

A β Produced as a Fusion to Maltose Binding Protein Can Be Readily Purified and Stably Associates with Copper and Zinc

J. Caine^{a,*}, I. Volitakis^{b,c}, R. Cherny^{b,c}, J. Varghese^a and I. Macreadie^a

^aCSIRO Molecular and Health Technologies, Parkville, Australia; ^bOxidation Disorders Laboratory and Alzheimer's Disease Division, Mental Health Research Institute, Parkville, Australia; ^cDepartment of Pathology, The University of Melbourne, Victoria, Australia

Abstract: The 42 amino acid Alzheimer's A β peptide has been produced in *E. coli* as a soluble fusion to maltose binding protein (MBP). Affinity purification on amylose columns of MBP-A β and MBP led to the recovery of proteins at purities that were suited for physicochemical analyses. MBP-A β was able to bind approximately 2 mole equivalents of copper or 4 mole equivalents of zinc, while MBP alone bound negligible amounts of zinc or copper. We conclude that A β can bind 2 copper or 4 zinc ions in its fusion format. Because MBP-A β is a convenient protein to work with, this system is well suited for further studies on the structure of A β and its interactions with metals.

Keywords: Alzheimer's disease; amyloid peptide; copper binding; zinc binding.

INTRODUCTION

Plaques in the brains of individuals with Alzheimer's disease (AD) contain significant amounts of a 42 amino acid peptide known as A β as well as metals such as zinc, iron and copper [1-4]. Metals may have a major role in the pathology of AD, in particular in the production of reactive oxygen species (ROS) [5,6].

The central role of A β in AD has ensured an on-going attention to the direct association of A β with metals through studies using synthetic peptides. Mantyh et. al. first reported an analysis of metal ion-induced aggregation of an iodinated A β 1-40 with a range of metal ions [7]. However, subsequent work of Bush et al. [8] showed that this was an invalid reagent affected greatly by the iodination. The addition of iodine to tyrosine 10 blocked access of transition metals to the histidine rich domain.

Bush and colleagues demonstrated the zinc-induced aggregation of A β 1-40 [8] and showed that at lower pH there was an even higher affinity for copper leading to increase aggregation [9]. Aggregated A β 1-40 bound three to four Cu²⁺ ions and a comparison with rat A β demonstrated the involvement of histidine and tyrosine residues located toward the N-terminus of the peptide [9].

In this study, we examined the ability of A β to bind metals *in vitro* when fused to a protein that would aid the solubility and isolation of A β . A β was expressed in *E. coli* as a fusion to maltose binding protein (MBP) to produce MBP-A β . The purified MBP-A β , as well as MBP prepared in the same way, was subjected to protein and metal analyses. The results show that MBP-A β can bind zinc and copper ions.

MATERIAL AND METHODS

E. coli Strains and Growth Conditions

The bacterial strain employed in this study was *Escherichia coli* strain BL21(DE3) *F- ompT hsdSB (rB-, mB-) gal dcm* (DE3). The growth medium used was 2 x YT (0.5% yeast extract, 0.8% tryptone, 0.5% NaCl). Transformants were grown in 2 x YT + 50 μ g/ml ampicillin + 0.2% glucose at 37°C until the OD₆₀₀ reached 0.8. For induction, isopropyl β -D-thiogalactopyranoside (IPTG) was added to a level of 0.3 mM and the cells were grown a further 3 hr at 30°C.

Construction of Expression Vectors

Plasmids designed to encode the A β 1-42 fused to MBP with a 5-alanine residue linker, were constructed as follows [10]. A sequence encoding a portion of MBP was amplified by PCR using the primer pair f1-5 (5'-CTGATT TATAACAAAGATCTGCTGCCG), containing a *Bgl*III site (underlined), and r1-5 (5'-GTCATGTCGGAATTCATCCGCTGCAGCAGCTGCATTAGT CTGCGCGGCTGCC AGGGCTGCATCGACAGT), containing a *Pst*I site (underlined), and sequences to encode a five alanine linker and part of the N-terminal sequence of A β . pMALc2 (New England Biolabs) was used as the template to give the PCR product with the sequence encoding MBP-5ala. In addition, a gene fragment of A β (residues 1-42) was amplified using primer pair f2-5 (5'-GACTAATG CAGC TGCTGCAGCGGATGC AGAATTCGACATGACTCAGGATA), containing a *Pst*I site (underlined), and r2-5 (5'-GCGCGGGATCCTTACTAC GCTATGACAACACCGCCACCATGAGTCC), containing a *Bam*HI site (underlined), and encoding part of the C-terminal MBP plus 5 alanine sequence. A vector, pBSAPP (a gift from Dr. R. Cappai, Department of Pathology, University of Melbourne, Vic., Australia), containing A β 1-42 residue was used as the template to give a PCR product containing the A β gene sequence.

*Address correspondence to this author at the CSIRO Molecular and Health Technologies, Parkville, Australia; Tel./Fax: (61-3) 03-96627260; E-mail: jo.caine@csiro.au

A gene fragment containing MBP-5ala and A β was generated by mixing the two PCR products mentioned above, MBP-5ala and 5ala-A β , and subjecting the mix to 25 cycles with primers f1-5 and r2-5, giving a product of ~0.9 kb. The PCR product was inserted into pMALC2 vector cut with *Bgl*III and *Bam*HI to produce pMAL-5ala-A β . The sequence in Figure 1 shows the changes to the MBP sequence around the linker as compared to the original MBP-linker sequence from pMALc2. To obtain a control vector of MBP-5ala without the A β , a stop codon was inserted between the last of the 5 alanines and the A β sequence using Quikchange Mutagenesis (Stratagene). Positive clones were sequenced and correct ones used in a test expression.

Protein Expression and Purification

Production of the fusion protein was induced in cells in exponential growth phase by the addition of IPTG. Following growth and IPTG induction of a 1000 mL culture, bacterial cells were centrifuged and resuspended in 20 mM Tris-HCl pH 7.5 + Complete Protease Inhibitor (Roche). The cell suspension was then sonicated, centrifuged, and the supernatant was run on a column (10mm x 10mm) of Amylose-Sepharose, equilibrated in Tris buffer at 1 ml/min. For metal-exposed samples, the column was washed with the Tris buffer, then washed with 2 column volumes of either a solution of 1 mM CuSO₄ + 2 mM glycine pH 7.4 or 1 mM ZnCl₂ + 2 mM glycine pH 7.4. The metal buffers were washed away with at least 3 column volumes of Tris buffer before elution of the fusion protein with 20 mM Tris pH 7.5 + 10 mM maltose. In the case of copper this could be followed by OD280 as the copper/glycine absorbs at 280 nm. The column was washed until the OD was back to baseline and then two extra column volumes were put through the column. The same volumes were used for zinc assuming that the zinc washes would be similar. For the control MBP-5ala, the above procedure was also followed. All column procedures were performed at 4°C.

Protein Analyses by SDS-PAGE and Western Blotting and Estimation of Protein Concentration

The MBP-A β peak from the affinity column was analysed by SDS-PAGE with Coomassie staining. Samples were also blotted onto nitrocellulose membranes and probed with mouse monoclonal antibodies specific for MBP and A β . The MBP antibody was 8G1 (Cell Signaling Technology, Danvers, MA) and the A β antibody, WO2, which recognizes

residues 5-8 of A β , was a gift from Prof Colin Masters (Department of Pathology, University of Melbourne, Vic., Australia). Antibody binding to MBP or A β was detected by reaction with sheep anti-mouse Ig conjugated to horse radish peroxidase (HRP) (Silenus). The complex was visualised by reaction with the substrate 3,3',5,5'-tetramethylbenzidine (TMB) [11] except that the 1% dextran sulphate was added to the TMB solution instead of pretreating the membrane. Protein concentration was estimated using the modified Bradford method [12].

Estimation of Metal Concentration by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS)

Samples of the MBP-A β fusion protein and the control MBP-5ala were subject to metal analysis with and without the metal exposure step in the purification. The protein was subjected to Inductively Coupled Plasma - Mass Spectroscopy (ICPMS) after dilution with 1% nitric acid (HNO₃) (Aristar, BDH Chemicals). Measurements were made using an Ultramass 700 (Varian, Australia) in peak hopping mode with spacing at 0.100 atomic mass unit, 1 point per peak, 50 scans per replicate and 3 non-consecutive replicates per sample. Plasma flow was 15.0 L/min with an auxiliary flow of 1.5 L/min. Radio frequency power was 1.2 kW. Samples were introduced using a glass nebulizer at a flow rate of 0.95 L/min. The instrument was calibrated using a blank, 10, 50 and 100 ppb in 1% HNO₃ of a certified multi-element ICPMS standard solution (ICP-MS-CA12-1, AccuStandard Inc., New Haven, CT) containing each element of interest. A certified internal standard solution (ICP-MS-IS-MIX1-1, AccuStandard Inc, New Haven, CT) containing 100 ppb of Yttrium (Y89) was added via a T-piece as an internal matrix and instrument performance control.

RESULTS

Production and Purification of MBP-A β

Cells transformed with pMAL-5ala-A β and pMAL-5ala were induced with IPTG as described in Materials and Methods. Soluble and insoluble fractions were fractionated by SDS-PAGE and examined by Coomassie staining (Figure 2A) and Western blot (Figure 2B). MBP and MBP-A β are identified by the arrows in Figure 2A. The MBP antibody reacted with both proteins but antibody to A β reacted specifically with MBP-A β , which has an *Mr* 4 kDa greater than MBP (Figure 2B). The Western blots reveal that each protein

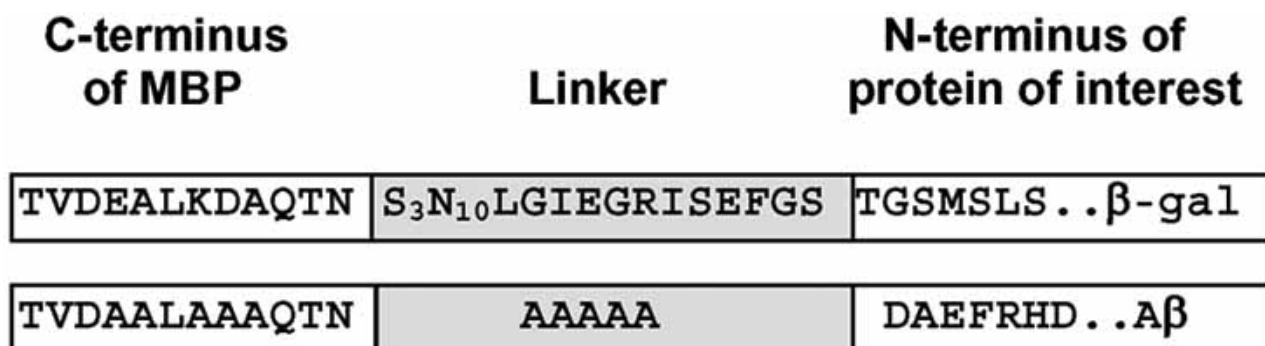


Figure 1. Comparison of the sequence differences of MBP-A β (bottom) and the fusion protein of pMALc2 (top).

tion procedure. The addition of a 5 alanine linker and changes of some of the residues preceding the linker were incorporated to aid in the proper folding of the fused A β [10].

From stoichiometric analyses, we have estimated that the highly purified MBP-A β binds two copper ions or four zinc ions. Since MBP alone exhibits no metal binding it is reasonable to expect that the observed metal binding of MBP-A β is due to the A β component. This is in general agreement with published results for soluble A β by Atwood et. al. who found that at pH 7.4, A β 42 bound Cu²⁺ at a stoichiometry of 2.2 and A β 40 bound Zn²⁺ at a stoichiometry of 2.9 [13]. When both Cu²⁺ and Zn²⁺ were added, a total of 3.6 bound metal ions (pH 7.4) per A β molecule were reported [13]. It is notable that higher amounts of metals appear bound at pH 6.6 where aggregation also occurs [9]. In our study A β 42 fused to MBP exhibits excellent solubility and would be suitable for studies on the structure and function of A β *in vitro* and *in vivo*. The purified fusion presents an alternative to the A β peptide which has solubility and aggregation problems in physiological solutions. We have found the MBP-A β remains soluble through storage periods of several months.

REFERENCES

- [1] Lovell, M.A., Robertson, J.D., Teesdale, W.J., Campbell, J.L. and Markesbery, W.R. (1998) *J. Neurol. Sci.*, 158, 47-52.
- [2] Lee, J.Y., Mook-Jung, I., Koh, J.Y. (1999) *J. Neuro. Sci.*, 19, RC10.
- [3] Suh, S.W., Jensen, K.B., Jensen, M.S., Silva, D.S., Kesslak, P.J., Danscher, G. and Frederickson, C.J. (2000) *Brain Res.*, 852, 274-278.
- [4] Smith, M.A., Harris, R.L., Sayre, L.M. and Perry, G. (1997) *Proc. Natl. Acad. Sci. USA*, 94, 9866-9868.
- [5] Huang, X., Cuajungco, M.P., Atwood, C.S., Hartshorn, M.A., Tyndall, J.D., Hanson, G.R., Stokes, K.C., Leopold, M., Multhaup, G., Goldstein, L.E., Scarpa, R.C., Saunders, A.J., Lim, J., Moir, R.D., Glabe, C., Bowden, E.F., Masters, C.L., Fairlie, D.P., Tanzi, R.E. and Bush, A.I. (1999) *J. Biol. Chem.*, 274, 37111-37116.
- [6] Huang, X., Atwood, C.S., Hartshorn, M.A., Multhaup, G., Goldstein, L.E., Scarpa, R.C., Cuajungco, M.P., Gray, D.N., Lim, J., Moir, R.D., Tanzi, R.E. and Bush, A.I. (1999) *Biochemistry*, 38, 7609-7616.
- [7] Mantyh, P.W., Ghilardi, J.R., Rogers, S., DeMaster, E., Allen, C.J., Stimson, E.R. and Maggio, J.E. (1993) *J. Neurochem.*, 61, 1171-1174.
- [8] Bush, A.I., Pettingell, W.H., Multhaup, G., d Paradis, M., Vonsattel, J.P., Gusella, J.F., Beyreuther, K., Masters, C.L. and Tanzi, R.E.. (1994) *Science*, 265, 1464-1473.
- [9] Atwood, C.S., Moir, R.D., Huang, X., Scarpa, R.C., Bacarra, N.M., Romano, D.M., Hartshorn, M.A., Tanzi, R.E. and Bush, A.I. (1998) *J. Biol. Chem.*, 273, 12817-12826.
- [10] Smyth, D.R., Mrozkiewicz, M.K., McGrath, W.J., Listwan, P and Kobe, B. (2003) *Protein Science*, 12, 1313-1322.
- [11] McKimm-Breschkin, J.L. (1990) *J. Immunol. Methods*, 135, 277-280.
- [12] Spector, T. (1978) *Anal. Biochem.*, 86, 142-146.
- [13] Atwood, C.S., Scarpa, R.C., Huang, X., Moir, R.D., Jone, W.D., Fairle, D. P., Tanzi, R.E. and Bush, A.I. (2000) *J. Neurochem.*, 75, 1219-1233.