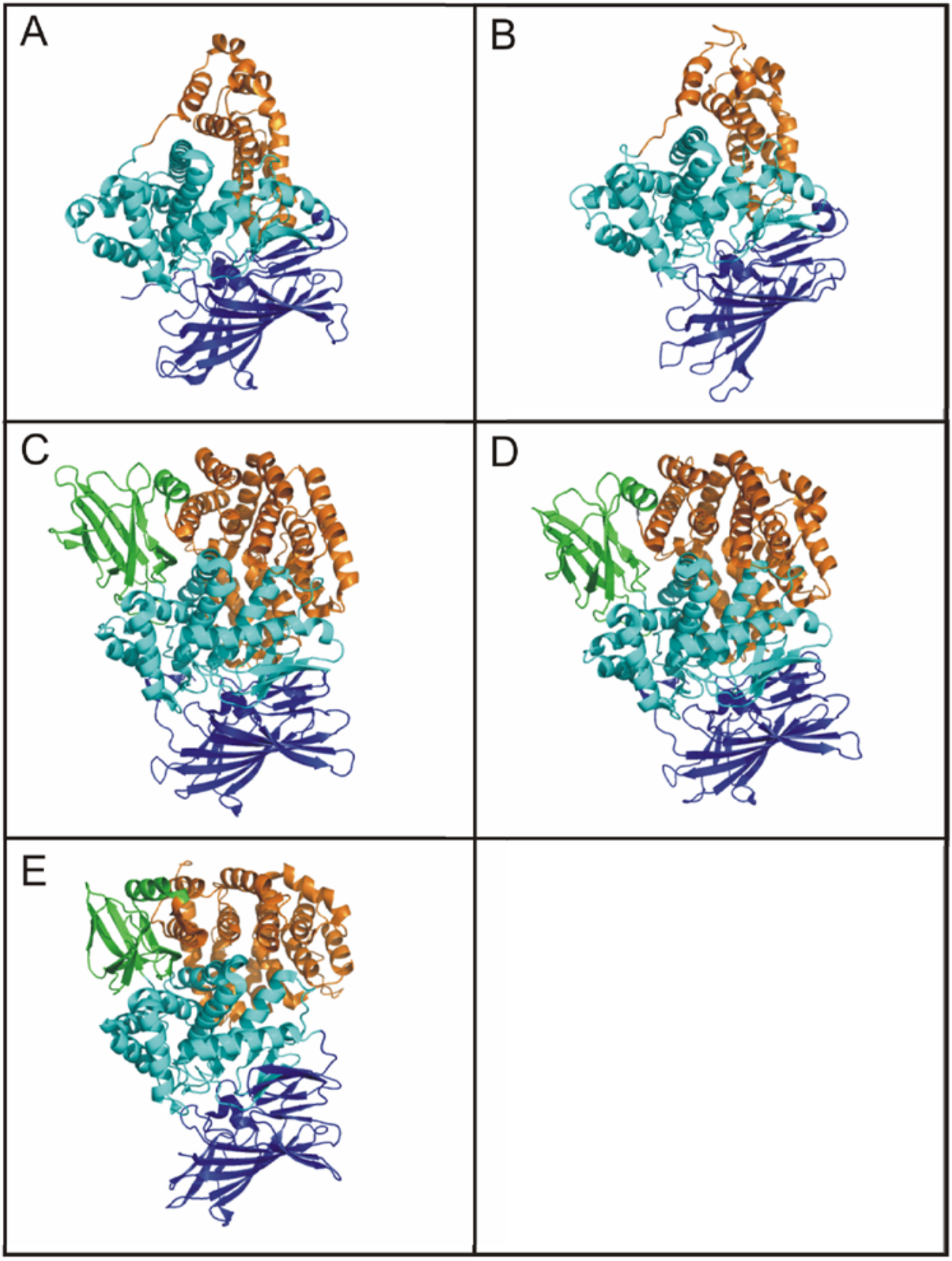

Figure 5: Cartoon representation of the known structures from members of the M1 aminopeptidase family. Structures are coloured by domain (using the notation in Fig. 4). (A) LTA4H. (B) ColAP. (C) *E. coli* APN. (D) *N. meningitides* APN. (E) Tricorn.

D1 and 41% and 1.2 Å for D2, respectively).

While APN (Fig. 5C and 5D) and Tricorn interacting factor F3 (Fig: - 5E) share the three domains described for ColAP and LTA4H, they contain an additional, small, barrel-like domain of β-strands (equivalent to D3 in the proposed domain structure of IRAP (Fig. 4)) located between the catalytic domain, D2, and the C-terminal domain, D4. This arrangement more closely resembles the predicted secondary structure of IRAP. As with LTA4H and ColAP, the major differences at both the sequence and 3D structure levels are found in D4. One of the most striking differences is in respect to the relative positioning of this domain with respect to D1 and D2. With LTA4H, ColAP and APN this C-terminal domain is folded back upon D2, such that they are situated quite close to each other. By contrast the overall structure of the tricorn protease more closely resembles that of a hook, with D4 positioned further away from D2, opening up the catalytic site. This means that Tricorn protease has a deep and wide cleft with its active site largely exposed to solvent, although it has been proposed that it may undergo a conformation change in D4 that may generate a closed-structure similar to those seen for the other M1 family members (Kyrieleis et al., 2005; Addlagatta et al., 2006). While these closed structures may only result from crystal contacts the ability of crystals of *E. coli* APN to absorb small molecules in crystal-soaking experiments suggests that the active site is still accessible. Movement between open and closed conformations may be a mechanism of regulating activity or substrate specificity (Ito et al., 2006).

Homology Modelling

For such large proteins with relatively low overall sequence identity, the crystal structures determined to date superimpose quite well, particularly over the catalytic region. This provides confidence in the reliability of homology models for other members of the M1 family developed using these structures as templates. To date the LTA4H structure has been used to generate models of the catalytic sites for APA (Rozenfeld et al., 2002; Goto et al., 2007; Claperon et al., 2008), APB (Pham et al., 2007), pyroglutamyl peptidase II (TRHDE) (Chavez-Gutierrez et al., 2006) and IRAP (Ye, et al., 2008) to try and identify key residues important in the biological activity of these enzymes. One example where this has been quite spectacularly successful is in the identification of residues responsible for the modulation of APA activity by calcium (Claperon et al., 2008). Earlier studies had identified other residues that exerted similar effects (Iturrioz et al., 2000; Goto et al., 2007), but with the model the authors were able to pinpoint the exact residues responsible and propose a mechanism by which calcium exerts its effects on APA. Another example of note is where models of TRHDE were used to try and explain the structural differences between standard M1 aminopeptidases and TRHDE, which is an omegapeptidase but still classed in the M1 family (Chavez-Gutierrez et al., 2006). By using their model to identify key differences between the omegapeptidase and aminopeptidase, they were able to mutate TRHDE such that it lost its omegapeptidase activity and displayed instead alanyl-aminopeptidase activity.

The lower sequence identity over D4 reduces the reliability of models built upon this domain. Coupled with this lower reliability is the variable orientation and close interaction of D4 with the active site seen in the published structures, meaning that models built using the structures as templates can only paint a limited picture of the regulation of enzyme activity. With the availability of the new structures of M1 aminopeptidases, however, more detailed models and improved insights will hopefully be gained.

Mechanism of activity and understanding substrate specificity

A detailed mechanism of action for M1 aminopeptidase activity was proposed by Ito et al. (2006) and computational docking of the synthetic substrate Leu-MCA into a molecular model of IRAP generates highly favourable solutions showing interactions consistent with this mechanistic description (Ye et al., 2008). Proton donation by Tyr 529 of IRAP completes hydrolysis of the N-terminal peptide bond of the substrate with interactions with the zinc ion stabilising the transition (Figure 6).

The crystal structures provide structural support for other biochemical data in proposing a mechanism for cleavage. These structures by themselves, however, have not provided significant insight into the substrate specificity of the M1 family, particularly considering the active site region shows high sequence and structural similarity. It is apparent that only small changes are responsible for determining substrate specificity. For example, only two mutations resulted in the change of TRHDE from an omegapeptidase to an aminopeptidase (Chavez-Gutierrez et al., 2006). Models of other members of the M1 family can used to provide insights into this question and to help design mutants to further understand substrate specificity.

With this aim in mind we compared our model of IRAP to LTA4H. Two key residues, Ala 427 and Leu 483, were identified which presented a more open arrangement of the S1 subsite of IRAP compared to LTA4H, where the corre-

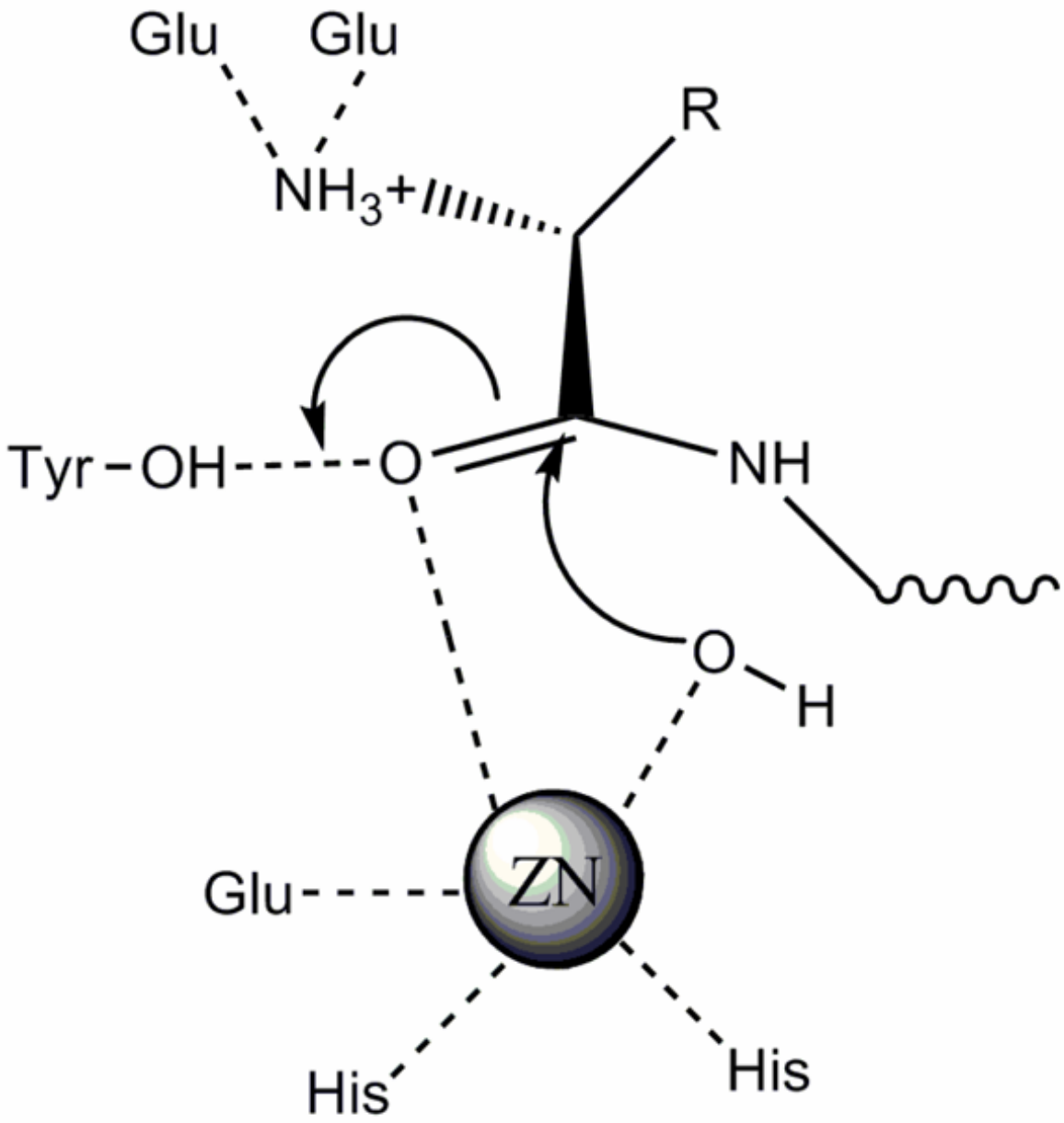

Figure 6: Schematic showing the mechanism for M1 aminopeptidase activity proposed by Ito and colleagues (2006).

sponding residues (Tyr 267 and Phe 314) are bulky aromatics (Ye et al., 2008). Alteration of the S1 subsite of IRAP by the corresponding mutations resulted in altered activity and substrate specificity. The IRAP mutant L483F, although capable of efficiently cleaving the N-terminal Cys from vasopressin, was unable to cleave the tyrosine residue from either leu-enkephalin or cyt6-vasopressin. The mutant also showed a dramatic reduction in the affinity of the inhibitor norleucine[1]-angiotensin IV whereas the affinity of Ang IV remained unaltered. Introducing an A427Y mutation to IRAP, by contrast, resulted in increased binding affinity of the peptide substrates without a corresponding increase in the rate of cleavage. This not only suggests a specific role for the two residues, Ala 427 and Leu 483, in defining the catalytic cleft of IRAP but confirms that substrate specificity is likely a result of small differences imparted from the chemical nature of the residues between the family members.

Inhibitors

The development of new, specific M1 aminopeptidase inhibitors is a difficult and complicated task due to the high sequence identity and structural homology of the catalytic sites of these proteins. The multiple biological roles of members of the M1 aminopeptidase family, however, require highly specific inhibitors for use both therapeutically and for

physiological investigations. The existing crystal structures of family members, however, do provide a basis for attempting the rational development of selective inhibitors. An example of this is the use of the structure of LTA4H computationally docked to a known inhibitor to develop a pharmacophore model then used to identify a subset of compounds that inhibited LTA4H activity (Grice et al., 2008). One of these compounds was a high affinity inhibitor *in vitro*, orally bioavailable and showed *in vivo* activity.

Most physiological studies of IRAP activity have utilised the two known peptide inhibitors, Ang IV and LVV-H7, and their modified forms (Albiston et al., 2007). These have some key limitations, including their short half-life and promiscuous inhibitory activity. One way of overcoming some of these limitations has been the development of cyclic peptide inhibitors that have higher IRAP affinity and longer half-lives (Axen et al., 2006; Axen et al., 2007). Unfortunately these cyclic peptides are still not selective enough for therapeutic use or detailed physiological investigation; for example the best cyclic inhibitor also inhibits APN.

We have taken a slightly different approach to our attempts to identify new inhibitors. Using a model of the active site of IRAP based upon the crystal structure of LTA4H, we used *in silico* screening and *in vitro* assays, some of the latter performed in the lab of our collaborators, Drs Anthony Albiston and Siew Yeen Chai, to identify a family of highly specific, competitive, non-peptide inhibitors (Albiston et al., 2008). Since these compounds are not peptidic but instead small molecule inhibitors they are amenable to medicinal chemistry modification to tune their activities and pharmacokinetic properties. Members of the family of compounds show negligible activity against some of IRAP's closest relatives, including ERAP1, ERAP2, APN and LTA4H. When administered intracranially they showed the same memory enhancement effects seen for classic IRAP inhibitors Ang IV and LVV-H7, providing further support for the effects being mediated directly through IRAP. These inhibitors are providing the basis for ongoing research into the development of a new class of therapeutics for the treatment of memory amelioration associated with Alzheimer's and more generally, other dementias.

Acknowledgments

We would like to thanks our many collaborators in our structural biology studies of Alzheimer's disease proteins. In particular we thank Kevin Barnham, Roberto Cappai, Colin Masters, Anthony Albiston, Siew Yeen Chai and Fred Mendelsohn. This work was supported by a grant from the National Health and Medical Research Council of Australia (NHMRC). D.B.A. is an Australian Postgraduate Award Scholar and a recipient of a St Vincent's Institute Foundation Scholarship sponsored by Colin North and Major Engineering. M.W.P. is an Australian Research Council Federation Fellow and an NHMRC Honorary Fellow.

References

1. Addlagatta A, Gay L, Matthews BW (2006) Structure of aminopeptidase N from Escherichia coli suggests a compartmentalized, gated active site. Proc Natl Acad Sci USA 103: 13339-13344.» CrossRef » Pubmed » Google Scholar

2. Albiston AL, Morton CJ, Ng HL, Pham V, Yeatman HR, et al. (2008) Identification and characterization of a new cognitive enhancer based on inhibition of insulin-regulated aminopeptidase. FASEB J 22: 4209-4217.» CrossRef » Pubmed » Google Scholar

3. Albiston AL, Mustafa T, McDowall SG, Mendelsohn FA, Lee J, et al. (2003) AT4 receptor is insulin-regulated membrane aminopeptidase: potential mechanisms of memory enhancement. Trends Endocrinol Metab 14: 72-77.» CrossRef » Pubmed » Google Scholar

4. Albiston AL, Peck GR, Yeatman HR, Fernando R, Ye S, et al. (2007) Therapeutic targeting of insulin-regulated aminopeptidase: Heads and tails? Pharmacol Ther 116: 417-427.» CrossRef » Pubmed » Google Scholar

5. Axen A, Andersson H, Lindeberg G, Ronnholm H, Kortesmaa J, et al. (2007) Small potent ligands to the insulin-regulated aminopeptidase (IRAP)/AT(4) receptor. J Pept Sci 13: 434-444.» CrossRef » Pubmed » Google Scholar

6. Axen A, Lindeberg G, Demaegdt H, Vauquelin G, Karlen A, et al. (2006) Cyclic insulin-regulated aminopeptidase (IRAP)/AT4 receptor ligands. J Pept Sci 12: 705-713. » CrossRef » Pubmed » Google Scholar

7. Barnham KJ, McKinstry WJ, Multhaup G, Galatis D, Morton CJ, et al. (2003) Structure of the Alzheimer's disease amyloid precursor protein copper binding domain. J Biol Chem 278: 17401-17407. » CrossRef » Pubmed » Google Scholar

8. Bauvois C, Jacquamet L, Huston AL, Borel F, Feller G, et al. (2008) Crystal structure of the cold-active aminopeptidase from *Colwellia psychrerythraea*, a close structural homologue of the human bifunctional leukotriene A4 hydrolase. J Biol Chem 283: 23315-23325. » CrossRef » Pubmed » Google Scholar

9. Birks J (2006) Cholinesterase inhibitors for Alzheimer's disease. Cochrane Database Syst Rev CD005593. » CrossRef » Pubmed » Google Scholar

10. Braszko JJ (2004) Involvement of D1 dopamine recep-

tors in the cognitive effects of angiotensin IV and des-Phe6 angiotensin IV. Peptides 25: 1195-203. » CrossRef » Pubmed » Google Scholar

11. Braszko JJ, Kupryszewski G, Witczuk B, Wisniewski K (1988) Angiotensin II-(3-8)-hexapeptide affects motor activity, performance of passive avoidance and a conditioned avoidance response in rats. Neuroscience 27: 777-783. » CrossRef » Pubmed » Google Scholar

12. Chauhan NB, Siegel GJ (2005) Efficacy of anti-Abeta antibody isotypes used for intracerebroventricular immunization in TgCRND8. Neurosci Lett 375: 143-147. » CrossRef » Pubmed » Google Scholar

13. Chvez-Gutirrez L, Matta-Camacho E, Osuna J, Horjales E, Joseph-Bravo P, et al. (2006) Homology modeling and site-directed mutagenesis of pyroglutamyl peptidase II. Insights into omega-versus aminopeptidase specificity in the M1 family. J Biol Chem 281: 18581-18590. » CrossRef » Pubmed » Google Scholar

14. Claperon C, Rozenfeld R, Iturrioz X, Inguimbert N, Okada M, et al. (2008) Asp-218 participates with aAsp-213 to bind a Ca2+ atom into the S1 subsite of aminopeptidase A: a key element for substrate specificity. Biochem J 416: 37-46. » Pubmed » Google Scholar

15. Dauge V, Lena I (1998) CCK in anxiety and cognitive processes. Neurosci Biobehav Rev 22: 815-825. » CrossRef » Pubmed » Google Scholar

16. Dauge V, Pophillat M, Crete D, Melik PS, Roques BP (2003) Involvement of brain endogenous cholecystokinin in stress-induced impairment of spatial recognition memory. Neuroscience 118: 19-23. » CrossRef » Pubmed » Google Scholar

17. de Wied D, Diamant M, Fodor M (1993) Central nervous system effects of the neurohypophyseal hormones and related peptides. Front Neuroendocrinol 14: 251-302. » CrossRef » Pubmed » Google Scholar

18. DeNoble VJ, Hepler DJ, Barto RA (1989) Cysteamine-induced depletion of somatostatin produces differential cognitive deficits in rats. Brain Res 482: 42-48» CrossRef » Pubmed » Google Scholar

19. Engelmann M, Wotjak CT, Neumann I, Ludwig M, Landgraf R (1996) Behavioral consequences of intracerebral vasopressin and oxytocin: focus on learning and memory. Neurosci Biobehav Rev 20: 341-358. » CrossRef » Pubmed » Google Scholar

20. Fink H, Rex A, Voits M, Voigt JP (1998) Major biological actions of CCK - a critical evaluation of research findings. Exp Brain Res 123: 77-83. » CrossRef » Pubmed » Google Scholar

21. Frenkel DI, Dewachter I, Van Leuven F, Solomon B (2003) Reduction of beta-amyloid plaques in brain of transgenic mouse model of Alzheimer's disease by EFRH-phage immunization. Vaccine 21: 1060-1065. » CrossRef » Pubmed » Google Scholar

22. Gardberg AS, Dice LT, Ou S, Rich RL, Helmbrecht E, et al. (2007) Molecular basis for passive immunotherapy of Alzheimer's disease. Proc Natl Acad Sci USA 104: 15659-15664. » CrossRef » Pubmed » Google Scholar

23. Goto Y, Hattori A, Mizutani S, Tsujimoto M (2007) Aspartic acid-221 is critical in the calcium-induced modulation of the enzymatic activity of human aminopeptidase A. J Biol Chem 282: 37074-37081. » CrossRef » Pubmed » Google Scholar

24. Grice CA, Tays KL, Savall BM, Wei J, Butler CR, et al. (2008) Identification of a potent, selective, and orally active leukotriene A4 hydrolase inhibitor with anti-inflammatory activity. J Med Chem 51: 4150-4169. » CrossRef » Pubmed » Google Scholar

25. Hall KL, Venkateswaran S, Hanesworth JM, Schelling ME, Harding JW (1995) Characterization of a functional angiotensin IV receptor on coronary microvascular endothelial cells. Regul Pept 58: 107-115. » CrossRef » Pubmed » Google Scholar

26. Holmes C, Boche D, Wilkinson D, Yadegarfar G, Hopkins V, et al. (2008) Long-term effects of Abeta42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. Lancet 372: 216-223. » CrossRef » Pubmed » Google Scholar

27. Huang S, Czech MP (2007) The GLUT4 glucose transporter. Cell Metab 5: 237-252» CrossRef » Pubmed » Google Scholar

28. Huston JP, Schildein S, Gerhardt P, Privou C, Fink H, et al. (1998). Modulation of memory, reinforcement and anxiety parameters by intra-amygdala injection of cholecystokinin-fragments Boc-CCK-4 and CCK-8s. Peptides 19: 27-37. » CrossRef » Pubmed » Google Scholar

29. Ito K, Nakajima Y, Onohara Y, Takeo M, Nakashima K, et al. (2006) Crystal structure of aminopeptidase N (proteobacteria alanyl aminopeptidase) from *Escherichia coli* and conformational change of methionine 260 involved in substrate recognition. J Biol Chem 281: 33664-33676. » CrossRef » Pubmed » Google Scholar

30. Iturrioz X, Rozenfeld R, Michaud A, Corvol P, Llorens-Cortes C (2001) Study of asparagine 353 in aminopeptidase A: characterization of a novel motif (GXMEN) implicated in exopeptidase specificity of monozinc aminopeptidases. Biochemistry 40: 14440-14448. » CrossRef » Pubmed » Google Scholar

31. Iturrioz X, Vazeux G, Celerier J, Corvol P, Llorens-Cortes C (2000) Histidine 450 plays a critical role in catalysis and, with Ca2+, contributes to the substrate specificity

of aminopeptidase A. Biochemistry 39: 3061-3068. »CrossRef » Pubmed » Google Scholar

32. Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, et al. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325: 733-736.» CrossRef » Pubmed » Google Scholar

33. Kanzaki M (2006) Insulin receptor signals regulating GLUT4 translocation and actin dynamics. Endocr J 53: 267-293. » CrossRef » Pubmed » Google Scholar

34. Keller SR, Scott HM, Mastick CC, Aebersold R, Lienhard GE (1995) Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from GLUT4 vesicles. J Biol Chem 270: 23612-23618. »CrossRef » Pubmed » Google Scholar

35. Kong GKW, Galatis D, Barnham KJ, Polekhina G, Adams JJ, et al. (2005) Crystallization and preliminary crystallographic studies of the copper-binding domain of the amyloid precursor protein of Alzheimer's disease. Acta Crystallogr Sect F Struct Biol Cryst Commun 61: 93-95. »CrossRef » Pubmed » Google Scholar

36. Kong GK, Adams JJ, Harris HH, Boas JF, Curtain CC, et al. (2007a) Structural studies of the Alzheimer's amyloid precursor protein copper-binding domain reveal how it binds copper ions. J Mol Biol 367: 148-161. »CrossRef » Pubmed » Google Scholar

37. Kong GK, Adams JJ, Cappai R, Parker MW (2007b) Structure of Alzheimer's disease amyloid precursor protein copper-binding domain at atomic resolution. Acta Crystallogr F 63: 819-824. » Pubmed

38. Kong GK, Miles LA, Crespi GA, Morton CJ, Ng HL, et al. (2008) Copper binding to the Alzheimer's disease amyloid precursor protein. Eur Biophys J 37: 269-279. »CrossRef » Pubmed » Google Scholar

39. Kyrieleis OJ, Goettig P, Kiefersauer R, Huber R, Brandstetter H (2005) Crystal structures of the tricorn interacting factor F3 from *Thermoplasma acidophilum*, a zinc aminopeptidase in three different conformations. J Mol Biol 349: 787-800.» CrossRef » Pubmed » Google Scholar

40. Lee J, Albiston AL, Allen AM, Mendelsohn FA, Ping SE, et al. (2004) Effect of I.C.V. injection of AT4 receptor ligands, NLE1-angiotensin IV and LVV-hemorphin 7, on spatial learning in rats. Neuroscience 124: 341-349.» CrossRef » Pubmed » Google Scholar

41. Lew RA, Mustafa T, Ye S, McDowall SG, Chai SY, et al. (2003) Angiotensin AT4 ligands are potent, competitive inhibitors of insulin regulated aminopeptidase (IRAP). J Neurochem 86: 344-350. » CrossRef » Pubmed » Google Scholar

42. McLaurin J, Cecal R, Kierstead ME, Tian X, Phinney AL, et al. (2002) Therapeutically effective antibodies against amyloid-beta peptide target amyloid-beta residues 4-10 and inhibit cytotoxicity and fibrillogenesis. Nat Med 8: 1263-1269. » CrossRef » Pubmed » Google Scholar

43. Miles LA, Wun KS, Crespi GA, Fodero-Tavoletti MT, Galatis D, et al. (2008) Amyloid-beta-anti-amyloid-beta complex structure reveals an extended conformation in the immunodominant B-cell epitope. J Mol Biol 377: 181-192.» CrossRef » Pubmed » Google Scholar

44. Nocek B, Mulligan R, Bargassa M, Collart F, Joachimiak A (2008) Crystal structure of aminopeptidase N from human pathogen *Neisseria meningitidis*. Proteins 70: 273-279. » CrossRef » Pubmed » Google Scholar

45. Olson ML, Olson EA, Qualls JH, Stratton JJ, Harding JW, et al. (2004) Norleucine1-Angiotensin IV alleviates mecamylamine-induced spatial memory deficits. Peptides 25: 233-241.» CrossRef » Pubmed » Google Scholar

46. Patton RL, Kalback WM, Esh CL, Kokjohn TA, Van Vickle GD, et al. (2006) Amyloid-beta peptide remnants in AN-1792-immunized Alzheimer's disease patients: a biochemical analysis. Am J Pathol 169: 1048-1063.» CrossRef » Pubmed » Google Scholar

47. Pederson ES, Harding JW, Wright JW (1998) Attenuation of scopolamine-induced spatial learning impairments by an angiotensin IV analog. Regul Pept 74: 97-103. »CrossRef » Pubmed » Google Scholar

48. Pereira C, Ferreiro E, Cardoso SM, de Oliveira CR (2004) Cell degeneration induced by amyloid-beta peptides: implications for Alzheimer's disease. J Mol Neurosci 23: 97-104.» CrossRef » Pubmed

49. Pham VL, Cadel MS, Gouzy-Darmon C, Hanquez C, Beinfeld MC, et al. (2007) Aminopeptidase B, a glucagon-processing enzyme: site directed mutagenesis of the Zn2+-binding motif and molecular modelling. BMC Biochem 8: 21.» CrossRef » Pubmed » Google Scholar

50. Rogi T, Tsujimoto M, Nakazato H, Mizutani S, Tomoda Y (1996) Human placental leucine aminopeptidase/oxytocinase. A new member of type II membrane-spanning zinc metallopeptidase family. J Biol Chem 271: 56-61.» CrossRef » Pubmed » Google Scholar

51. Rossjohn J, Cappai R, Feil SC, Henry A, McKinstry WJ, et al. (1999) Crystal structure of the N- terminal growth factor-like domain of Alzheimer's amyloid precursor protein. Nat Struct Biol 6: 327-331. » CrossRef » Pubmed » Google Scholar

52. Rozenfeld R, Iturrioz X, Maigret B, Llorens-Cortes C (2002) Contribution of molecular modeling and site-directed mutagenesis to the identification of two structural residues, Arg-220 and Asp-227, in aminopeptidase A. J Biol Chem 277: 29242-29252. » CrossRef » Pubmed » Google Scholar

53. Rozenfeld R, Muller L, El Messari S, Llorens-Cortes C (2004) The C-terminal domain of aminopeptidase A is an intramolecular chaperone required for the correct folding, cell surface expression, and activity of this monozinc aminopeptidase. J Biol Chem 279: 43285-43295. » CrossRef » Pubmed » Google Scholar

54. Schettini G, Florio T, Magri G, Grimaldi M, Meucci O, et al. (1988) Somatostatin and SMS 201-995 reverse the impairment of cognitive functions induced by cysteamine depletion of brain somatostatin. Eur J Pharmacol 151: 399-407. » Pubmed » Google Scholar

55. Sebret A, Lena I, Crete D, Matsui T, Roques BP, et al. (1999) Rat hippocampal neurons are critically involved in physiological improvement of memory processes induced by cholecystokinin-B receptor stimulation. J Neurosci 19: 7230-7237. » CrossRef » Pubmed » Google Scholar

56. Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. Science 298: 789-791. » CrossRef » Pubmed » Google Scholar

57. Solomon B (2004) Alzheimer's disease and immunotherapy. Curr Alzheimer Res 1: 149-163. » CrossRef » Pubmed

58. Thunnissen MM, Nordlund P, Haeggstrom JZ (2001) Crystal structure of human leukotriene A(4) hydrolase, a bifunctional enzyme in inflammation. Nat Struct Biol 8: 131-135. » CrossRef » Pubmed » Google Scholar

59. Tomizawa K, Iga N, Lu YF, Moriwaki A, Matsushita M, et al. (2003). Oxytocin improves long-lasting spatial memory during motherhood through MAP kinase cascade. Nat Neurosci 6: 384-390. » CrossRef » Pubmed » Google Scholar

60. Voits M, Hasenohrl RU, Huston JP, Fink H (2001) Repeated treatment with cholecystokinin octapeptide improves maze performance in aged Fischer 344 rats. Peptides 22: 1325-1330. » CrossRef » Pubmed » Google Scholar

61. Wallace AC, Laskowski RA, Thorton JM (1995) LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. Protein Eng 8: 127-134. » CrossRef » Pubmed » Google Scholar

62. Weksler ME (2004) The immunotherapy of Alzheimer's disease. Immun Ageing 1: 2. » CrossRef » Pubmed » Google Scholar

63. Wisniewski K, Borawska M, Car H (1993) The effect of angiotensin II and its fragments on post-alcohol impairment of learning and memory. Pol J Pharmacol 45: 23-29. » CrossRef » Pubmed » Google Scholar

64. Wolfe MS (2002) Therapeutic strategies for Alzheimer's disease. Nat Rev Drug Discov 1: 859-866. » CrossRef » Pubmed » Google Scholar

65. Wright JW, Clemens JA, Panetta JA, Smalstig EB, Weatherly LA, et al. (1996) Effects of LY231617 and angiotensin IV on ischemia-induced deficits in circular water maze and passive avoidance performance in rats. Brain Res 717: 1-11. » CrossRef » Pubmed » Google Scholar

66. Wright JW, Harding JW (1994) Brain angiotensin receptor subtypes in the control of physiological and behavioral responses. Neurosci Biobehav Rev 18: 21-53. » CrossRef » Pubmed » Google Scholar

67. Wright JW, Miller-Wing AV, Shaffer MJ, Higginson C, Wright DE, etal. (1993) Angiotensin II(3-8) (ANG IV) hippocampal binding: potential role in the facilitation of memory. Brain Res Bull 32: 497-502. » CrossRef » Pubmed » Google Scholar

68. Wright JW, Stubley L, Pederson ES, Kramar EA, Hanesworth JM, et al. (1999) Contributions of the brain angiotensin IV-AT4 receptor subtype system to spatial learning. J Neurosci 19: 3952-3961. » CrossRef » Pubmed » Google Scholar

69. Wun KS, Miles LA, Crespi GA, Wycherley K, Ascher DB, et al. (2008) Crystallization and preliminary X-ray diffraction analysis of the Fab fragment of WO2, an antibody specific for the Abeta peptides associated with Alzheimer's disease. Acta Crystallogr Sect F Struct Biol Cryst Commun 64: 438-441. » Pubmed » Google Scholar

70. Ye S, Chai SY, Lew RA, Ascher DB, Morton CJ, et al. (2008) Identification of modulating residues defining the catalytic cleft of insulin-regulated aminopeptidase. Biochem Cell Biol 86: 251-261. » CrossRef » Pubmed » Google Scholar

71. Zirah S, Kozin SA, Mazur AK, Blond A, Cheminant M, et al. (2006) Structural changes of region 1-16 of the Alzheimer disease amyloid beta-peptide upon zinc binding and *in vitro* aging. J Biol Chem 281: 2151-2161. » CrossRef » Pubmed » Google Scholar