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BMAA-protein interactions: A possible new mechanism of toxicity

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ABSTRACT

β-*N*-methylamino-L-alanine (BMAA) has been shown to accumulate in organisms by associating with host proteins. It has been proposed that this association is the result of misincorporation of BMAA into the primary structure of proteins, specifically in the place of L-serine, and that this misincorporation causes protein misfolding resulting in the tangle formation typically associated with neurodegenerative diseases. However, more recent studies have questioned the validity of the BMAA misincorporation hypothesis. Furthermore, BMAA association with proteins in the absence of *de novo* protein synthesis has been demonstrated although the nature of these associations has not yet been characterized. We therefore sought to investigate the effects of these undescribed interactions on protein functioning, and to identify the site(s) of these interactions. We present data here to show that BMAA can inhibit the activity of certain enzymes, interfere with protein folding in the absence of *de novo* protein synthesis, and associate *in vitro* with commercial proteins to such an extent that it cannot be removed by protein precipitation or denaturation. Based on the observed effects of these interactions on protein functioning, we suggest that this might constitute an additional mechanism of toxicity that could help to explain the role of BMAA in the development of neurodegenerative diseases.

1. Introduction

Exposure to the cyanobacterial (Downing et al., 2011), non-canonical amino acid β-*N*-methylamino-L-alanine (BMAA), has been suggested to contribute to the development of sporadic neurodegenerative diseases. Specifically, it has been proposed to be the main etiological agent of the 100 times higher incidence of Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC) prevalent on the island of Guam from the 1950's to 1980's (Arnold et al., 1953; reviewed in Banack et al., 2009). This neurotoxin bioaccumulates and biomagnifies in food chains (Banack and Cox, 2003; Brand et al., 2010; Jonasson et al., 2010), and this property within the unique Guam trophic system resulted in high levels of exposure in the Chamorro people living on Guam (reviewed in Banack and Murch, 2009). The cosmopolitan distribution of BMAA-containing cyanobacteria (Cox et al., 2005; Banack et al., 2007; Esterhuizen and Downing, 2008; Metcalf et al., 2008; Craighead et al., 2009; Cervantes et al., 2012) and the increasing number of water bodies that suffer persistent cyanobacterial blooms, suggest that people outside of Guam are also at risk of exposure to BMAA.

It has been suggested that the association of BMAA with proteins that results in bioaccumulation, is due to misincorporation of BMAA into protein backbones, the main evidence for which is the requirement for overnight acid hydrolysis, or treatment with proteases, to remove BMAA from proteins (Murch et al., 2004; Dunlop et al., 2013). It has also been proposed that this hypothetical misincorporation of BMAA into proteins, specifically in place of L-serine, might constitute a mechanism of toxicity of BMAA (Dunlop et al., 2013; Glover et al., 2014) since it could result in protein misfolding and aggregation, and ultimately in the protein tangles that are characteristic of neurodegenerative pathologies. This hypothesis was based on a reported concentration-dependent increase in the amount of radiolabel associated with proteins when exposed to ^{3}H-BMAA, and the toxicity observed in neuronal cell culture following BMAA exposure (Dunlop et al., 2013). However, previous studies have attributed neuronal toxicity of BMAA to excitotoxicity of the carbamate adduct of BMAA (β-*N*-carboxy BMAA) that forms spontaneously in the presence of bicarbonate (Weiss and Choi, 1988; Weiss et al., 1989; Myers and Nelson, 1990), or to the accumulation of reactive oxygen species (ROS) as a result of inhibition of cystine uptake by BMAA via the cystine/glutamate

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antiporter (Liu et al., 2009). High levels of toxicity, and specifically the accumulation of aggregated proteins as a result of misincorporation of BMAA, should be more pronounced in cell lines with high protein turnover rates such as liver cell lines, but toxicity in non-neural cell lines has yet to be reported. In addition, Van Onselen et al. (2015, 2017a, b) have shown that misincorporation of BMAA could not be demonstrated in either bacteria or in a rat phaeochromocytoma cell line, and that BMAA can be removed from proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by immobilized metal ion affinity chromatography, suggesting that BMAA-protein interactions are non-covalent in nature.

In 2011, Esterhuizen-Londt et al. reported that BMAA inhibits glutathione reductase activity *in vitro*, but not that of alcohol dehydrogenase, alkaline phosphatase or acetylcholinesterase. This inhibitory association could not be attributed to misincorporation of BMAA since the exposure and the enzyme reactions were conducted in the absence of *de novo* protein synthesis. This concurs with the observations of Van Onselen et al. (2015) that there are BMAA-protein associations not related to misincorporation that potentially play a role in BMAA toxicity. Butterfield et al. (1993) reported interactions between BMAA and membrane cytoskeletal proteins that were not related to protein misincorporation, and in Karlsson et al. (2009) also demonstrated strong *in vitro* interactions between BMAA and melanin, a polymer essentially composed of oxidized tyrosine residues. These data all add to the growing body of evidence suggesting that BMAA is not misincorporated into proteins, and that the nature of the observed protein-BMAA interactions warrants further investigation.

What sets BMAA apart from the other canonical amino acids that contain secondary amines in their side chains, is the unique pK_a values of BMAA. The pK_1 value for BMAA is 2.1, which is similar to all of the other canonical amino acids (Vega and Bell, 1967; Vega et al., 1968). However, the pK_2 for BMAA was determined to be 6.6, which is much lower than any of the other basic amino acids (Vega and Bell, 1967; Vega et al., 1968). This property renders BMAA a neutral, polar, zwitterion at physiological pH, with a deprotonated carboxyl and a protonated amine primarily at the β-position (Nunn and O'Brien, 1989). These characteristics make BMAA a reactive molecule at physiological pH. This reactivity of BMAA is demonstrated in the spontaneous reaction of BMAA with CO_2, a reaction that has been shown to be essential for the neurotoxicity of BMAA (Weiss and Choi, 1988; Weiss et al., 1989). It is therefore conceivable that the inherent reactivity of BMAA will allow it to interact with proteins and/or free amino acids without being incorporated into proteins, albeit via electrostatic interactions. We therefore sought to investigate the effects of BMAA-protein associations, other than the hypothesized misincorporation, on the normal functioning of a variety of proteins and to identify the target site(s) of these interactions.

2. Materials and methods

2.1. Investigating the effects of BMAA on enzyme functioning

The effect of BMAA on enzyme activity was investigated using six different commercial enzyme preparations. For β-amylase, a 0.65 mg ml^{-1} solution (β-amylase from barley, Sigma) was incubated with 300 μM BMAA (β-*N*-methylamino-L-alanine hydrochloride, Sigma, Steinheim, Germany, Lot # SLBG1185V), giving a molar ratio of 100 BMAA:enzyme in 20 mM sodium phosphate buffer, pH 7.4 for 3 h at 20 °C with continuous agitation. As controls, L-serine, L-arginine and L-alanine (all Sigma) were incubated with β-amylase in the same way, and controls without added amino acids were also prepared. The enzyme activity after each treatment was assayed spectrophotometrically as described by Bernfield (1955) using starch as a substrate and subsequently measuring absorbance at 540 nm. Negative controls were prepared in the same way except that they contained a heat-inactivated enzyme solution. All test and control samples were repeated in replicates of five.

In order to determine the effect of BMAA on catalase function, a commercial catalase solution (from human erythrocytes, Sigma) was diluted ten times in 0.01 M sodium phosphate buffer pH 7.0 containing BMAA to a final concentration of 88 μM, yielding a 100 BMAA:enzyme molar ratio. For control reactions, the enzyme solution was diluted in sodium phosphate buffer without BMAA. Following 1 h of pre-incubation with continuous agitation at 20 °C, the catalase activity of each solution was determined as per Sinha (1972) by measuring the formation of chromic acetate spectrophotometrically at 570 nm.

The effect of BMAA on glutathione S-transferase (GST) was determined by using a commercial preparation of an S9 cytosolic fraction from human liver. The extract, a pooled sample of 50 individuals purchased from XenoTech (Germany), was diluted to a final concentration of 0.05 mg ml^{-1} protein in 0.1 M sodium phosphate buffer pH 6.5 containing 20% glycerol, 1.4 mM dithioerythritol, and 1 mM ethylenediaminetetraacetic acid. BMAA was co-incubated with the diluted S9 fraction to a final concentration of 500 μM for 6 h at 4 °C. Controls without BMAA were prepared and incubated in the same way. Following incubation, the GST activity for each sample was determined as described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene as the substrate.

To investigate the effect of BMAA on peroxidase activity, a 1 μg ml^{-1} peroxidase solution (peroxidase from horseradish, Sigma) was incubated with 3 μM of BMAA, yielding a molar concentration of 1000 BMAA:enzyme, in 100 mM potassium phosphate buffer pH 7.0 with continuous agitation for 3 h at 20 °C. Following pre-incubation of the peroxidase with BMAA, peroxidase activity was determined as described by Chance and Maehly (1955) using pyrogallol as the hydrogen donor and subsequently monitoring the formation of purpurogallin spectrophotometrically at 420 nm.

The effect of BMAA on superoxide dismutase (SOD) activity was determined using the Calbiochem Superoxide Dismutase Assay Kit II. All reagents were prepared as described by the manufacturer and standard curves were prepared for each assay as per kit instruction, with the SOD concentration ranging from 0 to 0.125 U.ml^{-1} prepared from the stock SOD solution that was supplied. The stock SOD enzyme solution was incubated with 650 nM BMAA, giving a molar concentration of 1000 BMAA:enzyme, in the provided sample buffer for 3 h at 20 °C with continuous agitation. Controls without BMAA supplementation were incubated in the same way. The enzyme activities were subsequently assayed as per manufacturer's instructions in microtiter plates using a Biotek® PowerWaveXS spectrophotometer and measuring absorbance at 450 nm.

Ribonuclease H activity, and the effect of BMAA on that activity was determined as described below.

2.2. Investigating the effect of BMAA on in vitro protein folding

A fresh stock solution (2 mg ml^{-1}) of RNase H (bovine pancreatic, Sigma) was reconstituted in dissolving buffer (20 mM Tris-HCl, 100 mM NaCl, 8 M urea, pH 7.4) to allow unfolding of the enzyme. A 25 μL aliquot of this solution was subsequently added to 10 ml of refolding buffer (100 mM Tris-acetate, 100 mM NaCl, 0.5 M arginine, 1 mM GSH, 0.4 mM GSSG, pH 8) containing different BMAA concentrations (0, 50, 100 and 500 μM). The refolding solution was