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ORIGINAL ARTICLE

The natural non-protein amino acid *N*-β-methylamino-L-alanine (BMAA) is incorporated into protein during synthesis

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Abstract *N*-β-methylamino-L-alanine (BMAA) is an amino acid produced by cyanobacteria and accumulated through trophic levels in the environment and natural food webs. Human exposure to BMAA has been linked to progressive neurodegenerative diseases, potentially due to incorporation of BMAA into protein. The insertion of BMAA and other non-protein amino acids into proteins may trigger protein misfunction, misfolding and/or aggregation. However, the specific mechanism by which BMAA is associated with proteins remained unidentified. Such studies are challenging because of the complexity of biological systems and samples. A cell-free in vitro protein synthesis system offers an excellent approach for investigation of changing amino acid composition in protein. In this study, we report that BMAA incorporates into protein as an error in synthesis when a template DNA sequence is used. Bicinchoninic acid assay of total protein synthesis determined that BMAA effectively substituted for alanine and serine in protein product. LC–MS/MS confirmed that BMAA was selectively inserted into proteins in place of other amino acids, but isomers *N*-(2-aminoethyl)glycine (AEG) and 2,4-diaminobutyric acid (DAB) did not share this characteristic. Incorporation of BMAA into proteins was significantly higher when genomic DNA from post-mortem brain was the template. About half of BMAA in the synthetic proteins was released with denaturation with sodium dodecylsulfonate and dithiothreitol, but the remaining BMAA could only be released by acid hydrolysis. Together these data demonstrate that BMAA is incorporated into the amino acid backbone of proteins during synthesis and also associated with proteins through non-covalent bonding.

Keywords *N*-β-methylamino-L-alanine · *N*-(2-Aminoethyl)glycine · 2,4-Diaminobutyric acid · Neurodegeneration · Protein synthesis · Protein misfolding

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Introduction

N-β-methylamino-L-alanine (BMAA) is a natural non-protein amino acid that accumulates through food webs and eventually appears as a contaminant in some human diets (Cox et al. 2003; Murch et al. 2004a; Banack et al. 2006; Banack and Murch 2009; Jonasson et al. 2010; Brandt et al. 2010; Masseret et al. 2013; Fig. 1). Interest in BMAA has grown in recent years because of a proposed link with progressive neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) (Cox et al. 2003, 2005; Bradley 2009; Bradley et al. 2013; Field et al. 2013). BMAA was first discovered in the traditional diets of the Chamorro people of Guam and specifically in flour made from seeds of cycads (*Cycas micronesica* Hill) (Vega and Bell 1967). Variability in results of analysis of the flour made interpretation of the importance of BMAA difficult and, in 2004, it was reported that hydrolysis of the protein in the flour significantly increased the amount of BMAA detected (Murch et al. 2004a). It was not determined, however, whether the BMAA was incorporated into the protein or whether the BMAA was associated with proteins by some other mechanism such as electrostatic

interactions, carrier proteins or non-specific binding (Murch et al. 2004a). This was unknown BMAA mechanism was termed "protein-associated BMAA" and most subsequent researchers have included protein hydrolysis in BMAA analysis Murch et al. 2004a; Banack et al. 2011). To confound matters further, two biologically occurring isomers, *N*-(2-aminoethyl)glycine (AEG) and 2,4-diaminobutyric acid (DAB), are produced alongside BMAA (Banack et al. 2011)

There are several previous examples of non-protein amino acids incorporated into proteins during synthesis (reviewed in Rodgers 2014). Dunlop et al. (2013) described detection of ^{3}H-labeled BMAA in proteins in human cell cultures (Dunlop et al. 2013) and changes in protein structure have been reported in rats fed BMAA (Karlsson et al. 2014). It is possible that the misincorporation of BMAA into proteins may trigger progressive neurodegenerative diseases including ALS, AD and ALS-Parkinsonian Dementia Complex among the Chamorro people of Guam (Rodgers 2014; Murch et al. 2004b). The current work was designed to investigate the hypothesis that BMAA is incorporated into the amino acid backbone of proteins via errors in protein synthesis. We used an in vitro cell-free protein expression system for transcription/translation (PURExpress® In Vitro Protein Synthesis Kit, New England Biolabs), limiting amino acids to demonstrate selective incorporation of BMAA. This ideal system provides all the biological components required for protein synthesis to occur, but does not allow for breakdown of the protein or for other cellular and biological processes to interfere.

Methods and materials

In vitro protein synthesis

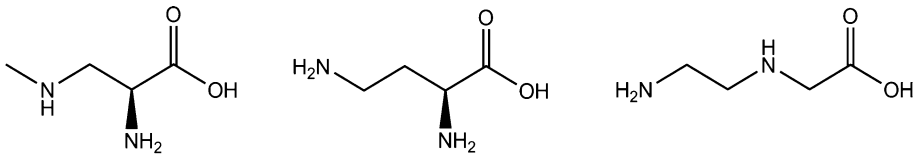
The PURExpress protein synthesis kit (P/N E6840S; New England Biolabs) was used for all protein synthesis reactions according to the instructions included with no variation. All kit components were kept on ice prior to experiments as per the instructions. In preliminary reactions, the template DNA (DHFR) supplied with the kit was used for both experimental groups as well as the positive and negative controls. The 20 classic protein amino acids (60 mM; Sigma, St. Louis, MO) are positive control for all reactions. The final concentration of each amino acid in the mixed solutions was 3 mM as per the kit instructions. The negative control contained only the enzymes and template DNA with no added amino acid solutions to provide a baseline protein concentration for the reaction. Once combined, all kit components were gently mixed and incubated for 16 h at 37 °C to facilitate protein synthesis. After incubation, all sample tubes were placed on ice to halt the synthesis reaction, and 25 μL of ice cold nuclease free water was added to each reaction. Each sample was then split into two 25 μL aliquots to measure the total amount of protein and the BMAA content of the protein product (Fig. 1).

Incorporation of BMAA

The positive control contained all 20 standard protein amino acids, but not BMAA. In preliminary experiments, amino acids were eliminated from the reaction solution individually to determine which amino acids were limiting for protein synthesis. BMAA was added to the positive control (all AA + BMAA) or substituted for limiting amino acids alanine (-ala + BMAA), glutamate (-glu + BMAA), glutamine (-gln + BMAA), isoleucine (-ile + BMAA), cysteine (-cys + BMAA), phenylalanine (-phe + BMAA), proline (-pro + BMAA), serine (-ser + BMAA), and threonine (-thr + BMAA). To determine whether the reactions with BMAA were unique or could be induced by the amino acid isomers, 2,4-diaminobutyric acid (DAB) and *N*-(2-aminoethyl)lglycine (AEG) were incorporated in the same manner (Fig. 1).

Human DNA templates

Genomic DNA was extracted from post-mortem brain tissues of three human patients (see Table 1 for patient information) using the Invitrogen PureLink Genomic DNA Extraction kit (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. In brief, 25 mg of tissue were weighed, in triplicate, into a sterile microcentrifuge tube, and 200 μL of a 90:10 mixture of the supplied digestion buffer and proteinase K solutions were added to each sample. After vortexing and incubation at 55 °C, samples were centrifuged and the supernatant removed. A 20-μL aliquot of ribonuclease was added to each tube prior to incubation for 2 min at room temperature. A 400 μL mixture of 50:50 binding buffer:ethanol was added to each sample; the lysate was then loaded on a supplied spin column and centrifuged. Two 500-μL washes were performed, with elution completed using 100 μL of the supplied elution buffer. Quantitation and purity determination of extracted DNA was performed using a nanodrop spectrophotometer (NanoDrop ND-1000, Fisher Scientific, Mississauga, ON); the $A_{260/280}$ and $A_{260/230}$ values were between 1.8 and 2.1 in all cases, indicating all extracts were quite free of protein and salt contamination. For protein synthesis reactions with human genomic DNA, exactly 125 ng of DNA was used to match the recommended concentration of the template DNA. Protein synthesis reactions were carried out using identical conditions and procedures as initial experiments.



L-BMAA L-DAB AEG

Fig. 1 *N*-β-methylamino-L-alanine (BMAA) and its biologically occurring isomers *N*-(2-aminoethyl)glycine (AEG) and 2,4-diaminobutyric acid (DAB)

Table 1 Patient information for analysis of protein containing BMAA

Patient	Age	Race	Gender	Neuropathological diagnosis
AD	81	Caucasian	M	Alzheimer's disease
ALS	70	Caucasian	M	Amyotrophic lateral sclerosis
Control	78	Caucasian	F	Non-neurologic control

Protein quantification

The total protein in each reaction mixture was quantified by BCA analysis (Pierce BCA Kit, Fisher Scientific, Mississauga, ON). Reagents A and B of the BCA assay kit were combined at ratio of 8:1, respectively, and vortexed vigorously to create the working reagent as per the instructions. Total protein was quantified by comparison to a bovine serum albumin (BSA) standard curve in triplicate at concentrations ranging from 10 to 0.5 mg/mL. For all analyses, 200 μL of the working reagent was mixed with 5 μL of each standard in a microcentrifuge tube. The solution was incubated at 30 °C for 30 min, after which each sample was transferred to a 96-well microplate (Corning Costar) and the absorbance was measured at 540 nm (BioTek Synergy HT microplate reader).

Analysis of BMAA

Protein was precipitated by addition of 25 μL of 0.1 N trichloroacetic acid (TCA), vortexing and centrifuging at 16,000*g* for 5 min. The resulting protein-free supernatant was transferred to a clean microcentrifuge tube. To ensure complete separation of the protein, an additional 25 μL of 0.1 N TCA was added to the protein pellets, vortexed and centrifuged. The supernatants were pooled and analyzed separately from the protein pellets as below.

Each protein pellet was dissolved in 500 μL of 6 N HCl in a 4 mL glass vial, and hydrolyzed overnight under nitrogen at 110 °C. A 100 μL subsample of each protein hydrolysate was lyophilized overnight (Labconco Freezone). When dry, 20 μL of 20 mM HCl and 60 μL of 0.2 M borate buffer were added and samples were vortexed. Protein samples were derivatized with 20 μL of AccQ-Fluor.

Liquid chromatography

A 10 μL aliquot from each sample was injected onto a reverse phase column (150 × 2.1 mm, 1.7 μm C18 BEH, Waters) using a Waters I-Class UPLC. Column temperature was set to 55 °C and the flow rate set to 0.650 mL/min. Mobile phase A was 20 mM ammonium acetate (adjusted to pH 5.0 using glacial acetic acid) and B 100 % methanol. The gradient was set as follows: 0.0 min = 90.0 % A; 5.0 min = 50 % A curve 6; 5.1 min = 15 % A curve 6; 5.5 min = 15 % A curve 6; 5.6 min = 90 % A curve 6; 7.0 min = 90 % A curve 6. The eluent was split so that only one-third of the eluent was directed to the ESI source of a Waters Xevo TQ-S triple quadrupole mass spectrometer. In order to control contamination buildup on the sample cone, largely resulting from nonvolatile borate buffer salts in the derivatization mixture, a divert valve was used to modify the flow state throughout the run. From 0.85 to 5.6 min, eluent was sent to the mass spectrometer; for the remainder of the run, the eluent was shunted to waste.

Mass spectrometry

The instrument was run in ES+ mode, with a cone voltage of 16 V, a capillary voltage of 750 V and a source offset of 20 V. The desolvation temperature was set to 550 °C, with corresponding gas flow of 800 L/h and a cone gas flow of 150 L/h. Data were acquired in MRM mode, with ultra-high purity argon, regulated to 7 psi, used as the collision gas. The instrument was configured such that a resolution of 0.75 amu was achieved across each quadrupole, with an applied span of 0.2 amu. A dwell time of 15 ms was used for all MRM transitions. All channels were monitored from 1.0 to 5.5 min. Data were acquired using the MRM transitions outlined in Table 1. Lysine was used to monitor derivatization reaction to ensure data integrity since lysine can accept up to two derivative tags with the Accq-Fluor reagent used in these experiments. Incompletely derivatized lysine gives three distinct chromatographic peaks; the two mono-derivatized forms and the double-derivatized lysine. Quantification of all three peaks provides a measure of derivatization reaction completeness and ensured results are not influenced by incomplete derivatization (Table 2).

Table 2 MRM transitions monitored in the analysis of BMAA, AEG, DAB and lysine

Analyte name	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (*V*)
Mono-derivatized BMAA	230.0	171.0	30
Mono-derivatized lysine	317.3	171.0	18
AEG/BMAA/DAB	459.0	119.0	18
AEG/BMAA/DAB	459.0	171.0	30
DAB qualifier	459.0	188.0	20
BMAA qualifier	459.0	214.0	20
AEG qualifier	459.0	258.0	20
AEG/BMAA/DAB	459.0	289.1	15
Derivatized lysine	487.3	171.0	18

Data analysis

Acquired data were processed using MassLynx V4.1 (Waters). Chromatograms were smoothed using a 3 × 2 Mean smooth. Detector responses were recorded as the analyte peak area as a function of peak area of the internal standard.

Denaturation of protein

To determine whether BMAA was carried by proteins or incorporated into proteins, the amount of BMAA released by protein denaturation was determined in subsamples of the protein pellet. This pellet was denatured with 1.5 % sodium dodecylsulfonate (SDS) and 2 mM dithiothreitol (DTT), then re-precipitated and washed with 0.1 N TCA. The free fraction supernatant and the protein pellet were prepared as described above.

Results

Controls

The negative control samples contained all kit components in the absence of amino acids, and therefore provide a measure of the protein content if de novo synthesis did not occur (Fig. 2). The positive controls contained the standard 20 amino acids and provided a measure of the expected protein synthesis for each reaction set (Fig. 2). Positive and negative controls were included with each set of samples and the other treatments were randomized across the sample sets. Substitution of isoleucine, glutamine or cysteine with BMAA did not result in de novo protein synthesis and the total protein was not significantly different from the negative control (Fig. 2).

Amino acid substitution

When DAB, AEG or BMAA were added to the positive control including all 20 protein amino acids, the total amount of protein produced was significantly less than the protein produced by the solutions containing normal protein amino acids, but significantly greater than the negative controls indicating that protein synthesis was partially but not completely inhibited by the non-protein amino acids (Fig. 2). Substitution of serine, phenylalanine, threonine, proline, glutamate, and alanine with BMAA resulted in significant de novo protein synthesis (Fig. 2). Substitution of alanine with BMAA resulted in no significant reduction in de novo protein synthesis as compared to the positive control (Fig. 2). When the human DNA templates were used and BMAA was substituted for serine, there was no difference in the amount of protein synthesized (Fig. 2).

Detection of BMAA in synthetic proteins

BMAA was not detected in proteins from the negative or positive control samples as a check of method accuracy and efficiency (Fig. 3). Small amounts of BMAA were detected in the protein fractions of the cysteine, threonine, glutamine, and isoleucine-limited treatments in spite of limited de novo protein synthesis (Fig. 3). When phenylalanine, proline, alanine, glutamate, and serine were limiting in the reaction, between 10 and 20 % of the BMAA added was found in the protein fraction (Fig. 3). Interestingly, when the human DNA templates were used in place of the standard bacterial template and serine was limiting, the incorporation of BMAA was increased to about 35–70 % with significant differences between individuals (Fig. 3). The patient diagnosed with ALS had the highest rate of BMAA incorporation into protein, significantly greater than the matched control patient (Fig. 3).

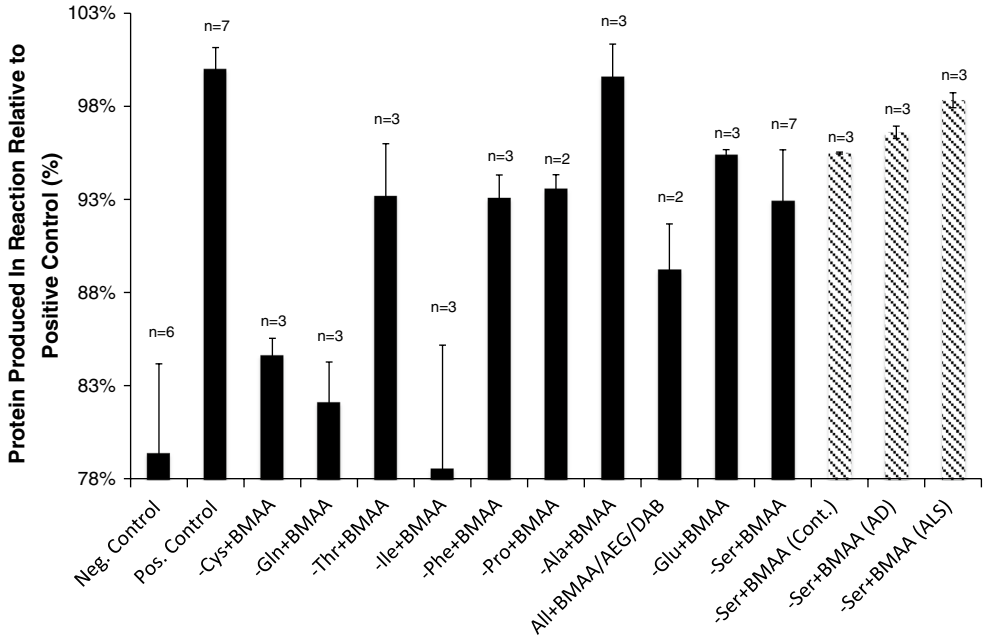


Fig. 2 Total protein in each cell-free synthesis reaction. Negative controls represent the protein present if no de novo protein synthesis occurs. Positive controls indicate the de novo protein synthesis with 20 amino acids present. Limiting amino acids are indicated by *hyphen*. Reactions containing the human DNA template are indicated by *patterned bars*. *Error bars* represent the standard error of the individual mean. Samples were randomized across multiple batches with individual replicates indicated as "n" for each bar

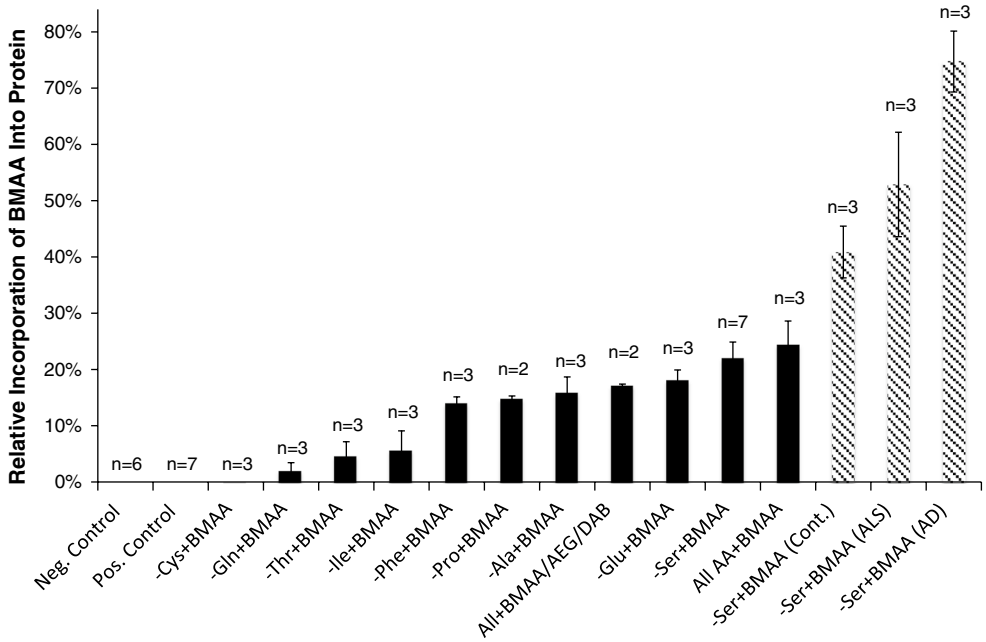


Fig. 3 Percent of BMAA incorporated into de novo protein as measured by LC–MS/MS. Limiting amino acids are indicated by *hyphen*. Reactions containing the human DNA template are indicated by *patterned bars*. *Error bars* represent the standard error of each individual mean. Samples were randomized across multiple batches with individual replicates indicated as "n" for each bar

Protein-associated vs. free BMAA

To determine whether the protein-associated BMAA was actually incorporated into the proteins during synthesis or attached to the proteins through some form of non-covalent interaction, proteins were denatured prior to analysis. About 50 % of the BMAA was released by denaturation indicating some non-covalent binding, but an equivalent

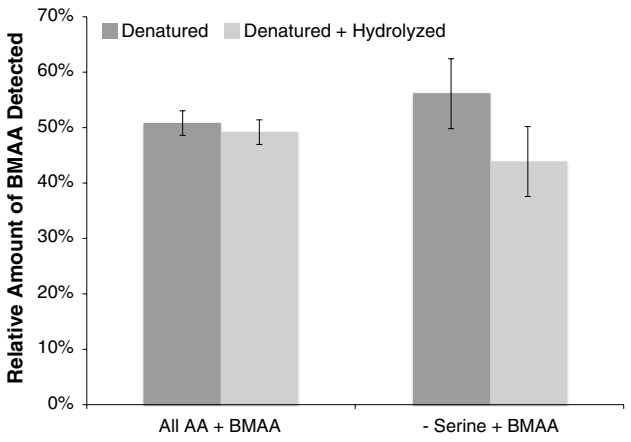


Fig. 4 BMAA recovered after denaturation of the protein and hydrolysis of the protein pellet after denaturation. *Error bars* represent the standard error of the individual means

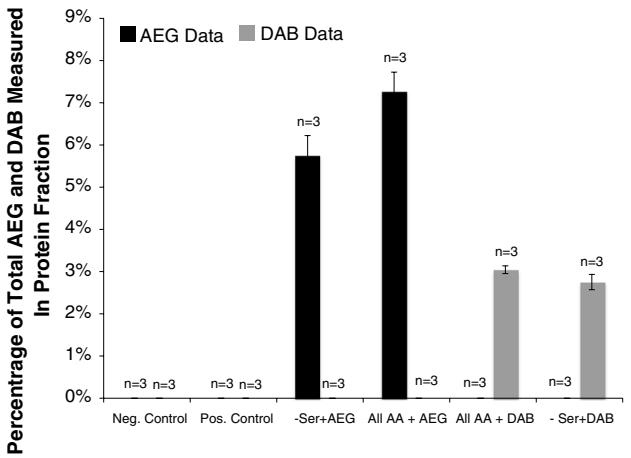


Fig. 5 Incorporation of AEG (*left*) and DAB (*right*) into protein as measured by LC–MS/MS. *Error bars* represent the standard error of individual means

amount was only released by hydrolysis after denaturation confirming covalent incorporation of BMAA into the amino acid chain (Fig. 4).

Isomers

To determine whether the incorporation of non-protein amino acids into proteins was a common mechanism or a unique characteristic of BMAA, the relative rate of association of other non-protein amino acids was determined in studies with BMAA isomers, DAB and AEG. Only a small amount of protein was made in the isomer reactions and small fractions of the isomers were recovered in the protein fractions with about 7 % of DAB and about 3 % of AEG associated with proteins (Fig. 5).

Discussion

The hypothesis that the non-protein amino acid BMAA could be "protein associated" has been controversial and efforts to understand the mechanisms of BMAA–protein association were required. The in vitro PURExpress protein synthesis system offered an ideal experimental design for these studies because protein synthesis was easily monitored and controlled. Our data show that BMAA is both incorporated directly into the protein backbone during de novo synthesis and attracted to the three-dimensional structure.

The idea that non-protein amino acids can be misincorporated into proteins during synthesis is relatively new. Selenocysteine, while not one of the 20 amino acids normally identified as a protein constituent, is found in approximately 25 human proteins, largely those involved in redox reactions (Nauser et al. 2012) and is now considered the 21st proteinogenic amino acid. Similarly, canavanine, an arginine analogue and plant non-protein amino acid, is incorporated into proteins by pests that feed on the plant tissues (Rosenthal and Dahlman 1986; Huang et al. 2011). In each of these cases, the amino acid that was substituted into protein for another, such as canavanine for arginine, is structurally quite similar to the original (Melangeli et al. 1997). Recently researchers have proposed that mischarging of the transfer RNA is one potential mechanism for amino acid misincorporation (Yadavalli and Ibba 2013). Our data demonstrate that incorporation of BMAA into the protein structure through peptide bonding occurs when structurally similar amino acids such as serine or alanine are deficient and that this occurs in human systems.

The incorporation or association of BMAA with proteins, specifically human neuroproteins, is significant for several reasons. BMAA has been proposed as a potential cause of progressive neurodegeneration (Cox et al. 2003, 2005; Bradley 2009; Bradley et al. 2013; Field et al. 2013). The incorporation of BMAA into proteins and re-release with protein turnover would result in a continual low recirculating dose that could result in a long latency period or the "slow" toxin mechanism that has previously been proposed (Spencer et al. 1991; Murch et al. 2004a, b). Our data provides a preliminary indication of variability between individuals but further research is required to determine whether the misincorporation of BMAA into proteins is a targeted or a random process. It is also possible that "intrinsically disordered proteins" such as α-synuclein or tau may be more prone to errors (Rodgers 2014; Uversky et al. 2008) and further investigations will determine the specific responses of different proteins. Neuronal cells may be less able to breakdown these malformed proteins (Rodgers 2014) and therefore the misincorporation of non-protein amino acids such as BMAA may be an environmental

factor that leads to protein misfolding and aggregation in neurodegenerative diseases.

Conclusion

We have used a cell-free synthesis system to investigate protein-associated BMAA and determined that BMAA is inserted into protein as an error in synthesis. Interestingly, human DNA templates seem more prone to error than the standard *E. coli* sequence. Further investigation with specific human DNA sequences of neuroproteins will determine whether specific proteins are more prone to amino acid substitution and will determine a specific mechanism for misincorporation. Together, these data suggest a new paradigm for understanding protein synthesis in which proteins are made up primarily of the standard 20 amino acids with other amino acids potentially included. Such a model may provide insight into proteomics and sequencing data that falls outside of the expected parameters. Further investigation is required to fully understand the impact of non-protein amino acids such as BMAA on protein structure and function.

Conflict of interest The authors declare that they have no conflict of interest.

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