Native Ubiquitin Structural Changes Resulting from Complexation with β-methylamino-L-alanine (BMAA)

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ABSTRACT: β-methylamino-L-alanine (BMAA) has been linked to the development of neurodegenerative (ND) symptoms following chronic environmental exposure through water and dietary sources. The brains of those affected by this condition, often referred to as amyotrophic lateral sclerosis-parkinsonism-dementia complex (ALS-PDC), have exhibited the presence of plaques and neurofibrillary tangles (NFTs) from protein aggregation. Although numerous studies have sought to better understand the correlation between BMAA exposure and onset of ND symptoms, no definitive link has been identified. One prevailing hypothesis is that BMAA acts as a small molecule ligand, complexing with critical proteins in the brain and reducing their function. The objective of this research was to investigate the effects of BMAA exposure on the native structure of ubiquitin. We hypothesized that formation of a Ubiquitin+BMAA noncovalent complex would alter the protein’s structure and folding and ultimately affect the ubiquitin-proteasome system (UPS) and the unfolded protein response (UPR). Ion mobility-mass spectrometry revealed that at sufficiently high concentrations BMAA did in fact form a noncovalent complex with ubiquitin, however similar complexes were identified for a range of additional amino acids. Collision induced unfolding (CIU) was used to interrogate the unfolding dynamics of native ubiquitin and these Ubq-amino acid complexes and it was determined that complexation with BMAA led to a significant alteration in native protein size and conformation, and this complex required considerably more energy to unfold. This indicates that the complex remains more stable under native conditions and this may indicate that BMAA has attached to a critical binding location.
INTRODUCTION

β-methylamino-L-alanine (BMAA) is a non-canonical amino acid synthesized by cyanobacteria (Figure 1A).\textsuperscript{1} Concern regarding environmental BMAA exposure and its potential toxicity dates back to the 1950s and 1960s when a significant increase in neurodegenerative symptoms was observed among the native Chamorro people of Guam and neighboring islands.\textsuperscript{2–4} The incidence of this neurodegenerative disease, referred to as amyotrophic lateral sclerosis-parkinsonism-dementia complex (ALS-PDC) due to its similarity to those diseases, was noted to be as much as 150 times higher than in other populations elsewhere\textsuperscript{3,5} and exhibited a strong familial factor but with no observed pattern of Mendelian inheritance.\textsuperscript{6} Researchers later suggested that compounds present in cycad seeds, a major component of diets on the affected islands, may have been linked to the incidence of the disease.\textsuperscript{7–9} Isolation of BMAA from cycads was first achieved by Vega and Bell in 1967, following which those researchers injected the compound into rats and observed neurotoxic symptoms that included convulsions, general weakness, and a dragging gait.\textsuperscript{10,11}
Figure 1. Molecular structure of (A) the non-canonical amino acid β-methylamino-L-alanine, (B) serine, and (C) alanine.

This correlation led to research showing that BMAA was produced by nearly all cyanobacteria in freshwater, brackish water, and ocean water. Although the levels of BMAA present in most water sources were lower than would be expected to cause the observed symptoms, Cox et al. hypothesized that biomagnification of BMAA could result in significantly increased concentration in cycad flour and flying foxes, another dietary staple of the Chamorro people.
Since this revelation, and because of mounting concerns over its toxicity, numerous studies have analyzed for BMAA in a variety of environmental sources ranging from cyanobacterial blooms to marine mollusks and sea scallops.\textsuperscript{13,14} Increasing ocean temperatures resulting from climate change have directly led to eutrophication and more intense, longer lasting cyanobacterial blooms.\textsuperscript{15,16} An associated increase in environmental BMAA concentration is likely to follow, potentially exposing a larger population to this toxic amino acid.

Despite the correlation between BMAA and development of neurodegenerative disease in Guam, little was known about the biological effects of BMAA. Controversy has since surrounded the exact role, or lack thereof, in the onset and progression of ALS-PDC and potentially other neurodegenerative diseases such as Alzheimer’s. It has been known for several decades that changes to protein primary, secondary, and tertiary structure can potentially lead to protein misfolding and loss of function.\textsuperscript{17,18} Protein misfolding has been implicated in many neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease.\textsuperscript{19,20} Because of the correlation between BMAA exposure and onset of neurodegenerative symptoms, it was hypothesized that BMAA toxicity occurs because of misincorporation into proteins in place of serine and/or alanine residues. Complementary studies showed that BMAA was directly misincorporated into proteins, in the place or serine or alanine (Figure 1B-C), during translation when introduced into the growth media of several different cell lines (MRC-5 fibroblasts, SH-SY5Y neuroblastoma, etc.),\textsuperscript{21} and that this substitution could promote misfolding and aggregation;\textsuperscript{22} it is important to note that other studies have disputed this theory and were not able to detect similar changes.\textsuperscript{23}

Still other studies have indicated that BMAA indeed interacts with proteins, but instead through a non-covalent association rather than translational misincorporation.\textsuperscript{24} These interactions
could lead to BMAA influencing other biochemical pathways including ER stress, protein ubiquitination, the unfolded protein response, and mitochondrial dysfunction. Early investigation of the brains of those affected with ALS-PDC in Guam revealed the presence of neurofibrillar tangles (NFT, hyperphosphorylated tau protein), results that were later attributed to dietary exposure to BMAA resulting in NFT and β-amyloid deposits. The potential effects of BMAA exposure on the ubiquitination pathway constitutes any interesting area of research. Ubiquitin (Figure 2) is responsible for tagging misfolded proteins to be degraded by the 26S proteasome. Failure of this process can lead to accumulation of misfolded proteins, a hallmark feature of neurodegenerative diseases. This prompts the cell to activate the unfolded protein response (UPR), a mechanism that has shown to be upregulated in systems exposed to high concentrations of BMAA. Despite these correlations, specific protein structural effects have yet to be studied thoroughly. It is a necessity that we better understand the biological effects of BMAA exposure on the etiology and progression of neurodegenerative diseases. This study aims to investigate the hypothesis that noncovalent complexation of BMAA with ubiquitin affects the structure (and thus the activity) of this critical protein, ultimately disrupting the ubiquitination and unfolded protein response mechanisms and leading to a buildup of these proteins to form plaques in the brain.
Figure 2. Crystal structure of human erythrocytic ubiquitin, refined at 1.8 Å resolution. (Reprinted from Structure of ubiquitin refined at 1.8 Å resolution, 194, Senadhi Vijay-Kumar et al., 531-544, with permission from Elsevier).

EXPERIMENTAL METHODS

Sample Preparation

Ubiquitin from bovine erythrocytes and all amino acids were purchased from Sigma-Aldrich (St. Louis, MO). All samples were prepared using Fisher Optima LC-MS grade water with 10 mM ammonium acetate buffered to pH 7.0; both were purchased from Fisher Scientific (Pittsburgh, PA). Ubiquitin solutions were prepared at 36 μM. BMAA and other amino acids were added individually at 1:1 or 10:1 molar ratio to ubiquitin (as noted in the text).

Instrumentation and Tuning
All analysis was performed using an Agilent Technologies (Santa Clara, CA) 6560 IM-QTOF instrument. Samples were direct infused via syringe pump at 10 µL/min into an Agilent Jet Stream (AJS) dual ESI source. All experimental ion source conditions can be found in the Supporting Information (Table S1). All instrument parameters were tuned to achieve native mass spectrometry conditions according to the guidelines by Gabelica et al.⁴² These conditions were deemed appropriate when the collision cross section (CCS) and conformational profile for native ubiquitin were in agreement with other published values obtained using similar Agilent 6560 instrumentation.⁴²,³³ Briefly, ions were accumulated in the trapping ion funnel for 1000 µs and released for 250 µs into the drift tube maintained at approx. 4 Torr nitrogen gas under ambient temperature (~27 °C). The drift tube was operated with a field strength of 19.1 V/cm. CCS were calculated using the single-field method, which involves measurement of the CCS for Agilent Tune Mix ions (m/z 322-2721) to determine both β and T-Fix values. All IM-MS data was visualized and processed using Agilent IM-Browser 10.0.

**Collision Induced Unfolding (CIU)**

Collision induced unfolding (CIU) was performed on the ions during accumulation in the trapping ion funnel (Figure S1) according to a method developed by Gabelica et al.⁴² This involves ramping the Entrance Grid Delta between 2.5-15 V, in increments of 0.5 V. Data was acquired at each voltage for 30 seconds, while all other experimental parameters were maintained constant. Raw data was processed using CIU Suite 2, which allows for direct upload of raw Agilent .d files to create drift time/CCS vs. Entrance Grid Delta CIU plots.³⁴ Default parameters were used (i.e., no additional smoothing or processing).
RESULTS AND DISCUSSION

To test our hypothesis of BMAA exposure causing ubiquitin structural changes, we analyzed samples of native ubiquitin in the presence of BMAA and a series of other amino acid standards. Native MS conditions were achieved and confirmed both by the charge state distribution observed (Figure 3A) and by measurement of CCS using ion mobility spectrometry (Figure 3B). Under these optimized conditions, the base peak at m/z 1428 represents the +6 charge state, while other minor peaks were also observed for the +4, +5, +7, and +8 charge states. The ion mobility spectrum for the +6 charge state shows two major conformations at CCS ~1200 and 1400 Å², which is in good agreement with other published results obtained on the same Agilent 6560 platform.32,33
**Figure 3.** (A) Native mass spectrum for ubiquitin showing the base peak for the +6 charge state at m/z 1428. (B) Native ion mobility spectrum showing the primary conformation at ~1200 Å² and a larger, secondary conformation at ~1400 Å².

Collision induced unfolding (CIU) was performed on native ubiquitin by ramping the trapping funnel entrance grid delta from a low value of 2.5 V (i.e., native conditions) up to a high value of 15 V. Note that on this instrumental platform, mass selection cannot be performed prior to ion accumulation and CIU; as such, all charge states for ubiquitin are exposed to the same increasing energy conditions. In agreement with previous literature for native ubiquitin CIU, there was a major transition from the compact state to the first unfolded state at approx. 8 V (Figure 4). This unfolded state was stable over a wide energy range, although an additional unfolded state was observed at very high voltage (~13 V).

**Figure 4.** Collision induced unfolding (CIU) plot for the +6 charge state of native ubiquitin, showing CCS (Å²) as a function of increased entrance grid delta (V).
We then analyzed mixtures of native ubiquitin with BMAA and several other amino acids. The relative concentration of these amino acids was quite high (36-360 µM), but this is in good agreement with the concentrations at which neurodegenerative symptoms are observed in vivo in animal studies. The full mass spectrum for the Ubiquitin+BMAA sample showed an abundance of free protonated BMAA monomer and dimer (Figure S2). A zoomed mass spectrum for native ubiquitin (Figure 5A) and Ubiquitin+BMAA (Figure 5B) shows the new formation of the noncovalent Ubq-BMAA complex [Ubq+BMAA+6H]^6 at m/z 1448. Unlike the native ubiquitin, the ion mobility spectrum for the Ubq-BMAA complex displayed only a single mobility conformation, for the compact conformer at CCS ~1200 Å² (Figure S3). Mass spectra were also acquired for solutions of ubiquitin containing several different classes of amino acids: those with hydrophobic side chains (isoleucine, phenylalanine, tyrosine, and valine), polar uncharged side chains (serine, threonine, and glutamine), positively charged side chains (arginine and histidine), negatively charged side chains (aspartic acid), proline, and glycine. The formation of the noncovalent Ubq-AA complex was observed in the mass spectrum for each amino acid mixture, primarily as the +6 charge state. Zoomed mass spectra for Ubq+Ile and Ubq+Tyr are shown in Figure 5C-D. CCS values for the primary conformer of each noncovalent complex are listed in Table 1.
Figure 5. Zoomed mass spectra (m/z 1420-1460) for (A) the +6 charge state of native ubiquitin at m/z 1428; (B) ubiquitin+BMAA mixture, showing the Ubq-BMAA noncovalent complex [M+BMAA+6H]+ at m/z 1448; (C) ubiquitin+isoleucine mixture, showing the Ubq-Ile noncovalent complex [M+Ile+6H]+ at m/z 1450; and (D) ubiquitin+tyrosine mixture, showing the Ubq-Tyr noncovalent complex [M+Tyr+6H]+ at m/z 1458.
Table 1. Collision cross section (CCS) for the noncovalent complex formed between ubiquitin and several amino acids from different classes.

| Complex           | m/z  | CCS (Å²)     |
|-------------------|------|--------------|
| [Ubq+6H]^6        | 1428 | 1207.2 ± 1.2 |
| [Ubq+BMAA+6H]^6   | 1448 | 1237.0 ± 4.4 |
| [Ubq+His+6H]^6    | 1454 | 1193.6 ± 1.1 |
| [Ubq+Ile+6H]^6    | 1450 | 1250.5 ± 2.5 |
| [Ubq+Asp+6H]^6    | 1450 | 1211.1 ± 1.7 |
| [Ubq+Gln+6H]^6    | 1453 | 1239.6 ± 1.7 |
| [Ubq+Arg+6H]^6    | 1457 | 1202.0 ± 4.4 |
| [Ubq+Gly+6H]^6    | 1441 | 1196.8 ± 0.8 |
| [Ubq+Thr+6H]^6    | 1448 | 1207.7 ± 1.1 |

CIU was performed on each of the Ubq-amino acid mixtures to investigate the effect of complexation on unfolding dynamics for these complexes (Figure 6C-F). The CIU plots for all Ubq-AA complexes (excluding BMAA) resembled that which was observed for native ubiquitin alone, indicating that the structure for Ubq is not largely changed through complexation. Additionally, the unfolding transitions were nearly identical, with a major transition from compact to unfolded at ~8 V for all Ubq-AA complexes. The spectrum for Ubq-BMAA (Figure 6B),
however, was considerably different than either native Ubq or the other Ubq-AA complexes. Notably, the CIU spectrum showed that the compact conformation was stable up to a considerably higher energy (~10-11 V) indicating the relative stability of the protein in its compact form. This information supports the hypothesis that BMAA, as a small molecule ligand capable of binding tightly with ubiquitin, disrupts the structure and unfolding dynamics of the protein; this could potentially disrupt the ubiquitination and unfolded protein response mechanisms and lead to a buildup of these proteins to form plaques.
Figure 6. Collision induced unfolding (CIU) plots for (A) native ubiquitin, and noncovalent complexes with (B) BMAA; (C) isoleucine (hydrophobic side chain); (D) serine (polar side chain); (E) proline; and (F) glycine. The white dashed line in each plot highlights the unfolding transition point.
CONCLUSION

In this work, we investigated the effects of the proposed cyanobacterial neurotoxin β-methylamino-L-alanine (BMAA) on the native structure and unfolding dynamics of ubiquitin, an important component in tagging misfolded proteins for degradation. We used native mass spectrometry to identify noncovalent complexes for ubiquitin with BMAA as well as other amino acids in each major class, the dominant species being the +6 charge state for each. We observed that the conformational distribution for the Ubq-BMAA complex shifts to the smaller conformer, but more importantly it displays a significantly different collision induced unfolding (CIU) behavior. Native ubiquitin and its complexes with other amino acids resemble the unfolding dynamics of native ubiquitin alone. Studies are underway using high-resolution protein NMR to further probe the BMAA binding location(s) to ubiquitin. Additional studies will also expose *C. elegans*, as a model organism, to high levels and BMAA, after which both bottom-up and native proteomics approaches can be used to study the effects of BMAA on ubiquitin *in vivo*.

ASSOCIATED CONTENT

**Supporting Information.** The Supporting Information is available free of charge. Agilent 6560 schematic; Full mass spectrum of Ubiquitin+BMAA; CCS plot for native ubiquitin and Ubiquitin+BMAA; Instrumental parameters for native proteomics experiments on Agilent 6560 (.docx)

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Author Contributions

K.M.W, A.B.M, and S.W.M prepared and analyzed the samples. K.M.W. and C.D.C processed the data and wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

BMAA, β-methylamino-L-alanine; ND, neurodegenerative; NFT, neurofibrillary tangle; UPS, ubiquitin-proteasome system; CIU, collision-induced unfolding; ALS-PDC, amyotrophic lateral sclerosis-parkinsonism-dementia complex; UPR, unfolded protein response.
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