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REVIEW

Copper binding to the Alzheimer's disease amyloid precursor protein

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Abstract Alzheimer's disease is the fourth biggest killer in developed countries. Amyloid precursor protein (APP) plays a central role in the development of the disease, through the generation of a peptide called Aβ by proteolysis of the precursor protein. APP can function as a metalloprotein and modulate copper transport via its extracellular copper binding domain (CuBD). Copper binding to this domain has been shown to reduce Aβ levels and hence a molecular understanding of the interaction between metal and protein could lead to the development of novel therapeutics to treat the disease. We have recently determined the three-dimensional structures of apo and copper bound forms of CuBD. The structures provide a mechanism by which CuBD could readily transfer copper ions to other proteins. Importantly, the lack of significant conformational changes to CuBD on copper binding suggests a model in which copper binding affects the dimerisation state of APP leading to reduction in Aβ production. We thus predict that disruption of APP dimers may be a novel therapeutic approach to treat Alzheimer's disease.

Keywords Alzheimer's disease · Amyloid precursor protein · Copper binding · Crystal structures · Receptor signalling

Introduction

Alzheimer's disease (AD) is characterised pathologically by the presence of amyloid plaques, extensive neuronal

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death and shrinkage of the brain. The symptoms of the disease begin with short-term memory disturbances and disorientation, and as the disease progresses judgement and reasoning become impaired and the sufferer dies after several years of dementia. Currently there is no treatment for the disease, although acetylcholinesterase inhibitors have displayed some moderate benefit in treatment.

There is increasing evidence that the neurotoxic Aβ peptide can compromise neuronal functions and trigger cell death (Selkoe 2002). The peptide can aggregate into small soluble oligomers, eventually leading to the amyloid plaques observed in AD diseased brains. Small soluble oligomers of Aβ have been shown to cause damage to cultured neurones (Haass and Selkoe 2007). 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). APP belongs to a larger family of APP-like proteins (APLPs) and homologues have been identified in a range of higher organisms including worms, insects and rodents (Coulson et al. 2000). These proteins exhibit some functional redundancy but only APP cleavage gives rise to the Aβ peptide. The cleavage occurs during trafficking and maturation through the protein secretory pathway by membrane-bound proteases. Aβ arises through the sequential cleavage by the BACE enzyme (Sinha et al. 1999; Yan et al. 1999) at a site just outside the plasma membrane and the γ-secretase protein complex (Haass and Steiner 2002) within the membrane (Fig. 1). An alternative APP proteolytic pathway exists in which cleavage by α-secretase results in the release of soluble APP. The secreted APP has been shown to possess growth-promoting activities and can bind to extracellular matrix components (De Strooper and Annaert 2000). This major pathway is non-amyloidogenic since it cleaves close to the middle of the Aβ peptide and therefore prevents intact Aβ peptide formation.

APP as a signalling molecule

Amyloid precursor protein is a Type-I transmembrane protein with a large extracellular region that can be subdivided into several distinct domains (Fig. 1). At the N-terminus is a cysteine-rich region consisting of the growth factor domain (GFD), which binds heparin and can stimulate neurite outgrowth (Ohsawa et al. 1997; Rossjohn et al. 1999) and a copper-binding domain (CuBD) (Barnham et al. 2003). The cysteine-rich region is followed by an acidic-rich region, a Kunitz-type protease inhibitor (KPI) domain and an OX2 domain (Tanaka et al. 1988; Tanzi et al. 1988). The KPI and OX2 domains can be spliced out, to produce three main variants: APP_{770} (with 770 amino acid residues), APP_{751} and APP_{695}. The latter is the most common isoform in the brain (Haass et al. 1991). Following these domains is a glycosylated domain referred to as E2 (Wang and Ha 2004) and an unstructured region immediately preceding the transmembrane domain. The E2 domain possesses a RERMS sequence that may contribute to APP's growth-promoting properties and it also has a heparin binding site. The C-terminal cytoplasmic tail is involved in various cellular functions through interaction with a multitude of proteins (De Strooper and Annaert 2000).

The function of normal APP remains largely unknown. There is appreciable evidence that APP acts as a cell surface receptor. It shares a similar architecture, cellular orientation and localisation to known (Type-1) cell surface receptors (Kang et al. 1987; Weidemann et al. 1989; Schubert et al. 1991). The APP cytoplasmic domain has been shown to bind to Fe65 protein, a protein related to oncogenic signal transducers, implicating a role for APP as a cell surface signalling receptor (Cao and Südhof 2001). Mouse knockouts of either APP, APLP1, APLP2 or their binding partner Fe65 cause the same phenotype of cortical

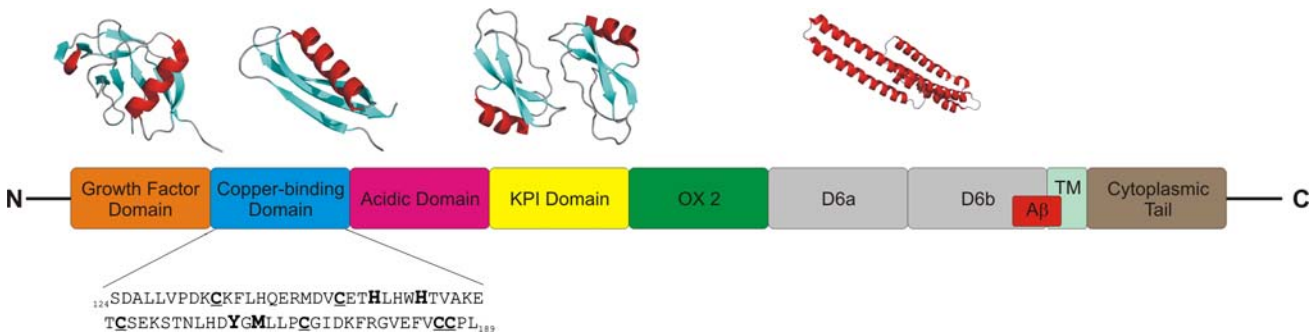

Fig. 1 Domain arrangement of APP highlighting some important regions of the molecule. Cartoon ribbon pictures of known 3D structures of domains are shown at the *top* of the picture. The N-terminal growth factor domain (GFD; PDB id: 1MWP) is followed by CuBD (PDB ids: 1OWT, 2FKL, 2FJZ and 2FK3), an acidic-rich region, Kunitz-type protease inhibitor (KPI; PDB id: 1AAP) and OX2 domains that occur in some APP isoforms, a couple of glycosylated domains (D6a, sometimes called the E2 domain or CAPPD; PDB ids: 1TKN, 1RW6; followed by an unstructured domain we refer to as D6b), a transmembrane region (TM) and a cytoplasmic tail. The location of the Aβ region, a major component of Alzheimer's disease plaques, is shown. The sequence of CuBD is shown with the copper binding ligands and Met_{170} in large *bold* type and the cysteines that are involved in disulfide bridges shown in *underlined bold* type

dysplasias suggesting the phenotype is caused by decreased transmission of an APP-dependent signal through the Fe65 protein (Guenette et al. 2006). APP hereditary mutations that are linked with Alzheimer's disease, can cause constitutive activation of G_o, a member of the heteromeric G protein family that serve as signal tranducers of cell surface receptors (Okamoto et al. 1995). An antibody directed towards the APP N-terminal domain stimulates G protein and MAP kinase activity (Okamoto et al. 1995). The antibody is presumably mimicking the action of a still-to-be-identified physiological ligand. There is evidence that APP exists in a monomer to dimer equilibrium in the membrane (Scheuermann et al. 2001; Soba et al. 2005) and the binding of high molecular weight heparin induces dimerisation (Gralle et al. 2006). Dimerisation may cause an initiation of signalling events (Sondag and Combs 2004) and play a role in modulating APP cleavage as the production of Aβ is increased when APP is forcibly dimerised (Scheuermann et al. 2001).

APP as a copper binding molecule

Copper is essential to life but is also toxic and as a result cells have developed elaborate systems for copper storage and transport (Bush 2000). The CuBD of APP can strongly bind Cu(II) ($K_d^{APP} \approx 10$ nM) and reduce it to Cu(I) in vitro (Hesse et al. 1994; Multhaup et al. 1996). This can lead to increased copper mediated neurotoxicity in cultured neurones (White et al. 1999a), presumably through increased oxidative stress or the generation of reactive oxygen species (Multhaup et al. 1997). APP appears to play a role in copper transport. In particular, a role for APP in copper efflux has been supported by a series of experiments which demonstrated an increased intracellular accumulation of copper in neuronal cultures (Bellingham et al. 2004) and in the brains of *APP/APLP2* double knockout mice (White et al. 1999b) whilst transgenic over-expression of APP had the opposite effects in cultured neurones and mice (Maynard et al. 2002; Bellingham et al. 2004). The involvement of CuBD in copper efflux has been demonstrated in yeast expressing an APP ectodomain fragment. Mutating the copper binding residues in CuBD significantly increases intracellular copper levels compared to yeast expressing a wild-type fragment (Treiber et al. 2004).

The interaction between copper ions and CuBD can modulate Aβ production. The treatment of CHO cells over-expressing APP with extracellular Cu(II) leads to reduced Aβ production and a shift of the cleavage equilibrium away from the amyloidgenic pathway (Borchardt et al. 1999). The effects are abolished when the copper binding residues of CuBD are mutated (Borchardt et al. 2000). The possible beneficial outcomes of Cu(II) binding to APP have been suggested in two independent transgenic mouse studies. The survival of APP23 mice (which overproduce Aβ) was improved along with reduced Aβ-levels in the brain when their drinking water was supplemented with Cu(II) ions (Bayer et al. 2003). Similar effects were achieved by crossing TgCRND8 mice (that overproduce Aβ) with mice that have raised brain copper levels due to the expression of a mutant copper transporter CuATPase7b. These mice had decreased amyloid plaque and plasma Aβ levels (Phinney et al. 2003).

A molecular target for the APP:Cu or APLP2:Cu complexes is the proteoglycan molecule glypican-1 (Cappai et al. 2005). APP and APLP2 can regulate the non-enzymatic metabolism of the glypican-1 heparin sulfate side-chains in a copper-dependent manner in vitro and in vivo. The APP : Cu complex stimulates glypican-1 autodegradation in the presence of either Cu(II) and Zn(II) ions, whereas the Cu(I) form of APP and the Cu(II) and Cu(I) forms of APLP2 inhibit autodegradation (Cappai et al. 2005).

In addition to copper binding to CuBD, copper is also known to bind the Aβ peptide fragment of APP, inducing peptide aggregation and leading to the production of reactive oxygen species via Fenton chemistry (Smith et al. 2007). The interested reader is referred to more in-depth reviews on this topic found elsewhere in this special issue.

CuBD structure

To understand the interaction of copper with APP at the molecular level, we initially determined the three-dimensional structure of CuBD (APP residues 124–189) by nuclear magnetic resonance spectroscopy (NMR) (PDB id: 1OWT; Barnham et al. 2003). This work revealed that the CuBD structure consists of an α-helix (residues 147–159) packed against a triple-stranded β-sheet (residues 133–139, 162–167 and 181–188) (Fig. 2a). A Cys_{144}–Cys_{174} disulfide bond connected two loops at one end of the molecule. On the other end a disulfide bond between Cys_{133} and Cys_{187} linked strands β1 and β3 and another between Cys_{158} and Cys_{186} linked the α-helix to strand β3. The surface of CuBD is highly charged with several areas of high negative (Glu_{156}, Glu_{160}, Glu_{183}, Asp_{167} and Asp_{131}) and positive (Lys_{132}, Lys_{134}, Lys_{161}, His_{147}, His_{151} and Lys_{155}) charge. CuBD belongs to the class of mixed α/β proteins, displays an architecture of a two-layered sandwich, with a topology that can be classified as either α-β plaits or defensin A-like (Pearl et al. 2005). Proteins that exhibit the latter topology include copper transport proteins such as yeast metallochaperone Atx1 (Arnesano et al. 2001), human metallochaperone HAH1 (Anastassopoulou et al. 2004) and the metal-binding domains of Menkes Protein ATP7A (Banci et al. 2004, 2005). These proteins contain an additional α-helix and β-strand after the $\beta\alpha\beta\beta$ motif of CuBD and

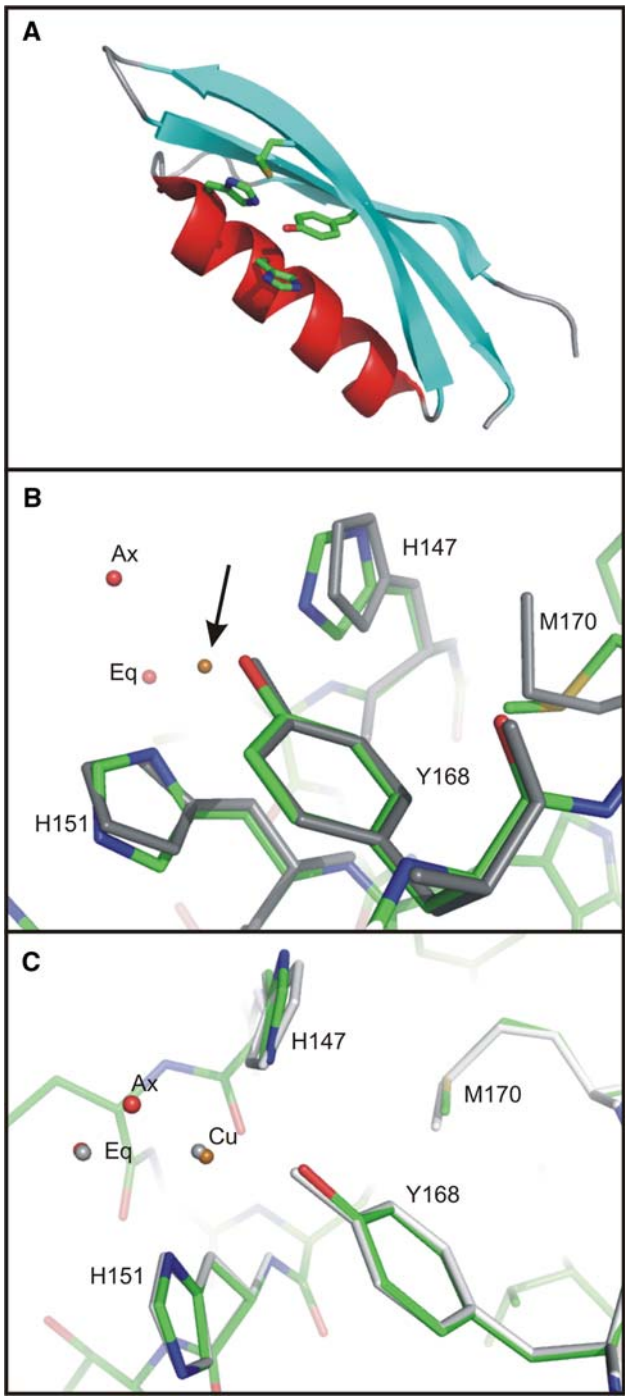

Fig. 2 Three-dimensional structure of CuBD (Kong et al. 2007a). **a** Ribbon picture of apo CuBD (PDB ids: 2FKL, 2FJZ and 2FK3) highlighting some of the side-chains that contribute to the copper binding site. **b** The Cu(II) coordination geometry in CuBD (PDB id: 2FK1) and comparison of the copper binding residues in the Cu(II)-bound and apo structures. The Cu(II)-bound structure is displayed in standard atomic colouring and the apo structure is in *grey*. The Cu(II) ion is indicated by the *arrow*, while 'eq' and 'ax' denote the equatorial and axial water molecules, respectively. **C** The Cu(I) coordination geometry in CuBD (PDB id: 2FK2) and a comparison of the copper binding site in the Cu(II)-bound and Cu(I)-bound CuBD structures. The Cu(II)- and Cu(I)-bound structures are shown in *standard atomic colouring* and in *grey*, respectively

display no sequence similarity to CuBD. Their copper binding sites are located on the surface, but unlike CuBD, in the loop joining strand β_1 and helix α_1, and use two cysteines in a CXXC motif as copper ligands.

More recently we determined the crystal structure of apo CuBD from three different crystal forms at high resolution (Fig. 2a) (PDB ids: 2FKL, 2FJZ, 2FK3; Kong et al. 2007a). These structures revealed eleven different views of the molecule due to multiple copies in the asymmetric unit in some crystal forms. Despite the different crystal lattice forces, these structures superimpose closely amongst each other and with the previously determined NMR structure (Barnham et al. 2003). Major deviations are largely restricted to loops and are attributable to various crystal packing contacts. Backbone atoms of the copper binding residues superimpose closely despite being involved in different types of crystal contacts amongst the crystal forms. The relative positions of those side-chains are also well conserved, although His_{147} is seen to adopt different side-chain orientations, which may be relevant for copper transport to and from other proteins (see below).

We have just reported the crystal structure of CuBD in metal-free form at ultra-high resolution (0.85 Å) (PDB id: 2FMA; Kong et al. 2007b). The structure shows that the copper binding residues of CuBD (see below) are rather rigid but Met_{170}, thought to be the electron source for Cu(II) reduction, adopts two different side-chain conformations. In one, the thioether is about 7 Å from the copper binding site and points away from the other copper binding residues. In the second conformation, the thioether group is closer to the copper binding site (6 Å) and points towards the copper ion, although Met_{170} itself is still buried from the surface. The flexibility of the Met_{170} side-chain may be important in facilitating copper ion binding or the reduction of Cu(II) to Cu(I) after Cu(II) is bound to the CuBD (White et al. 2002; Barnham et al. 2003).

Copper binding to CuBD

The conformation of Cu(II)-binding residues in CuBD could not be visualised by NMR as the paramagnetism of Cu(II) caused severe peak broadening arising from atoms in close proximity to the metal ion and addition of metals such as copper and zinc induced protein aggregation in solution (Barnham et al. 2003). However, this paramagnetic effect implicated His_{147}, His_{151}, Tyr_{168} and Met_{170} in Cu(II) binding. Copper ions could not be introduced to CuBD either by co-crystallisation nor by soaking preformed crystals at high concentrations of copper without causing crystal shattering, likely due to numerous copper binding sites on the surface of the protein. However, careful manipulation of the soaking conditions led to crystals in which the high affinity copper binding site was occupied without crystal destruction.

Our structure derived from such crystals revealed that the two histidines (His_{147}, His_{151}) and the tyrosine residue (Tyr_{168}) participate in Cu(II) binding but not the methionine residue. In addition, two water ligands were identified: an axial water and an 'equatorial' water molecule located ~1.5 Å above the plane of the other ligands (Fig. 2b) (PDB id: 2FK1; Kong et al. 2007a). The arrangement of ligands about the metal centre is typical of the square pyramidal geometry normally adopted by five coordinate Cu(II) and can be classified as a Type 2 non-blue Cu(II) centre in which the metal is normally bound by two or three nitrogen atoms and oxygen atoms (http://metallo.scripps.edu/PROMISE/CUMAIN.html). The copper binding geometry is consistent with observations suggesting that APP is capable of reducing Cu(II) to Cu(I) (Multhaup et al. 1996) as Type 2 sites are often associated with redox-driven catalysis (Messerschmidt et al. 2001). A similar Type 2 site has been described in nitrosocyanin which is also open to solvent and involves a water ligand (Arciero et al. 2002). The Cu(II) coordination geometry was further confirmed by electron paramagnetic resonance spectroscopy and extended X-ray absorption fine structure spectroscopy of Cu(II)-bound CuBD in solution (Kong et al. 2007a).

We also determined the crystal structure of the Cu(I) form of the protein through the use of a reductant on the Cu(II)-bound CuBD crystals (PDB id: 2FK2; Kong et al. 2007a). The Cu(I) binding geometry is almost identical to that of Cu(II) in CuBD (see above) with such small differences between oxidation states having been noted previously for other copper proteins (Gray et al. 2000).The only significant difference is the absence of the axial water ligand (Fig. 2c). Similar losses of a water ligand upon Cu(II) reduction have been observed in a couple of other copper redox proteins (Murphy et al. 1997; Basumallick et al. 2005). Normally four coordinate Cu(I) adopts a tetrahedral geometry and binds soft ligands (Casella and Gullotti 1993) rather than the distorted square planar arrangement and oxygen ligands seen for CuBD. Thus, the site appears unfavourable for Cu(I) suggesting the Cu(I)-bound state may not be stable, possibly facilitating the transfer of metal to other proteins following CuBD-mediated reduction of Cu(II).

The NMR Cu(II) titration experiments led to evidence of Met_{170} oxidation and peak broadening of its proton resonances suggesting that it was in the neighbourhood of the metal ion (Barnham et al. 2003). However, in the crystal structures we found that sulfur atom of Met_{170} is 7 Å from either the Cu(II) or Cu(I) ion (Fig. 2b,c) (Kong et al. 2007a, b). We propose that Met_{170} may play an important role as an electron donor to Cu(II) either through rapid conformational changes which would bring the sulfur atom in close contact with the metal ion, or through an electron transfer pathway which relays the electron from Met_{170} to Cu(II) through the copper binding residues. The proximity of Met_{170} and Tyr_{168} in CuBD provides a plausible electron transfer pathway; reminiscent of a similar mechanism proposed for Aβ peptide-induced Cu(II) reduction where a methionine residue has been suggested to provide the electron for Cu(II) reduction through a tyrosine ligand (Curtain et al. 2001; Barnham et al. 2004).

The surface location of the copper site in CuBD may facilitate the transfer of metal ions to and from other copper-binding proteins. This is, in a sense, similar to the case of the metal-binding domains of the human Menkes protein (ATP7a), where Cu(I) also binds in an exposed environment after it is delivered from the chaperone HAH1 although the ligands involved are exclusively cysteines (Banci et al. 2005). In the case of the yeast chaperone protein Atx1, its interaction with its downstream target Ccc2 ATPase (Ccc2a) involves bringing the Cu(I) ion from a more shielded environment to the surface where the cysteine ligands of Ccc2a would be able to chelate the Cu(I) from Atx1. The Atx1-Ccc2a interaction, although aided by other forces, is absolutely dependent on Cu(I) binding (Banci et al. 2006). We can similarly envisage the interaction of CuBD and its partners being driven by the presence of copper ions. The water ligands in CuBD could suggest where an endogenous ligand or binding partner of CuBD may dock by displacing these waters (Prigge et al. 2004). The putative ligand could come from a number of sources: (a) another ligand of the CuBD, (b) from an adjacent domain within the same protein molecule, (c) from another APP molecule to form an oligomer, or (d) a binding partner of APP, such as proteins that transport copper ions to or from APP. The third possibility would be consistent with the observation that CuBD may form part of the APP dimerisation interface (Scheuermann et al. 2001; Soba et al. 2005) and the positions of the water molecules may represent where the dimer interactions occur. The crystal packing interactions seen in some of the CuBD crystal forms suggest a propensity of the copper binding residues to take part in protein–protein interactions. However, we have not observed specific CuBD oligomerisation induced by Cu(II) (unpublished results).

Modelling CuBDs from other species

One of the consequences of Cu(II) binding to APP is thought to be neuronal toxicity which has been demonstrated so far in cultured neurones. The hypothesised role of CuBD in neuronal toxicity is that the bound Cu(II) is reduced to Cu(I), which can then lead to lipoprotein peroxidation and free radicals that damage the cell (White et al. 2002). CuBD is one of the best conserved domains within the APP superfamily (Fig. 3). Surprisingly, the effect of copper binding varies between the orthologues

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hAPP770     124  SDALLVPDKCKFLHQERMDVCETHLHWHTVAKETCSEK------STNLHDYGMLLPCGIDKFRGVEFVCCPL  189
rAPP770     124  SDALLVPDKCKFLHQERMDVCETHLHWHTVAKETCSEK------STNLHDYGMLLPCGIDKFRGVEFVCCPL  189
mAPP        124  SDALLVPDKCKFLHQERMDVCETHLHWHTVAKETCSEK------STNLHDYGMLLPCGIDKFRGVEFVCCPL  189
hAPLP2      140  SDVLLVPEKCQFFHKERMEVCENHQHWHTVVKEACLTQ------GMTLYSYGMLLPCGVDQFHGTEYVCCPQ  205
rAPLP-2     140  SDVLLVPENCQFFHQERMEVCEKHQRWHTVVKEACLTE------GMTLYSYGMLLPCGVDQFHGTEYVCCPQ  205
mAPLP-2     140  SDVLLVPDNCQFFHQERMEVCEKHQRWHTLVKEACLTE------GLTLYSYGMLLPCGVDQFHGTEYVCCPQ  205
hAPLP-1     147  SEALLVPEGCRFLHQERMDQCESSTRRHQEAQEACSSQ------GLILHGSGMLLPCGSDRFRGVEYVCCPP  212
mAPLP-1     146  SEALLVPEGCRFLHQERMDQCESSTRRHQEAQEACSSQ------GLILHGSGMLLPCGSDRFRGVEYVCCPP  211
dAPPL       134  SDALLVPEGCLFDHIHNASRCWPFVRWNQTGAAACQER------GMQMRSFAMLLPCGISVFSGVEFVCCPK  199
cAPL-1      126  SEALQVPHDCQFSHVNSRDQCNDYQHWKDEAGKQCKTKKSKGNKDMIVRSFAVLEPCALDMFTGVEFVCCPN  197
                 *:.* **. * * * .  . *    : :      *  .      .  : . .:* **. . * *.*:****
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Fig. 3 A sequence alignment of CuBD from selected members of the APP superfamily. The prefixes h, r, m, d and c denote human, rat, mouse, *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively. The N-terminal and C-terminal numbering of each CuBD are listed. Conserved residues are indicated by *asterisks* and *coloured in aqua*, *colons* indicate positions with very similar amino acid residues while *dots* indicate positions with some similarity. The copper binding residues of human APP CuBD and their equivalents in other homologues are coloured in *orange*

and paralogues. For example, it has been shown that the presence of Cu(II) salts in primary murine neuronal cultures causes greater cell death in wild-type neurones compared to $APP^{-/-}$ neurones whereas there is no difference between wild-type and $APLP2^{-/-}$ neurones (White et al. 1999a). Lipoprotein peroxidation is enhanced by Cu(II)-treated ectodomain of human APP and APLP2, whereas neither the non-metallated forms of those proteins nor Cu(II)-metallated human APLP1 show increased peroxidation above background (White et al. 2002). Cu(II)-bound CuBD of human APP appears sufficient in the generation of Cu(I) and causing neuronal cell death (White et al. 2002).

The behaviour of peptides derived from CuBDs of various APP homologues suggests that the toxicity may depend on the presence of the copper binding histidine residues. The replacement of one, and both of those histidines results in an inert and protective effect, respectively, when the peptides are added to cultured neurones incubated with lipoproteins. The peptides studied were derived from human APLP1 (one histidine replaced), *Caenorhabditis elegans* homologue APL-1 (two histidines replaced) and *Drosophila melanogaster* homologue APPL (two histidines replaced). However, these fragments do not cover the position corresponding to the copper binding residue Tyr168 and do not preserve the native disulfide bonds. Hence the structure, binding behaviour and Cu(II) reduction mechanism of the peptides are very likely to be different in intact CuBD.

We have produced three-dimensional atomic models of selected CuBDs from various organisms using homology modelling based on the sequence alignments shown in Fig. 3. The sequence alignment shows that the copper binding residues of human APP are not conserved in the APLP-1 family, *C. elegans* homologue APL-1 or *D. melanogaster* homologue APPL (Fig. 3). The replacement of the copper binding residues in human APLP-1 (Fig. 4b), *C. elegans* APL-1 (Fig. 4c) and *D. melanogaster* APPL (Fig. 4d) likely abolishes copper binding since residues are eliminated that are likely to be involved in copper coordination and the distance between the putative Cu(II) ion binding site to the surrounding residues increases (Fig. 4).

Those APP homologues where the CuBD models are predicted to lack the copper binding site will not be able to bind Cu(II) and thus reduce it to the more toxic Cu(I). This would explain why human APLP-1 is not able to oxidise lipoproteins and cause neuronal death (White et al. 2002). It is also consistent with the protective effect of *D. melanogaster* APPL and *C. elegans* APL-1 CuBD peptides against lipoprotein peroxidation by Cu(II) ions. If the hypothesis on Cu(II)-induced toxicity holds, the result of modelling would support the role of His_{147}, His_{151} and Tyr_{168} of human APP CuBD in copper binding.

The copper binding site of human APLP-2 CuBD is predicted to be very similar to human APP CuBD based on comparison of primary structures (Fig. 3) and the similarity of the APLP-2 CuBD homology model with the crystal structure (Fig. 4a). In view of the high similarity of the Cu(II)-bound, Cu(I)-bound and apo structures of human APP CuBD (see above), it is expected that the Cu(II)-bound and Cu(I)-bound structures of human APLP-2 CuBD would be very similar as well. The copper binding residues His_{147}, His_{151} and Tyr_{168}, are also conserved in APLP-2s in other organisms, but the toxicity mediated by APLP-2s appears to be lower than APPs. For example, Cu(II)-bound human APP is more effective than Cu(II)-bound human APLP-2 in oxidising lipoproteins and causing cell death (White et al. 2002). Wild-type murine hippocampal neurones suffer greater cell death compared to mouse $APP^{-/-}$ counterparts while mouse $APLP\text{-}2^{-/-}$ offers no protection (White et al. 1999a). The crystal structure of human APP CuBD and the model of human APLP-2 CuBD do not provide an obvious explanation for the different toxicity effects mediated by these two APP homologues.

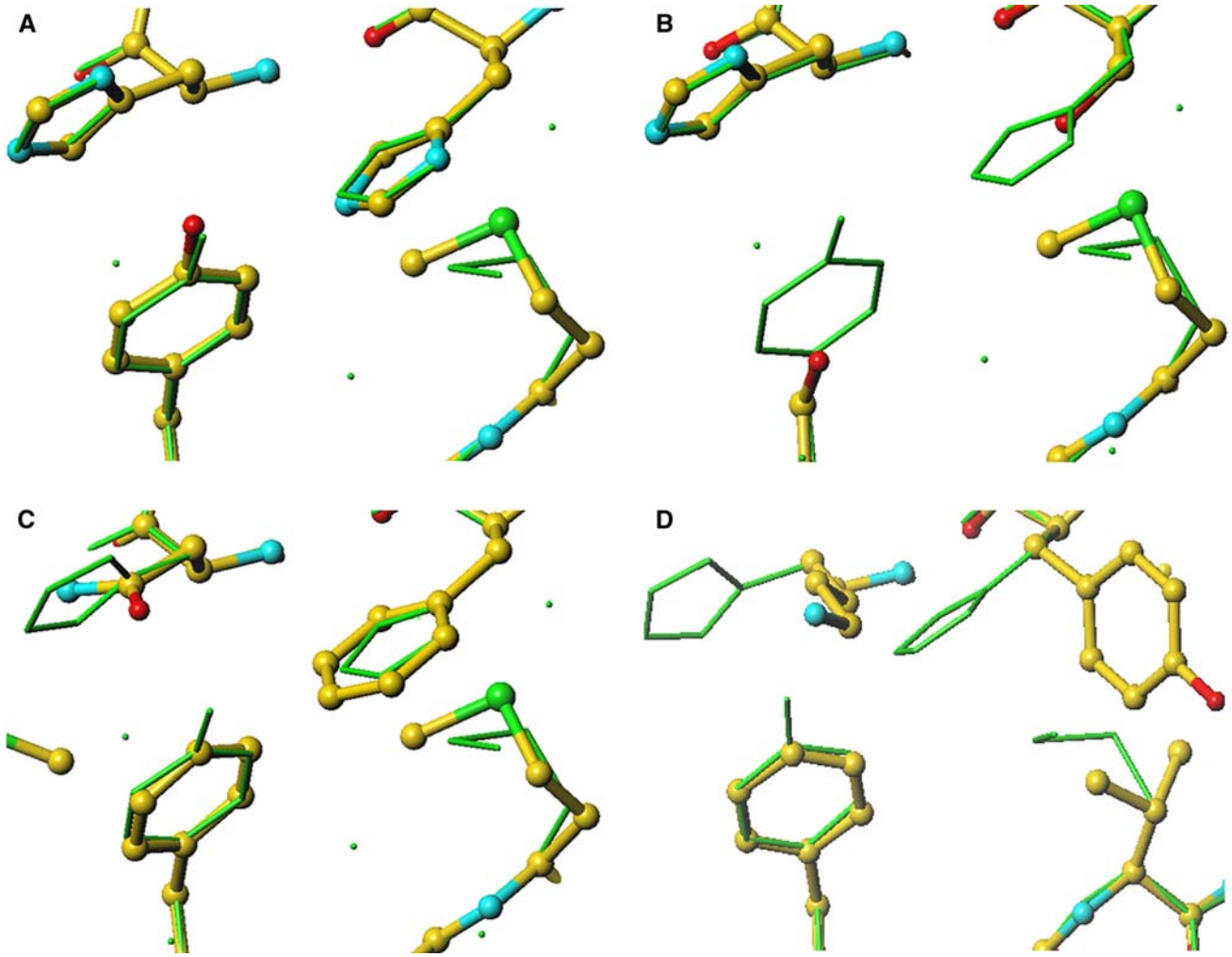

Fig. 4 A comparison of the human APP CuBD copper binding site to the equivalent site in orthologues and paralogues. a Human APLP-2, b human APLP-1, c *D. melanogaster* APPL and d *C. elegans* APL-1. The human APP residues are shown in *green sticks* whereas the homology models are in *ball*-and-*stick* with the standard atomic colouring

Modelling CuBD interactions with GFD

The N-terminal GFD is immediately upstream of CuBD (Fig. 1) and together they constitute the cysteine-rich region of the molecule. To determine if these two domains likely interact we used the GRAMM program (Vasker and Aflalo 1994; Vasker 1996) to dock CuBD to the crystal structure of GFD (PDB id: 1MWP; Rossjohn et al. 1999). Visual inspection indicated that the most plausible domain pair structure brought the C-terminus of GFD and the N-terminus of CuBD in close proximity while the arrangement of the domain interface was suggestive of a continuation of the β-sheet from domain 1 into the β-sheet of domain 2 (Fig. 5). The total surface area buried in forming the interface is ~200 Å^2. The residues forming the interface are primarily hydrophobic, including Val_{86}, Leu_{118}, Val_{119} and Gly_{120} from GFD and Ala_{126}, Leu_{127}, Leu_{128}, Val_{129} and Pro_{130} of CuBD with the ends of this hydrophobic region flanked by polar and charged residues that are exposed to solvent. In total there are nine potential hydrogen bonds and 28 van der Waals interactions in the domain interface. Both the heparin binding site of GFD and the copper binding site of CuBD, are exposed in the model at opposite ends of the cysteine rich region, separated by ~50 Å.

We have determined the crystal structure of a longer length fragment of $CuBD_{124–189}$ to 2.5 Å resolution (Kong et al. 2007a). This crystal from was found to have two CuBD molecules in the asymmetric unit. An interesting feature of this structure is that the two molecules interact head-to-head via new β-strand (β_0) involving residues 127–129 of one molecule hydrogen bonding onto strand β_2 of a neighbouring molecule to form an extended β-sheet (Fig. 6). The interaction buries about 1,550 Å^2 of surface area between the molecules. However, there are a few contacts between the two chains. The new strand β_0 of chain B provides some of those interactions through hydrogen

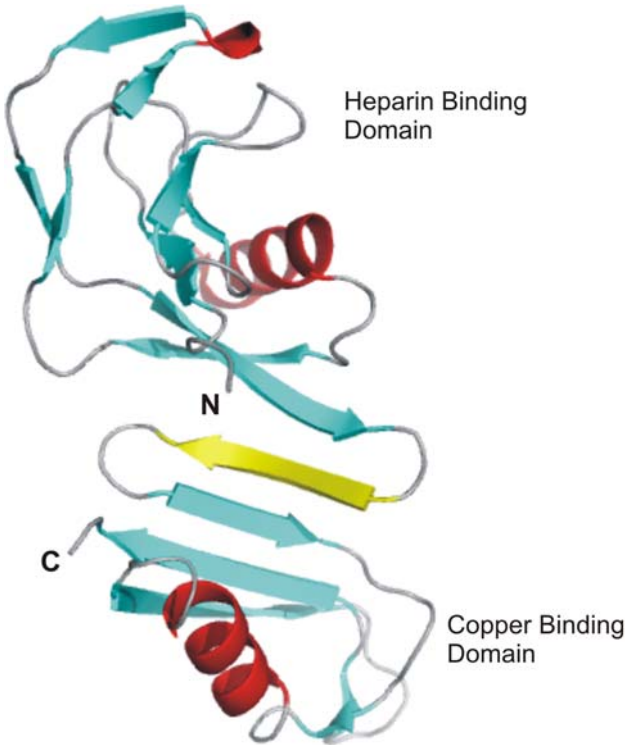

Fig. 5 Model of the APP growth factor domain docked onto CuBD. Note that an extra β-strand (coloured *yellow*), suggested by the modelling, has been inserted between the two domains to form an extended sheet

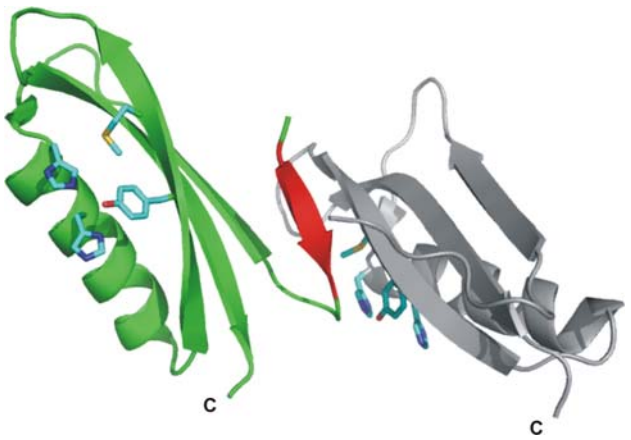

Fig. 6 A ribbon diagram of $CuBD_{124–189}$. Chains A and B are coloured in *grey* and *green*, respectively. Strand β_0 of chain B is highlighted in *red*. The side-chains of the copper binding residues and Met_{170} are shown as *blue sticks*

bonds from the backbone amides of Ala_{126} (B) and Leu_{127} (B) to the backbone carbonyl of Met_{170} (A), and that from the backbone carbonyl of Tyr_{168} (A) to backbone amide of Val_{129} (B). There are van der Waals contacts between the rings of His_{147} (A) and Phe_{135} (B), between the backbone of Gly_{175} (A) and side-chain of Leu_{171} (B), between the side-chains of Asp_{177} (A) and His_{137} (B), and a hydrogen bond between the backbone amide of Asp_{177} (A) and carboxylate group of Glu_{183} (B). Strand β_0 is also within van der Waals distance of the copper binding site of the latter molecule. However, it is unlikely that the dimer seen in this crystal form has a significant existence in solution because there is no evidence of dimerisation of $CuBD_{124–189}$ in the NMR experiment (Barnham et al. 2003). Nevertheless, the structure shows that N-terminal residues 124–132 of CuBD have an inherent propensity to form a β-strand and thus lends support to the suggestion discussed above that this region of CuBD could be induced to form a strand leading to the extended β-sheet with GFD in intact APP (Fig. 5).

In a recent collaboration an ab initio reconstruction of a molecular model of the complete extracellular region of APP (sAPP) was presented based on data from high-resolution solution X-ray scattering (SAXS) data together with the fitting of domains from published crystal structures (Gralle et al. 2006). In this model the N-terminal growth-factor domain and CuBD would lie at a distance of ~100 Å from the membrane. The results from SAXS, analytical ultracentrifugation (AUC) and size-exclusion chromatography (SEC) analysis indicated that sAPP is monomeric in solution. On the other hand, SEC, fluorescence, AUC and SAXS measurements showed that sAPP forms a 2:1 complex with heparin. A model for the sAPP:heparin complex was also derived from the SAXS data.

Copper binding to APP: implications for signalling

Our structures do not directly explain how copper binding leads to decreasing Aβ production since we see no evidence of significant conformational changes in the structure of CuBD upon metal binding or reduction. However, it must be mediated by some sort of signalling mechanism, for example, modulation of proteins that cleave APP (Angeletti et al. 2005), a change in conformation of APP, recruitment of other proteins or a change in the oligomerisation state of the APP. APP can associate with G_o as a G-protein coupled receptor (Brouillet et al. 1999) and signalling can be initiated upon binding of an antibody, 22C11, that binds to the GFD and is thought to act as a ligand mimetic (Okamoto et al. 1995). Scheuermann et al. (2001) were able to isolate endogenous membrane-bound APP as dimers and showed that Aβ production increased when APP was cross-linked to form dimers. However, it cannot be excluded that the effect on Aβ production could have been as a consequence of modifying APP near its α-secretase cleavage site. Putative dimerisation domains have been proposed. Wang and Ha (2004) suggested the E2 domain (Fig. 1) as a candidate. On the other hand, Scheuermann et al. (2001) demonstrated that a fragment encompassing the GFD, CuBD and the acidic-rich region existed in dimeric form by gel filtration, while Soba et al. (2005) showed that the GFD and CuBD

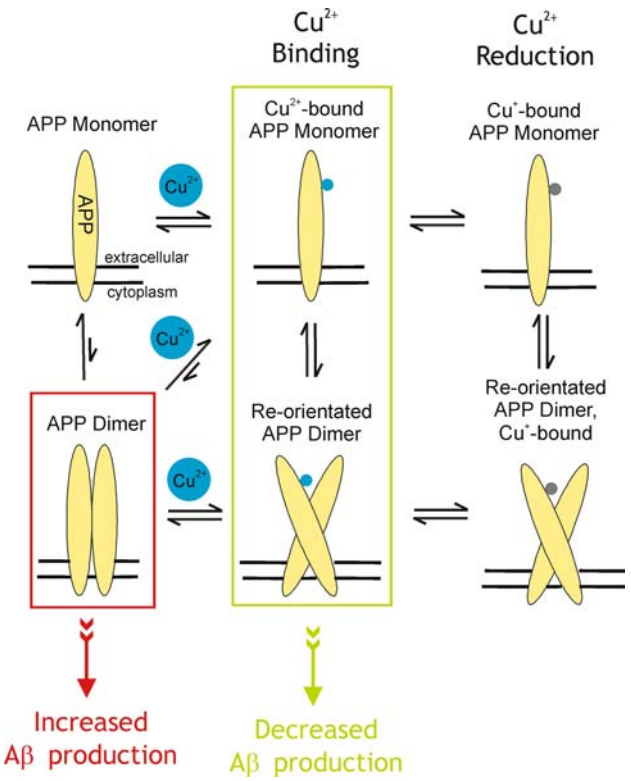

Fig. 7 A model of the putative effects of Cu(II) binding on APP dimerisation and Aβ production

might be a major site of dimerisation of APP in the cellular context. If the CuBD does contribute to APP dimerisation in the absence of Cu ions, we propose that the effect of Cu(II) in lowering Aβ production may perhaps be elicited through either a shift of monomer-dimer equilibrium to favour monomers or a re-orientation of the APP dimers to a form that disfavours Aβ production (Fig. 7). If the copper site is exposed in the monomeric or re-orientated dimer form, it could also aid the interaction of APP with other copper binding proteins in the transfer of copper ions. The copper binding pocket, perhaps together with other parts of CuBD or APP, will provide a site for the docking of interacting partners. Based on this hypothesis, new approaches to Alzheimer's therapeutics could be devised, as compounds that can disrupt the formation or organisation of pre-formed APP dimers may be beneficial in reducing Aβ production from APP.

Concluding remarks

Copper binding to APP can yield paradoxical outcomes. On the one hand copper binding to the CuBD of APP results in Aβ depletion whereas copper binding to the Aβ peptide generates toxic species. From a therapeutic point-of-view these observations suggest that a molecule that mimics the effect of copper binding on CuBD could be of significant clinical benefit.

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