Structure of the Alzheimer's Disease Amyloid Precursor Protein Copper Binding Domain

A REGULATOR OF NEURONAL COPPER HOMEOSTASIS

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A major source of free radical production in the brain derives from copper. To prevent metal-mediated oxidative stress, cells have evolved complex metal transport systems. The Alzheimer's disease amyloid precursor protein (APP) is a major regulator of neuronal copper homeostasis. APP knockout mice have elevated copper levels in the cerebral cortex, whereas APP-overexpressing transgenic mice have reduced brain copper levels. Importantly, copper binding to APP can greatly reduce amyloid β production in vitro. To understand this interaction at the molecular level we solved the structure of the APP copper binding domain (CuBD) and found that it contains a novel copper binding site that favors Cu(I) coordination. The surface location of this site, structural homology of CuBD to copper chaperones, and the role of APP in neuronal copper homeostasis are consistent with the CuBD acting as a neuronal metallotransporter.

Alzheimer's disease (AD) is characterized by progressive neuronal dysfunction, reactive gliosis, and the formation of amyloid plaques in the brain. The cause of the neuronal cell loss in AD is unclear but may be related to increased oxidative stress from excessive free radical generation (1–4). A major source of free radical production in the brain is from the transition metals copper and iron (3, 5). These metals are vital for life because of their high redox activity and have been utilized in a number of enzymatic pathways, including cellular respiration. However, if the redox reactivity of copper and iron is not strictly regulated, this can result in the generation of toxic reactive oxygen species (5). The potential for oxidative damage from reactive oxygen intermediates in the aging brain is further enhanced by the high oxygen consumption and relatively low antioxidant levels in brain tissue. To prevent transition metal-mediated oxidative stress, cells have evolved elaborate systems for copper storage and transport that deliver copper and iron to metalloenzymes and proteins. A number of studies have implicated cell surface metalloredoxcupases in the reduction of Cu(II) to Cu(I), which is the form of the metal ion that is delivered to the cytoplasm of eukaryotic cells via copper transporters (6). To avoid Cu(I) redox chemistry inside the cell, Cu(I) ions are escorted by specific cytosolic metalloproteins such as the copper chaperones that are involved in intracellular copper trafficking to Wilson's disease copper ATPase and the copper/zinc superoxide dismutase (7). This results in unbound copper being essentially absent in the intracellular environment (8). Therefore, cupro-proteins play an important role in maintaining cellular copper metabolism (9).

Both APP and Aβ, a proteolytic breakdown product of APP, play a central role in Alzheimer's disease and can strongly bind Cu(II) (K, APP ~ 10 nM) and reduce it to Cu(I) in vitro (10–15). The APP copper binding domain (CuBD) is located in the N-terminal cysteine-rich region next to the growth factor-like domain (10, 16) (Fig. 1). APP is a member of a multigene family, and the CuBD sequence is similar among the different APP family paralogs and orthologs, suggesting an overall conservation in its function or activity. In vivo studies show that APP expression is a key modulator of neuronal copper homeostasis since APP knockout mice have increased copper levels in the brain (17). Conversely, APP overexpressing transgenic mice have significantly reduced copper levels in transgenic mouse brain (18). The APP CuBD can also modulate Cu(I)-mediated neurotoxicity (19) and, depending on the ortholog, can either promote or inhibit copper neurotoxicity (20). The interaction between the APP-Cu(I) species with hydrogen peroxide can result in Cu(I) oxidation to Cu(II) and APP fragmentation (21). Of importance to Alzheimer disease pathology is the finding that increasing the copper concentration modulates APP processing, resulting in greatly reduced Aβ production and increased levels of the cell-bound and secreted forms of Aβ.

The Journal of Biological Chemistry
© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 278, No. 19, Issue of May 9, pp. 17401–17407, 2003
Printed in U.S.A.

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: AD, Alzheimer's disease; APP, amyloid precursor protein; CuBD, copper binding domain; EPR, electron paramagnetic resonance; PDB, Protein Data Bank; HSQC, heteronuclear single quantum correlation.

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3 The atomic coordinates and structure factors (code IOWT) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
APP (22, 23). Mutagenesis of histidine residues within CuBD inhibits the effects of copper on APP expression and proteolysis (23).

The importance of copper to Alzheimer’s disease is emphasized by the neurotoxic interaction between the Aβ peptide and copper. The Aβ peptide binds copper with a high affinity and reduces Cu(II) to Cu(I), resulting in the catalytic generation of hydrogen peroxide (H₂O₂) and Aβ aggregation (24). Aβ and copper can interact to form an oligomeric complex that binds copper at a copper/zinc superoxide dismutase-like binding site (25). The importance of copper in AD pathology has been demonstrated by the ability of a chelator (clioquinol) to disaggregate amyloid both in vitro and in a transgenic mouse model in vivo (26). It is plausible that copper binding to CuBD and Aβ are linked phenomenon. The modulation of copper levels by the APP CuBD would influence Aβ-Cu interactions, resulting in increased H₂O₂ from Aβ-Cu or an overall increase in neuronal reactive oxygen intermediate production. Changes in copper levels will also affect APP processing into Aβ, thus controlling the production of neurotoxic Aβ. Therefore, defining the interaction of copper with APP has important consequences for Aβ production and AD pathogenesis and subsequent therapeutic intervention. To understand the interaction of copper with the APP CuBD at the molecular level, we have determined the three-dimensional structure of the CuBD (APP residues 124–189) by NMR spectroscopy. The structure has led to the identification of a novel copper binding site. The CuBD has structural homology to copper chaperones, thus suggesting the APP CuBD functions as a neuronal metallotransporter and/or metallochaperone.

EXPERIMENTAL PROCEDURES

Expression and Purification—CuBD, encompassed by residues 124–189, was expressed using the methylotrophic yeast, Pichia pastoris. The N-terminal border was determined based on proteolysis of the whole extracellular domain (16). The C-terminal border was selected to end before the start of the acidic region (glutamate 191). APP-(124–189) was generated by PCR using the primers GCT CGA GAA AA GAG AGG CTA GTG ATO CCC TTC TCG and GAA TTC TTA CAG TGG GCA ACA CAC AAA CTC. The PCR product was cloned as a XhoI-EcoRI fragment into the P. pastoris vector pIC9 (Invitrogen). The constructs were transfected into P. pastoris strain GS115 as previously described (27).

Uniformly 15N- and 13C,15N-labeled CuBD proteins were prepared by a standard protocol (28) using 15NH₄Cl, [13C]glucose, and [13C]methanol as the isotope sources. The proteins were purified to homogeneity using a QHyperD 1.6 × 13-cm column (BioSera) followed by a Superdex 75 10/30 gel filtration column (Amersham Biosciences). N-terminal amino acid sequencing and mass spectrometry (matrix-assisted laser desorption/ionization-time of flight) analysis confirmed that the N terminus was intact, and the mass correlated with the predicted sequence. Protein concentration was determined using a Bio-Rad protein assay. Inductively coupled plasma mass spectrometry for metal analysis was performed using an Ultramass 700 (Varian, VIC, Australia).

NMR Spectroscopy—NMR spectra were acquired at 30 °C on a Bruker DRX-600 spectrometer equipped with triple-resonance pulsed-field gradient probes. Sequential resonance assignments were made using a series of triple-resonance spectra (29) acquired on either uniformly 15N- or 13C,15N-labeled CuBD using the methods described previously (30). Spectra were obtained on samples that were typically 0.5 mM protein in 20 mM phosphate buffer (pH 6.9), 10% 2H₂O, and 1 mM EDTA, which was either removed or titrated out in the metal binding studies. An essentially complete set of resonance assignments was determined from spectra acquired using 15N and 13C,15N-labeled protein. The structures were calculated with CNS (31) using protocols described elsewhere (30). The final 21 structures (see Fig. 2) were selected on the basis of their stereochemical energies. Structural statistics are presented in Table I.

Electron Paramagnetic Resonance (EPR) Spectroscopy—Continuous-wave X-band EPR spectra were obtained using a Bruker ECS106 spectrometer equipped with a temperature controller and flow-through liquid nitrogen cryostat. 65Cu(II) was added to CuBD (residues 133–189), and spectra were collected at 105 K from samples contained in 4-mm internal diameter Suprasil quartz EPR tubes (Wilmad). Measurements of spectral parameters were carried out using either the instrument software or, off-line, Bruker WinEPR™, and spectra were simulated with the aid of Bruker SimFonia™. For the calculation of g values the instrument was equipped with a Bruker EIPS548B frequency counter, and the magnetic field was calibrated using a sample of a,a′-diphenyl-

β-picrylhydrazyl.
Amyloid Precursor Protein CuBD Structure

Table I

Structural statistics for the 21 energy-minimized structures of CuBD

| r.m.s., root mean square. |        |
|--------------------------|--------|
| Total distance constraints| 1233   |
| Sequential (|i - j| = 1) | 321    |
| Short range (|i - j| < 5) | 229    |
| Long range (|i - j| ≥ 5) | 532    |
| Intra-residue            | 151    |
| Hydrogen bonds            | 10     |
| Total angle constraints   | 111    |
| ϕ                        | 59     |
| ψ                        | 21     |
| χ1                       | 31     |
| r.m.s. deviations from experimental distance restraints Å (1241) | 0.2016 ± 0.0009 |
| r.m.s. deviations from experimental dihedral restraints (degree) (109) | 0.83 ± 0.05 |
| r.m.s. deviations from idealized geometry                           |        |
| Bonds (Å)                | 0.004 ± 0.0009 |
| Angles (degrees)         | 0.50 ± 0.02 |
| Impropers (degrees)      | 0.41 ± 0.02 |
| Mean pairwise r.m.s. deviation (Å)                                  |        |

| Backbone heavy atoms | All heavy atoms |
|----------------------|-----------------|
| Residues 127–189    | 0.43 ± 0.16     |
| Residues 133–138, 147–159, 162–167, 181–188 | 1.13 ± 0.13 |
|                     | 0.17 ± 0.05     |
|                     | 0.78 ± 0.11     |

* The best 21 structures after energy minimization as described under “Experimental Procedures” are shown. 98.4% of the backbone ϕ, ψ angles lie in the allowed regions, and 100% lie in the generously allowed regions of the Ramachandran plot as determined by PROCHECK (33). The numbers of restraints are shown in parentheses. None of the structures had distance violations >0.2 Å or dihedral angle violations >5°.

RESULTS

Structure of CuBD—We previously identified the second domain of APP, residues 124–189 (CuBD) (Fig. 1), as the copper binding domain of APP (10, 20). The CuBD was expressed recombinantly from P. pastoris. The copper binding and redox activity of CuBD in isolation was very similar to that of the intact protein (20). Mass spectrometry revealed that the purified protein was essentially free of metal ions, indicating it was in the apo form. To characterize the interaction of copper with CuBD at the molecular level, we have determined the three-dimensional structure of the CuBD by triple resonance multidimensional NMR spectroscopy. Sequential resonance assignments were made using a series of triple-resonance spectra (29) acquired at pH 6.9 on either uniformly15N- or 13C,15N-labeled CuBD using methods described previously (30). Resonance assignments were essentially complete. We used an Ellman’s test and the NMR data to show there are six half-cystine residues in CuBD linked in three disulfide bonds. Klaus et al. show that CγH-CαH, and CβH-CβH nuclear Overhauser effects are predictive of disulfide pairings between half-cystines i and j (32). This method allowed the unambiguous assignment of pairings to 133/187, 144/174, and 158/186.

The structures were calculated with CNS (31) using protocols described elsewhere (30). The final 21 structures (Fig. 2) were selected on the basis of their stereochemical energies. Structural statistics are presented in Table I. The structures are well defined and have good stereochemical properties, with all residues falling in the allowed regions (33) of the Ramachandran plot. The 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. 2). A disulfide bond between Cys-133 and Cys-174 links strands β1 and β3 and another between Cys-158 and Cys-186 links the α-helix to strand β3. The Cys-144–Cys-174 disulfide bond connects two loops at the other end of the molecule. There are very few buried hydrophobic residues in the vicinity of this disulfide bond, and therefore, this bond is probably very important in stabilizing the structure in a region that does not have any secondary structure. In addition to the three disulfide bonds, there is a small hydrophobic core to assist in stabilizing the structure consisting of a small segment of residues (Leu-136, Trp-150, Val-153, Ala-154, Leu-165, Met-170, Val-182, and Val-185) from each of the secondary structure elements. With the exception of the unstructured residues near the N terminus, 1H,15N heteronuclear nuclear Overhauser effect data (data not shown) indicated that the molecule was rigid along the entire backbone. The surface of CuBD is highly charged with several areas of high negative (Glu-156, Glu-160, and Glu-183, Asp-167 and Asp-131), and positive (Lys-132, Lys-134, Lys-161, His-147, His-151, and Lys-155) potential.

Metal Binding to CuBD—To determine the metal binding site on CuBD, Cu(Gly)2 was titrated into a solution of CuBD at pH 6.9. This resulted in the broadening of some resonances in the NMR spectrum, as would be expected for resonances from nuclei close to a paramagnetic center such as Cu(II). Analysis of the pattern of peaks in the two-dimensional 1H, 13N HSQC spectrum that were broadened by Cu(II) (Fig. 3a) suggested the presence of two binding sites, one centered on His-147, His-151, Tyr-168, and Met-170 and the other involving the N terminus. This second copper binding site of CuBD is not physiologically relevant since the N terminus is connected to the growth factor domain in the intact protein (Fig. 1). This second site is identical to a copper binding site at the N terminus of thioredoxin (Protein Data Bank (PDB) code 2TRX) that is also thought to be physiologically irrelevant.

There was a general decrease in the signal-to-noise ratio of the spectrum after Cu(II) addition, suggesting that higher order aggregates formed. To further characterize the metal binding site, the diamagnetic ions, Zn(II) and Ni(II), were titrated separately into CuBD solutions, and similar changes in the NMR spectra were observed with either metal. Decreases in the signal-to-noise consistent with metal-induced aggregation were observed with all metals, the addition of Zn(II) leading to a visible precipitate. Resonances corresponding to the aromatic protons of Tyr-168 broadened and disappeared, as did CH3 resonances of Met-170 (Fig. 3b). The backbone amide resonances from His-147, His-151 and Tyr-168 in the two-dimensional 1H,15N HSQC spectrum broadened beyond detection on the addition of excess metal. The broadening of resonances upon the addition of zinc or nickel is evidence for chemical exchange between the metal-bound and apo forms of the protein at intermediate exchange kinetics, which implies a Kd in the micromolar range. The lack of change in resonances distant from the immediate metal binding sites indicated there was no
significant structural alteration upon metal binding. To demonstrate a role for Met-170 in the binding and reduction of Cu(II), changes in the two-dimensional H, C HSQC spectrum of CuBD upon the addition of Cu(II) were monitored with the H and C chemical shifts of the C/H3 and C/H1 of methionine being diagnostic. Initially, the S-methyl resonance of Met-170 broadened beyond detection, whereas the Met-141 resonance, the only other methionine in CuBD, was unaffected, indicating the close proximity of Met-170 to the Cu(II). After incubating the sample at 30 °C for 48 h a new resonance was observed with H and C chemical shifts typical of C/H3 and C/H1 from methionine (Fig. 3c). The top spectrum shows the S-methyl resonances of Met-141 on the left and Met-170 on the right. The middle spectrum shows that the effect of adding 1 eq of Cu(II). The bottom spectrum was measured after the protein was incubated with Cu(II) for 48 h. The signal-to-noise of the spectrum decreased (due to aggregation), which necessitated the use of lower contour levels to observe the modified Met-170 signal. The Met-141 peak appears to get larger because of the lower levels, but in fact the peak width at half-peak height remains constant.

DISCUSSION

APP has a copper binding domain located in the N-terminal cysteine-rich region that can strongly coordinate Cu(II) and...
reduce it to Cu(I) (Fig. 1). It has been demonstrated that this domain can modulate copper homeostasis and production of Aβ, a peptide that plays a central role in the progression of Alzheimer’s disease. Here we report the structure of this domain and identify the residues (His-147, His-151, Tyr-168, and Met-170) involved in coordinating copper and the possible mechanism for copper reduction. The nature and orientation of these residues constitute a novel copper binding site.

His-147 and His-151 were shown previously to be necessary for copper binding (11). The orientation of these residues in the three-dimensional structure indicates that, with very small side-chain movements, a tetrahedral metal binding site suitable for coordinating Cu(I) is formed (Fig. 4). Such a site is reminiscent of the blue copper proteins that bind copper with a tetrahedral arrangement of ligands consisting of two histidines, a methionine, and a cysteine residue (36). The binding site in CuBD appears novel; a search of the PDB failed to identify a copper site with the same ligands. The closest example was peptidylglycine monoxygenase (PDB code 1PHM), which contains a redox active Cu(II) binding site consisting of two histidine residues, a methionine residue, and a water molecule in a tetrahedral coordination about the metal (37). Beyond this, there was no sequence or structural similarities between the two proteins.

The coordination of Cu(II) to the tetrahedrally arranged His-147, His-151, Tyr-168, and Met-170 (Fig. 4) can explain the redox chemistry associated with Cu binding to APP. In general, four coordinate Cu(II) ions favor a square planar coordination sphere about the metal, whereas Cu(I) generally prefers a tetrahedral arrangement (38). The EPR data (Fig. 5) suggest that Cu(II) bound to APP CuBD is distorted away from the square plane toward a tetrahedral structure. Histidine residues are common ligands for Cu(I) sites, and thioether ligands are known to stabilize Cu(I) in model compounds (38). Oxygen ligands are more common in Cu(II) complexes, and an oxygen ligand in stellacyanin is thought to be a major factor in this protein having the lowest reduction potential of all blue proteins (39). Hence, the tyrosine ligand in APP may facilitate binding of Cu(II), and this is subsequently followed by redox reactions. Because the copper binding site of CuBD appears to be a relatively rigid tetrahedral site, Cu(I) binding would be preferred, and the geometry would facilitate the reduction of Cu(II), which in the absence of any exogenous reductants, results in Met-170 oxidation (Fig. 3c). The oxidation of Met-170 in vivo is unlikely because this would alter the characteristics of the binding site, making it less likely to stably bind Cu(I); the presence of exogenous reductants such as ascorbate and thiols would also render metal reduction via Met-170 redundant.

Cu(I) sites are normally sequestered inside proteins because exposure could lead to the generation of reactive oxygen species via Fenton chemistry. Indeed such chemistry is observed when
copper binds to this domain (21). The APP copper binding site described here is unusual in that it is surface-exposed but similar to copper chaperone proteins that also possess surface Cu(I) sites (40). It is thought that the surface location ensures that the metal can be sequestered on binding of the chaperone to its target. Because exposed Cu(I) sites are prone to Fenton chemistry it would seem imperative that copper binding to APP would result in a rapid response. One possible scenario is as follows (Fig. 6). 1) Membrane-bound APP acts as a copper sensor/scavenger (17, 18). Cu(II) binding to APP leads to Cu(II) reduction since the CuBD binding site is optimized for Cu(I) binding. 2) The Cu(I) binding signals APP processing or proteolytic breakdown via the non-amylloidogenic route (21, 22). The signal transduction pathway could be triggered by conformational changes or oligomerization caused by the reduction. This is supported by experimental evidence showing that copper binding causes such changes (10); our metal binding experiments were accompanied by varying degrees of protein aggregation, and APP oligomerization plays a major role in APP processing (41). 3) The release of the APP ectodomain from the membrane (21, 22) would allow this secreted form to transport the metal to a nearby copper transporter/receptor or for excretion from the body via the liver. This hypothesis would explain the need for a surface location of the Cu(I) ion and provide a molecular basis for the observed role of APP in copper homeostasis (17, 18) and copper modulation of APP processing (22, 23).

Interestingly a search of the Protein Data Bank for similar folds (42) yielded 51 structures with the same α-helix packed over a triple strand β-sheet topology. Three of these proteins are involved in copper chaperone activity including the Menkes copper-transporting ATPase fragment (PDB code 1AW0), metallochaperone Atx1 (PDB code 2U2F), and SOD1 copper chaperone (PDB code 1QUP). These all have a different metal coordination sphere compared with CuBD using two thiol residues in a CXXC motif to bind Cu(I). However, these proteins are intracellular, whereas APP is an extracellular protein with the cysteine residues involved in disulfide bonds and, therefore, are available for metal coordination. Although most copper chaperones identified to date have been shown to utilize the high affinity of the sulphydryl group of cysteine residues to coordinate Cu(I), it has been reported that CopB copper ATPase, a transmembrane protein from Enterococcus hirae that is responsible for exporting excess copper, has histidine-rich metal binding motifs (43). The metallochaperone Atx1 displays a number of Lys residues on its surface, and it is thought that these residues play a critical in Atx1 recognizing its partner (Cc2, a copper transporting P-type ATPase) (44). Intriguingly, CuBD also has conserved Lys residues at 155 and 158 that lie in a similar location on the structure as does Lys-24 and -28 for Atx1.

The observations that APP knockout mice show specific elevations in brain and liver copper levels (17), whereas APP overexpression in mice results in significantly reduced copper levels (18) highlights the important role that APP plays in modulating neuronal copper levels. The structure presented here defines how copper interacts with the extracellular region of APP at the atomic level. Modulation of neuronal copper is important because a large body of work has emerged that suggests copper has a significant role to play in a range of neurodegenerative disease (3) including AD, Creutzfeldt-Jakob disease (45), Parkinson's disease (46), and amyotrophic lateral sclerosis (47).

As a possible treatment for Alzheimer's disease it would be highly desirable to develop a drug with specific high affinity binding to APP that would interfere with amyloidogenic APP processing in vivo. The interaction of copper with the CuBD effects APP processing such that Aβ production is significantly reduced (22). This suggests that agonists of copper interaction with APP would have therapeutic potential. The design of such agonists is greatly assisted by the structural information presented here. In addition, the recently reported success in a small-scale phase II clinical trial of the metal chelator clioquinol in reducing Aβ levels of treated patients illustrates the potential benefits of targeting copper interactions with APP/Aβ (48).

Acknowledgments—We thank Irene Volitakis and Robert Cherny for inductively coupled plasma mass spectrometry analysis and Frosa Katsis for N-terminal sequencing.

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J. Biol. Chem. 2003, 278:17401-17407.
doi: 10.1074/jbc.M300629200 originally published online February 28, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300629200

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