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

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Abstract: The 42 amino acid Alzheimer's Aβ peptide has been produced in *E. coli* as a soluble fusion to maltose binding protein (MBP). Affinity purification on amylose columns of MBP-Aβ and MBP led to the recovery of proteins at purities that were suited for physicochemical analyses. MBP-Aβ 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 Aβ can bind 2 copper or 4 zinc ions in its fusion format. Because MBP-Aβ is a convenient protein to work with, this system is well suited for further studies on the structure of Aβ 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 Aβ 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 Aβ in AD has ensured an on-going attention to the direct association of Aβ with metals through studies using synthetic peptides. Mantyh et. al. first reported an analysis of metal ion-induced aggregation of an iodinated Aβ1-40 with a range of metal ions [7]. However, subsequent of 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 Aβ1-40 [8] and showed that at lower pH there was an even higher affinity for copper leading to increase aggregation [9]. Aggregated Aβ1-40 bound three to four Cu^{2+} ions and a comparison with rat Aβ 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 Aβ to bind metals *in vitro* when fused to a protein that would aid the solubility and isolation of Aβ. Aβ was expressed in *E. coli* as a fusion to maltose binding protein (MBP) to produce MBP-Aβ. The purified MBP-Aβ, as well as MBP prepared in the same way, was subjected to protein and metal analyses. The results show that MBP-Aβ can bind zinc and copper ions.

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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 μg/ml ampicillin + 0.2% glucose at 37°C until the OD_{600} 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 Aβ1-42 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*II site (underlined), and r1-5 (5'-GTCATGTCGGAATTCTGCAT CCGCTGCAGCAGCTGCATTAGT CTGCGCGGCTGCC AGGGCTGCATCGACAGT), containing a *Pst*I site (underlined), and sequences to encode a five alanine linker and part of the N-terminal sequence of Aβ. 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 Aβ (residues 1-42) was amplified using primer pair f2-5 (5'-GACTAATG CAGC TGCTGCAGCGGATGC AGAATTCCGACATGACTCAGGATA), containing a *Pst*I site (underlined), and r2-5 (5'-GCGCGGGATCCTTACTAC GCTATGACAACACCGCCCACCATGAGTCC), 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 Aβ 1-42 residue was used as the template to give a PCR product containing the Aβ gene sequence.

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A gene fragment containing MBP-5ala and Aβ was generated by mixing the two PCR products mentioned above, MBP-5ala and 5ala-Aβ, 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*II and *Bam*HI to produce pMAL-5ala-Aβ. 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 Aβ, a stop codon was inserted between the last of the 5 alanines and the Aβ 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 $CuSO_4$ + 2 mM glycine pH 7.4 or 1 mM $ZnCl_2$ + 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-Aβ 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 Aβ. The MBP antibody was 8G1 (Cell Signaling Technology, Danvers, MA) and the Aβ antibody, WO2, which recognizes residues 5-8 of Aβ, was a gift from Prof Colin Masters (Department of Pathology, University of Melbourne, Vic., Australia). Antibody binding to MBP or Aβ 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-Aβ 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 (HNO_3) (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% HNO_3 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-Aβ

Cells transformed with pMAL-5ala-Aβ 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-Aβ are identified by the arrows in Figure **2A**. The MBP antibody reacted with both proteins but antibody to Aβ reacted specifically with MBP-Aβ, which has an *Mr* 4 kDa greater than MBP (Figure **2B**). The Western blots reveal that each protein

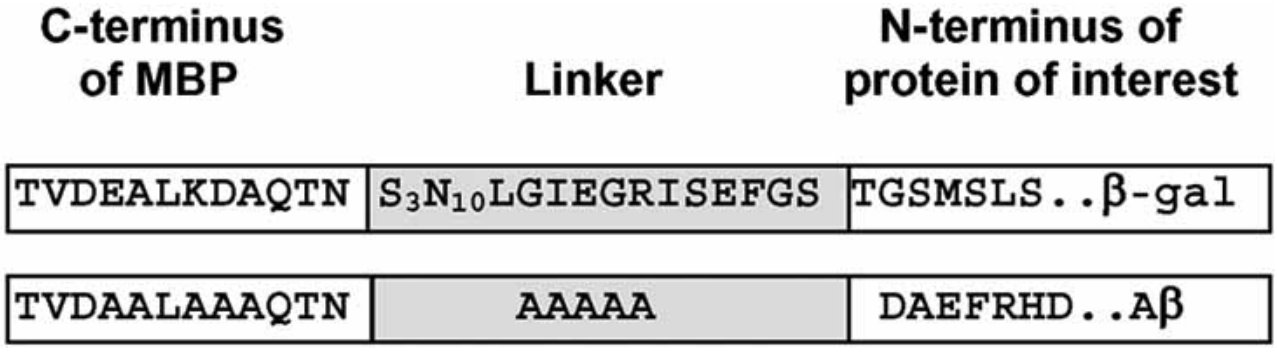

Figure 1. Comparison of the sequence differences of MBP-Aβ (bottom) and the fusion protein of pMALc2 (top).

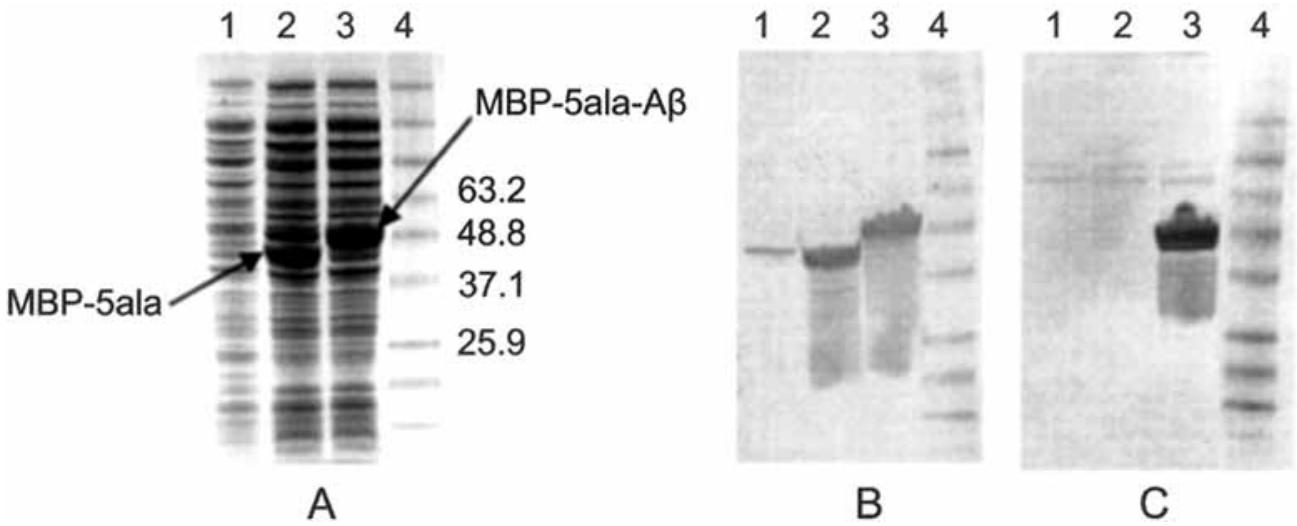

Figure 2. Production of MBP-Aβ. Panels are Coomassie–stained gel of the cell lysate (**A**) and Western blots probed with anti-MBP (**B**) and anti-Aβ (**C**). Lanes are: 1, uninduced MBP-5ala; 2, IPTG-induced MBP-5ala; 3, IPTG-induced MBP-5ala-Aβ; 4, Benchmark Prestained molecular weight standards in kDa (Invitrogen).

had no detectable breakdown products. From the Coomassie-stained gel (Figure **2A**) MBP and MBP-Aβ are estimated to represent around 5% of the soluble protein found in cell lysates.

The presence of both MBP and MBP-Aβ in the supernatant fraction (Figure **2A**) allowed affinity purification of both proteins through their ability to bind maltose. As shown in Figure **3**, procedures whereby MBP and MBP-Aβ were bound to the column, washed and then eluted, led to their recovery in ~95% pure form. In addition, the purified forms of both MBP and MBP-Aβ exhibited no breakdown and were of the expected size.

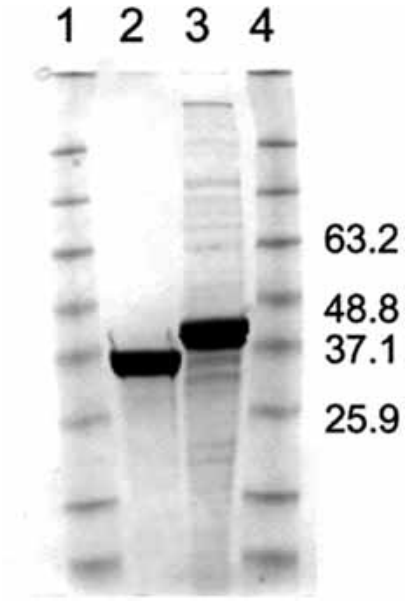

Figure 3. Purification of MBP-Aβ. Coomassie-stained gel after affinity purification as described. Lanes are: 1, 4, Benchmark Prestained molecular weight standards in kDa; 2, MBP-5ala; and 3, MBP-5ala-Aβ.

Metal Analyses

Purified MBP and MBP-Aβ were exposed to zinc and copper ions as described in Materials and Methods to determine metal binding to Aβ. The levels of metals bound were estimated using ICP-MS and the levels of protein were determined by Bradford assays. These molar ratios of metal to protein are shown in Figure **4**. Clearly there is negligible binding of metals to MBP but to MBP-Aβ, copper and zinc are bound at ratios of around 2 and 4 molar equivalents respectively.

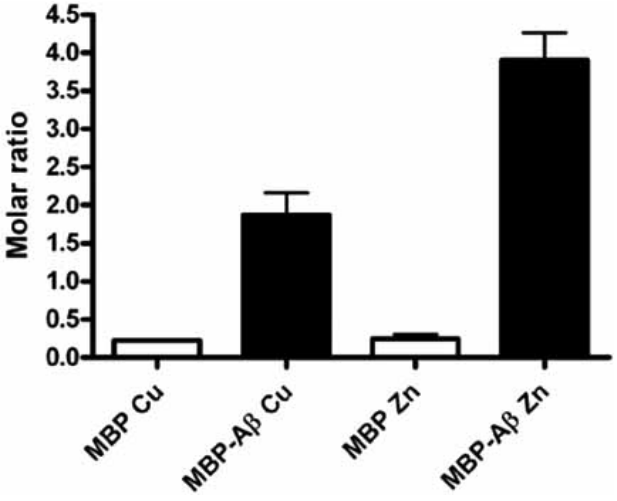

Figure 4. Metal bound to affinity-purified Aβ. Metal and protein concentrations were obtained by ICP mass spectrometry and Bradford assay, respectively. The molar ratios of metal to protein are represented on the graph after copper and zinc exposure.

DISCUSSION

In this study, Aβ has been produced in large quantities as a fusion to MBP, designated MBP-Aβ, making it soluble and easy to purify by the convenient amylose- affinity purifica-

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

From stoichiometric analyses, we have estimated that the highly purified MBP-Aβ 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-Aβ is due to the Aβ component. This is in general agreement with published results for soluble Aβ by Atwood et. al. who found that at pH 7.4, Aβ42 bound Cu^{2+} at a stoichiometry of 2.2 and Aβ40 bound Zn^{2+} at a stoichiometry of 2.9 [13]. When both Cu^{2+} and Zn^{2+} were added, a total of 3.6 bound metal ions (pH 7.4) per Aβ 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 Aβ42 fused to MBP exhibits excellent solubility and would be suitable for studies on the structure and function of Aβ *in vitro* and *in vivo*. The purified fusion presents an alternative to the Aβ peptide which has solubility and aggregation problems in physiological solutions. We have found the MBP-Aβ remains soluble through storage periods of several months.

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