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Copper Isotope Compositions of Superoxide Dismutase and Metallothionein from Post-Mortem Human Frontal Cortex

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Abstract: The natural copper isotopic compositions of superoxide dismutase and metallothionein from six post-mortem human frontal cortices were determined using a combination of size exclusion protein liquid chromatography, followed by anion exchange chromatography and multiple collector inductively-coupled plasma mass spectrometry. Superoxide dismutase was enriched in the heavier ^{65}Cu relative to the metallothionein fraction in all specimen pairs. The isotopic compositions were independent of copper content. This finding provides evidence that nitrogen ligands in protein copper binding sites will be enriched in heavy metal isotopes, and sulphur ligands will preferentially incorporate lighter isotopes in vivo. This in turn has implications for understanding isotopic distributions within different components in the body and the dominant ligands in different tissues. Differences in Cu isotope distributions between the two proteins were seen between Alzheimer's disease and healthy control samples, when normalised for sex.

Keywords: MCICPMS; Alzheimer's disease; isotope fractionation; copper; superoxide dismutase; metallothionein

1. Introduction

Trace metals are important for many biological functions and have a significant role in health and disease [1]. Loss of function in critical metalloenzymes results in an array of genetic diseases for example, Wilson's disease (mutation of ATP7B), Menkes (mutation of ATP7A) and aceruloplasminemia (mutation of ceruloplasmin). There is an increasing need for techniques to probe the role of trace elements in biology and provide further insight into metal–protein interactions in healthy and diseased states. Isotope compositions (e.g., the ratio of the amounts of ^{63}Cu to ^{65}Cu) are a relatively new tool to probe the pathway of trace metals in biological systems. In contrast, there have been numerous applications in the geosciences and the environment (e.g., [2–4]). Natural stable isotope fractionation is caused by mass-dependent differences in energy requirements and the fact that some chemical processes preferentially incorporate lower free energy states in different isotopologues, as occurs during a change in phase, ligand coordination, or oxidation state [2–5]. Differences in the isotopic fractionation of copper, zinc and iron have been found in an array of diseases relative to healthy controls [6–11], as well as in the ranges of "healthy" values in relation to age, sex and dietary intake [12–15]. These differences have been found in diverse tissue types including serum, urine

and breast tissue and are used to facilitate improved understanding of the mechanisms from the cellular level to the whole body. The interpretations of these results from humans are based on geochemical observations, computational simulations of metal–ligand interactions and a limited number of laboratory studies [4,12,16–21]. The isotopic compositions of trace metals in specific protein–metal complexes in the human body have not been studied, despite the fact that this is fundamental to the correct interpretation of such signatures (e.g., [22]). The objective of this study is to address this gap in knowledge, through a unique combination of metallo-biochemical techniques and high precision isotope ratio mass spectrometry.

The isotopic compositions of two copper binding metalloproteins, metallothionein (MT) and superoxide dismutase (SOD), enriched from seven specimens of human brain tissue from both healthy controls and those with Alzheimer's disease are presented. Metallothioneins and SODs are critical proteins for the mediation of oxidative stress [23,24]. Metallothioneins are important proteins with regards to the regulation of metal toxicity and transport, and various isoforms are found in many mammalian tissues including the liver and brain. The multiple cysteine residues bind metals with sulphur ligands, and a single molecule can contain up to seven bivalent metal ions and 20 monovalent ions [23]. Transition metals are key to SOD activity; two of the three human SOD isoforms contain copper and zinc. Of these isoforms, SOD1 is found in the cytosol of almost all human cells, and SOD3 is found extracellularly [24]. Unlike MT, the SOD copper-binding site is a nitrogen-rich histidine [25].

These two proteins have different, but critical roles in metal homeostasis, are spread widely throughout the body, and utilise different binding environments to interact with metals. Loss of MT function has been implicated in a range of diseases, including cancer [23], Parkinson's disease [26] and Alzheimer's disease [27]. Mutation of Cu– and Zn–SOD results in familial amyotrophic lateral sclerosis, and although the link with the metal cofactors is still being elucidated, there are promising therapeutic developments that implicate a causal role for Cu in the disease [28,29].

The aim of this study is to demonstrate a novel combination of HPLC and high precision isotopic analysis for the isolation and subsequent determination of copper isotope compositions in cortex samples, as a new tool to investigate copper metabolic changes in neurodegenerative diseases.

2. Results and Discussion

The copper isotope composition of SOD relative to metallothionein was found to be significantly heavier in all specimens analysed as part of this study regardless of disease state and gender, with the exception of the female Alzheimer's disease specimen (AD3, Table 1) for which no significant difference in isotope composition was found between the two complexes.

Table 1. The copper isotopic composition of metallothionein and superoxide dismutase isolated from brain homogenates of diseased and non-diseased states and similar values for the same bonding environments from other studies.

Sample	Sex	Total Cu_{SOD} (ng)	$\delta^{65}Cu_N$ (‰) [4]	Total Cu_{MT} (ng)	$\delta^{65}Cu_S$ (‰) [4]	$\Delta^{65}Cu_{N-S}$ (‰) [4]	Source
AD1 [1,2]	Male	180	0.34	43	−0.25	0.59	this study [5]
AD2 [1]	Male	86	0.36	30	−0.08	0.44	[5]
AD3 [1]	Female	40	−0.33	27	−0.39	0.06	[5]
CON1	Male	83	0.37	26	−0.42	0.79	[5]
CON2 [3]	Female	48	0.31	47	0.14	0.17	[5]
$Yeast_{SOD-MT}$						0.53	[4]
RBCs–serum	Female		0.46		−0.24	0.60	[12]
RBCs–serum	Male		0.67		−0.28	0.95	[12]

[1] National Institute of Aging (NIA) Reagan criteria; [2] mean values obtained from brain analysed in replicate analysis; [3] mean values of two female samples combined prior to isotopic analysis; [4] isotopic composition given in reference to the binding ligand. [5] this study. CON = non-diseased states. For this study and [4], N = superoxide dismutase (SOD), S = metallothionein (MT); for [12], N = erythrocytes (RBCs), S = serum.

A few other observations can be made from this dataset, which indicate that further study into this phenomenon is warranted. Despite the small sample set, the male specimens analysed appeared to have larger differences in copper isotope composition between the two metallo-protein complexes ($\Delta^{65}Cu_{SOD–MT}$) compared to the same values obtained from female specimens. All healthy control specimens had a larger $\Delta^{65}Cu_{SOD–MT}$ compared to the sex-matched Alzheimer's disease isotope composition.

The direction of isotopic offset between these two proteins is consistent with previous in vitro and computational studies. Ab initio modelling and laboratory-based investigations [12,16–18,21] of protein–metal interactions have indicated that, because of the stronger bonds formed, heavier isotopes will preferentially bond to amino acids with harder ligands, such as nitrogen and oxygen, whereas softer ligands such as sulphur, will show a preference for lighter isotopes. In addition, when applicable, the oxidation state of the metal in question is also important. For example, Cu^{+} will preferentially bind to soft ligands, whereas Cu^{2+} will be found in harder bonding environments [12]. Metallothionein binds metals through cysteine-based sulphur ligands [30], whereas SOD binds Cu through nitrogen ligands of histidine residues [31]. In general, the "rules" for isotopic fractionation due to bonding environment appear to translate well to the isotopic fractionation we observed for MT and SOD. The relative Cu isotopic fractionation between the two bonding environments found here ($\Delta^{65}Cu_{SOD–MT}$), however, was less than that calculated ab initio for cysteine–Cu and histidine–Cu bonds ($\Delta^{65}Cu_{his–cys}$ = 1‰) [10,17]. The calculated isotopic fractionation was for a simpler system, which may account for the differences. For example, it does not take into account sequential isotopic fractionation that occurs due to the amino acid residues that compose the active sites of the Cu-import/-transport proteins that move Cu from the gut throughout the body. In addition, due to the limited metal–protein exchange reactions and tight binding of metals to protein ligands, thermodynamic equilibrium may not have been reached in the biological system, and therefore, the calculated isotopic fractionation is in a different state to that measured. This highlights the importance of measuring isotopic compositions of the system of interest directly. Metallothionein and superoxide dismutase extracted from yeast cells cultured in a copper solution of known isotopic composition had a relative isotopic difference of 0.53‰ [4]. This is a similar difference compared with the values we report here of an average $\Delta^{65}Cu$ between SOD and MT of 0.41‰ (SD 0.3‰) (Table 1). Albarède et al. [12] found that the copper isotope compositions of erythrocytes were heavier than serum by 0.95‰ for males and 0.60‰ for females. The isotopically-light serum composition was attributed to a signature from liver metallothionein stores, whilst the erythrocyte SOD1 binding was deemed responsible for the heavier composition. The magnitude and direction of the isotopic difference between these two bonding environments in both studies echo the relative fractionation seen here for both males and females (Table 1). However, the isotopic compositions of the SOD and MT fractions were different relative to isotopic reference material NIST 976 Cu. This may indicate that the delivery and distribution of Cu in the brain occurs either (i) independently of the copper source in the erythrocyte and serum pools, which are isotopically heavy and, in the case of serum, predominately bound to ceruloplasmin via N, or (ii) the presence of additional processes that affect the isotopic composition of Cu available for incorporation to proteins between the blood pool and the brain (e.g., transport effects driven by copper transporter 1). Overall, the relative isotopic composition of metallothionein and SOD1 in these three studies indicates that this fractionation could be used to investigate upstream processes in biological systems and how diseases may disturb these processes.

Buchl et al. [32] found that the copper isotopic composition of whole mice brain samples was influenced by the presence or absence of a metal binding domain in prion protein (PrP). The change in isotopic composition was attributed to the triggering of an alternative, but so far unknown copper regulatory pathway. Interrogating the system with isotopic analysis of individual proteins in such brains, in combination with whole brain analysis, could help identify key mechanisms like these. Overexpression of PrP also had a limited effect on the copper isotopic composition, with some brain samples becoming heavier, isotopically speaking [32]. The overexpression of PrP is hypothesized to

upregulate the expression of SOD [33]. The isotopically-heavy SOD value obtained here supports this hypothesis when coupled with the data obtained for whole mouse brains by Buchl et al. [32]. Although a greater number of samples were required, the gender-normalised differences between Alzheimer's disease and healthy controls indicate that this technique can be a useful assay to assess how copper transport processes are disturbed in this disease.

The usefulness of this assay to probe the copper transport mechanisms in diseased states depends on its repeatability. The separation of three aliquots of one specimen into metallothionein and superoxide dismutase fractions and the comparison of these fractions with the original bulk brain homogenate tested the repeatability of the size exclusion fractionation. The copper isotopic composition of metallothionein was lighter and superoxide dismutase (SOD) heavier relative to bulk brain homogenate (Table 2). The mean difference between the two complexes was $\Delta^{65}Cu = 0.59‰$, which is significantly different from the 2SD across replicates ($\delta^{65}Cu_{MT}$ 2SD = ±0.21‰, $\delta^{65}Cu_{SOD}$ 2SD = ±0.33‰, *t*-test $p = 0.006$). The spread in the data obtained from biological sample sets is normally seen due to natural variation, and whilst this was still the case for high precision isotopic data, the resolution of these data was greater than typically seen for biomedical data. Harrington et al. [34] demonstrated that HPLC-mediated separation of metallo–protein species from bulk homogenate did not result in the exchange of the metal centres, which supports that the data obtained here are a true reflection of the isotopic composition of these proteins in vivo.

Table 2. The copper isotopic composition of metallothionein and superoxide dismutase as isolated from the brain homogenates of a male Alzheimer's disease patient.

Sample	Total Cu (ng)	δ^{65}Cu (‰)	Δ^{65}Cu (‰)
Bulk Brain	635	−0.09	
Metallothionein			
Replicate 1	43	−0.17	
Replicate 2	37	−0.21	
Replicate 3	45	−0.37	
Mean (SD)		−0.25 (0.11)	
Superoxide dismutase			
Replicate 1	168	0.50	
Replicate 2	130	0.17	
Replicate 3	166	0.34	
Mean (SD)		0.34 ** (0.17)	
SOD–MT Pair 1			0.67
SOD–MT Pair 2			0.38
SOD–MT Pair 3			0.73
Mean (SD)			0.59 (0.17)

Uncertainty (2 s.d.) for individual multiple-collector inductively-coupled plasma mass spectrometry (MC-ICP-MS) sessions <±0.15‰. In-house reference material Romil Cu [35] used for data collection. All data are expressed relative to NIST 976 Cu utilizing conversion factors presented by Moeller et al. [36]. ** Statistically heavier than the metallothionein fraction, $p = 0.006$, two-tailed *t*-test.

To develop a better understanding of the abundance of the available copper proteins present in the sample, we used size exclusion chromatography directly connected to an ICP-MS. Replicate injections of the same brain homogenate that was used for the isotopic ratio determination demonstrated that the SOD and metallothionein peaks together represented 84% of the total soluble copper (Figure 1). Reproducibility between size exclusion chromatography purification runs of the protein samples was <2% with regards to elution volume [37,38] and was typical for human-based populations. This is reflected in the replicates presented in Table 2. The copper in the MT peak is most likely bound to MT3 as MT1 and MT2 are mostly Zn-containing [39]. One caveat regarding the amount of Cu bound to MT is that Cu^+ is not air stable in an oxygen-rich environment, and the Cu could have migrated from other Cu proteins (e.g., ATOX1) after death or during the homogenisation process. The major source of this potentially migrated Cu is also sulphur binding sites; therefore, the isotopic signature is

unlikely to be affected by this process; however, future studies under anaerobic conditions or with model cellular systems will be required to determine the extent of Cu migration to MT3. Isotopic mass balance indicated that there are one or more sources of isotopically-light copper in the remaining 16% of bulk brain homogenate copper budget to account for the difference between the bulk brain (−0.09‰) and the sum of MT and SOD Cu (+0.21‰) for specimen AD1. The homogeneity of the brain sample, whilst sufficient for typical biological analysis and the associated uncertainty, such as total protein levels or total metal concentrations, may not be homogenous at the isotopic scale, which was detectable at the level of precision employed here. A subtle variation in each replicate injected into the FPLC might be responsible for the small differences observed. Western blot (Figure 2) of the isolated protein showed overlap in Fraction 10. This fraction was discarded due to this overlap; therefore, the protein collection was not quantitative, and uncertainty could have been introduced. In addition, the baseline level of Figure 1 indicates that there may be small contributions from other proteins in the fractions; however, the majority of the Cu-containing proteins in these fractions were MT and SOD, and therefore, they dominated the isotopic signature. Increased resolution of protein separation could avoid these two potential artefacts and will be the subject of future work.

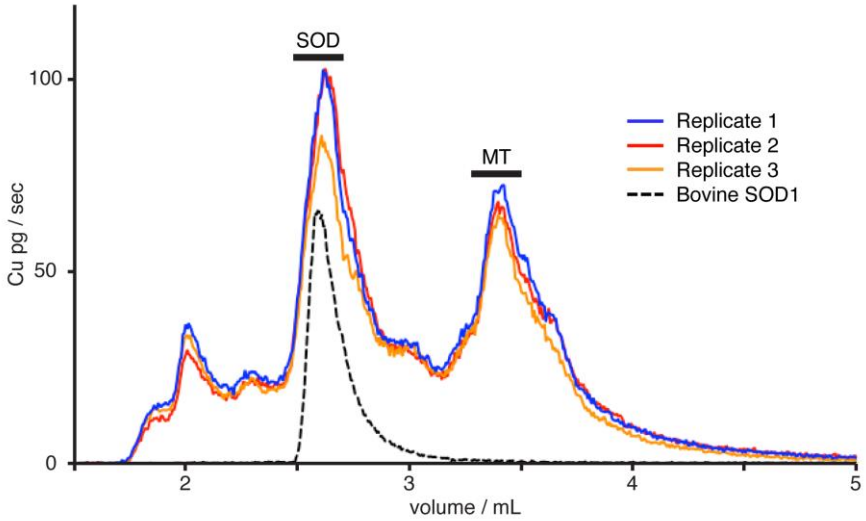

Figure 1. Size exclusion ICP-MS of copper proteins in human brain homogenate. The peak corresponding to the elution of Cu and Zn superoxide dismutase (SOD) and metallothionein (MT) are labelled. Bovine SOD was used as a standard (dashed line). The area of the SOD and the MT Cu peak comprise ca. 80% of the total soluble copper in human frontal cortex. Approximately 15% of the total Cu in the soluble fraction eluted between 1.5–2.4 mL.

This study indicates that the isotopic composition of metals bound in proteins could be resolved through purification of such proteins from the sample matrix. Increasing the resolution of HPLC separation to allow quantitative isolation of the proteins from one another coupled with increased sample numbers will provide novel information about the transport of metals in the human body. Comparisons of protein isotopic data between healthy candidates and those with conditions where metal metabolism is indicated, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, could supplement changes in metal concentrations and provide further insight into the mechanisms of disease. Overall, this workflow can be used as a guide to investigate the role of protein ligands and specific proteins on isotopic fractionation processes for copper and other essential elements such as zinc and iron.

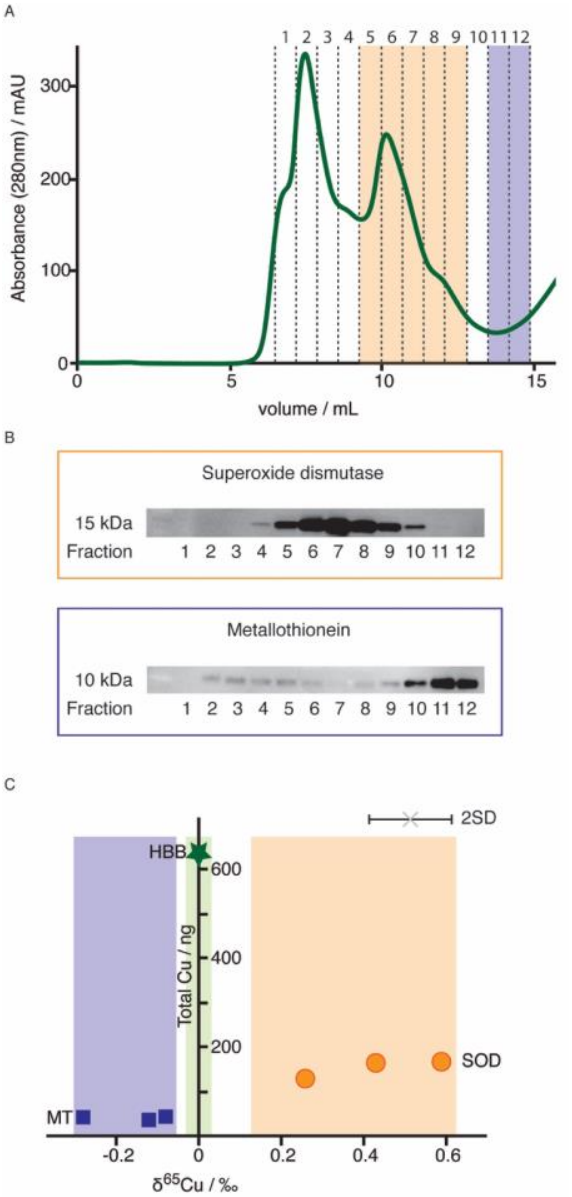

Figure 2. (**A**) Size exclusion chromatography fractionation of soluble human brain homogenate with the collected fraction indicated by dashed lines; elution of proteins was monitored at 280-nm absorbance; (**B**) the collected fractions were subjected to Western blot analysis to determine the fractions containing metallothionein (MT; ■) and superoxide dismutase (SOD; ●); (**C**) the replicates of the measured Cu isotopic ratio and the total amount of Cu in the MT or SOD fraction are shown relative to bulk brain homogenate (HBB; ★). Superoxide dismutase contains copper isotopically heavier than bulk brain and metallothionein.

3. Materials and Methods

Fresh frozen frontal cortex was prepared as previously described [40] from male and female patients with Alzheimer's disease (National Institute on Aging (NIA) Reagan criteria) and no brain disease (Table 3). The choice of samples was to gain a small snapshot into the variations that are seen in these conditions to aid the future choice of this precious specimen type for future study. For one specimen (AD1), triplicate samples were taken to test repeatability. Ethical approval for this study was provided by The University of Melbourne Human Research Ethics Committee (I.D. 1136882, 15 February 2017).

Table 3. Characteristics of donated specimens used in this study.

Sample Code	Disease State of Brain	Sex	Age	PMI [1]
AD1 [2]	Alzheimer's disease	Male	70	
AD2 [2]	Alzheimer's disease	Male	75.4	18.5
AD3 [2]	Alzheimer's disease	Female	76.8	31.0
CON1	Healthy	Male	76.9	49.0
CON2 [3]	Healthy	Female	76.8 [3]	40.3 [3]

[1] PMI: post-mortem index; [2] NIA Reagan criteria; [3] mean value of two female samples combined prior to isotopic analysis. Individual ages, 78.8 and 74.8; individual PMI, 19 and 61.5.

Briefly, 0.5 g of grey matter was dissected from fresh frozen tissue and homogenised in Tris-buffered saline (TBS, 50 mM Tris pH 8.0, 100 mM NaCl with EDTA-free protease inhibitors (Roche, Castle Hill, Australia)) at a ratio of 1:4 (tissue: buffer, *w*/*v*). Homogenisation was conducted with BioMasher (Omni International, Kennesaw, Australia) following the manufacturer's protocol. After centrifugation at 100,000× *g* for 30 min, the TBS supernatant was separated through a Superdex 75 10/300 size exclusion column using an Amersham Biosciences FPLC (injection volume 0.5 mL). The column was conditioned with trace metal-free 200 mM ammonium nitrate (Sigma, Castle Hill, Australia) buffer (pH 7.7), and calibrated with ribonuclease A (13.7 kDa, GE Healthcare Life Sciences, Silverwater, Australia), bovine serum albumin (67 kDa, Sigma) and bovine superoxide dismutase 1 (32 kDa, Sigma). A blank separation was performed by injecting the TBS buffer used for homogenisation followed by separations of brain homogenate. Fractions were collected in 0.7-mL aliquots in acid-cleaned centrifuge tubes. Western blot analysis was performed on 100-µL aliquots of the collected fractions to determine the elution of SOD1 (Abcam, ab16831, rabbit) and metallothionein (Dako, Clone E9, mouse) (Figure 2). Size exclusion ICP-MS was conducted as previously described [28,41]. For specimen AD1, Western blot analysis was only performed for Replicate 2.

Samples were freeze-dried prior to shipping to the UK. Samples were analysed for copper isotopic composition at the University of Oxford, Department of Earth Sciences. Quartz sub-boiled distilled HNO_3 and HCl were used throughout and diluted with 18.2 MΩ cm H_2O when required. Romil 30% (*v*/*v*) H_2O_2 was used for oxidation when needed. All sample handling was performed in pre-cleaned PFA, PTFE, or HDPE within a laminar flow hood to ensure minimum metal contamination. Fractions incorporating the same protein were combined into one PTFE inset vial and dissolved using microwave digestion [42]. Post-digestion, copper was quantitatively purified from the sample matrix using anion exchange chromatography [8]. The copper isotope composition was determined on a Nu Plasma HR instrument, in medium resolution [8,43]. The total procedural blank was <1 ng, which is <2% of the Cu measured.

4. Conclusions

This study indicates that the isotopic composition of metals bound in proteins could be resolved through purification of such proteins from the sample matrix. Increasing the resolution of HPLC separation to allow quantitative isolation of the proteins from one another coupled with increased sample numbers will provide novel information about the transport of metals in the human body. Comparisons of protein isotopic data between healthy candidates and those with conditions where metal metabolism is indicated, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, could supplement changes in metal concentrations and provide further insight into the mechanisms of disease. Overall this workflow can be used as a guide to investigate the role of protein ligands and specific proteins on isotopic fractionation processes for copper and other essential elements such as zinc and iron.

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