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Interaction of metal ions with glutaminase interacting protein (GIP): A potential role of GIP in brain diseases

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Abstract. Glutaminase interacting protein (GIP), a PDZ domain containing protein, mediates the distribution and clustering of proteins/peptides in membranes, acting as a scaffold where signaling molecules are linked to a multi-protein complex. GIP has been shown to play a key role in the glutamate signaling system. Some metals, particularly Pb^{2+}, Cu^{2+} and Zn^{2+}, have been implicated in a wide range of neurological disorders including Alzheimer's disease and Parkinson's disease, whose etiologies have been associated with dysfunction of the glutamate signaling system. Here, for the first time, the effects of lead, copper, and zinc on GIP were determined by using circular dichroism and fluorescence spectroscopy. All three metal ions significantly affected the conformational properties of GIP. The deconvolution of CD data showed that, with increasing amounts of Pb^{2+}/Cu^{2+}/Zn^{2+}, the α-helix percentage decreased while the β-sheet content increased. The binding constants of GIP to Pb^{2+}, Cu^{2+} and Zn^{2+} determined by fluorescence were found to be 1.4, 2.38 and 2.85 μM respectively, which indicated strong bindings between GIP and all three metal ions. We propose that the metal ion binding site of GIP is located on α-2 helix, where residues His90, Asp91 and Arg94 may coordinate the metal ions. The conformational change of GIP upon metal ion binding possibly results from the disruption of a salt bridge between Asp91 and Arg94.

Keywords: GIP, PDZ domain, brain diseases, metal ions interaction, fluorescence, CD

1. Introduction

PDZ domains, a common class of protein–protein interaction modules present in the human genome [11,16,25,27], act as ubiquitous protein recognition modules in the nervous system [7], playing a central role in assembling multiprotein signaling complexes, thereby coordinating and guiding the flow of regulatory information [10,13]. Glutaminase-interacting protein (GIP), initially discovered in the human brain, is a small soluble protein containing one PDZ domain. GIP was originally identified in a two-hybrid genetic selection system in yeast while looking for interactors of glutaminase in human brain [19]. It was found to interact with the C-terminus of LGA (glutaminase L), which is responsible for synaptic transmission and regulation of cerebral concentrations of glutamine and neurotransmitter glutamate [19]. Apart from glutaminase, a plethora of binding partners have been reported implicating GIP in key biological processes including the viral oncoproteins HTLV-1 Tax [21], HPV16 E6 [9], the Rho-activator rhotekin [20], the potassium channel Kir 2.3 [2], β-catenin [13] and FAS, which belongs to

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the Tumor Necrosis Factor (TNF) receptor family [20], and mediates cell apoptosis [2,12]. Hence inactivity of GIP could have profound physiological effects on certain key signaling pathway(s), the central nervous system (CNS), and seriously impact the glutamate signaling system.

Dysfunction of the glutamate signaling system has been associated with the development and progression of a variety of neurodegenerative brain disorders, including epilepsy, stroke, Parkinson's disease, ALS, and Alzheimer's disease [4]. It is also known that some metals such as lead, copper, and zinc play a vital role in the brain and olfactory system, causing a wide range of disorders including all the diseases listed above [5]. Preliminary relationships between the metals and the diseases listed above have been reported. In the case of lead, it is known that its neurotoxic effects are closely associated with interference in glutamate signaling; while for zinc, about 10% of CNS zinc is associated with pre-synaptic vesicles of glutaminergic neurons [22].

Considering the significance of GIP in glutamate metabolism, it is important to know whether these metals can interact and thus compromise GIP function directly and, as a result, lead to the dysfunction of the glutamate system. Recent studies have shown a decrease in LGA activity in the presence of lead acetate [15]. Besides, lead is a common metal toxin found in trace amounts in the environment including water, paint, insecticides, gasoline etc. Low levels of lead accumulate in the body causing cognitive effects primarily in small children. Studying the effect of metal ions on the conformation of GIP may contribute to our understanding of the deleterious effects of lead on the central nervous system (CNS).

We report here, the effects of lead, copper and zinc on the conformation of the human brain protein, GIP, using circular dichroism (CD), and fluorescence spectroscopy techniques. It is clear from our studies that these metals significantly alter the conformation of GIP. In addition, the binding mechanism is also proposed.

2. Materials and methods

2.1. Chemicals

Lead acetate (>95% pure) and zinc sulfate (>99% pure) were obtained from Sigma Aldrich. Copper chloride (>99% pure) was obtained from Mallinckrodt Chemical Works.

2.2. Overexpression and purification of GIP

Recombinant GIP containing 124 residues was overexpressed in BL21DE3plys *E. coli* cells using pET-3c vector [3]. Saturated overnight LB-ampicillin culture was diluted (1:100 v/v) in LB medium and grown at 37°C to an OD_{600} of 0.5–0.6. Expression was induced with 1 mM IPTG and cells were harvested by centrifugation after incubation at 30°C for 4 h. Bacterial cells were resuspended in phosphate buffer at pH 8 containing 200 mM NaCl, 4 mM EDTA, 4% glycerol, 1 mM PMSF and lysed using sonication. After centrifugation (14,000 rpm, 30 min), the supernatant containing soluble GIP was collected and were purified in a single step by size exclusion chromatography with a Sephacryl S-100 column (GE Healthcare) fitted to an FPLC system using 20 mM phosphate buffer containing 150 mM NaCl, 1 mM EDTA and 0.1% NaN_3 as the mobile phase. The activity of the protein was verified as described earlier [1].

2.3. Circular dichroism (CD)

All circular dichroism (CD) experiments were performed on a Jasco J-810 spectropolarimeter at 25°C. Far-UV CD spectra were measured in a 0.05 cm quartz cell. The buffer used was 10 mM phosphate buffer (pH 6.5). The protein concentration was 30 μM, which was determined by measuring the absorbance at 280 nm. Data were averaged over 100 scans for each protein sample and 50 scans for each control sample. Response time was 1 s and scan speed was 100 nm min^{-1}. In each titration experiment the concentration of the protein was corrected for volume dilution. Titrations of lead acetate, copper chloride and zinc sulfate were completed using a 60 μM stock solution of each compound.

2.4. Fluorescence

All fluorescence spectra were recorded on Perkin Elmer Precisely LS 55 Luminescence spectrofluorometer. Tryptophan fluorescence was excited at 280 nm and emission spectra were recorded over the range 300–500 nm with 1 nm steps. All fluorescence spectra were recorded at room temperature at pH 6.5. The buffer used in the titration and quenching experiments was 10 mM phosphate buffer (pH 6.5). Aliquots of the metal ions stock solutions were directly added to a cuvette containing 1 μM GIP. In each titration experiment the concentration of the protein was corrected for volume dilution, and observed emission was corrected for absorbance of the quencher.

2.5. Structure modeling

The GIP structure was modeled for the residues ranging from Leu29 to Leu108 using the program SWISS-MODEL [24] based on the coordinates of the structure of the second PDZ domain of human scribble protein (PDB codes 1 whaA) with sequence identity 43%.

3. Results

3.1. Overexpression and purification of GIP

GIP was overexpressed in BL21DE3pLys cell line using pET-3c vector. GIP was purified in a single step by gel filtration. Figure 1 shows that GIP was >95% pure after gel filtration.

3.2. Effect of metal binding on GIP by CD

The effect of metal binding on GIP secondary structure was investigated by CD spectroscopy. GIP was titrated with Pb^{2+}, Cu^{2+} and Zn^{2+}. Some white precipitation was observed with the addition of each metal ion, which could possibly be an indication of denaturation of protein sample. From the CD titration spectra of GIP with the metal ions (Fig. 2(a)–(c)), it is clear that all the three metal ions interact with GIP in a similar manner: inducing remarkable changes in the secondary structure of GIP. CD deconvolution data show that the percentage of helix in GIP decreases with increasing amount of the three metal ions, while the β-sheet content increases (Tables 1–3). In the case of each metal ion, there was no change observed in the secondary structure of GIP when the concentration exceeded 1.8 μM.

3.3. Effect of metal binding on GIP by fluorescence spectroscopy

The fluorescence emission spectra of GIP were recorded in presence and absence of various metal ions, viz., Pb^{2+}, Cu^{2+}, Zn^{2+} (Fig. 3). The intensity of GIP fluorescence, observed in the range 300–500 nm,

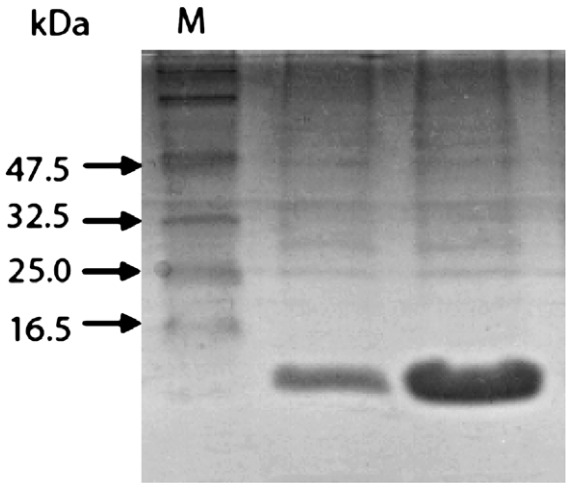

Fig. 1. SDS-PAGE analysis of purified GIP. Lane M, molecular mass markers with the masses indicated on the left side of the gel. GIP was concentrated and overloaded to check purity before further characterization (both lanes on the right).

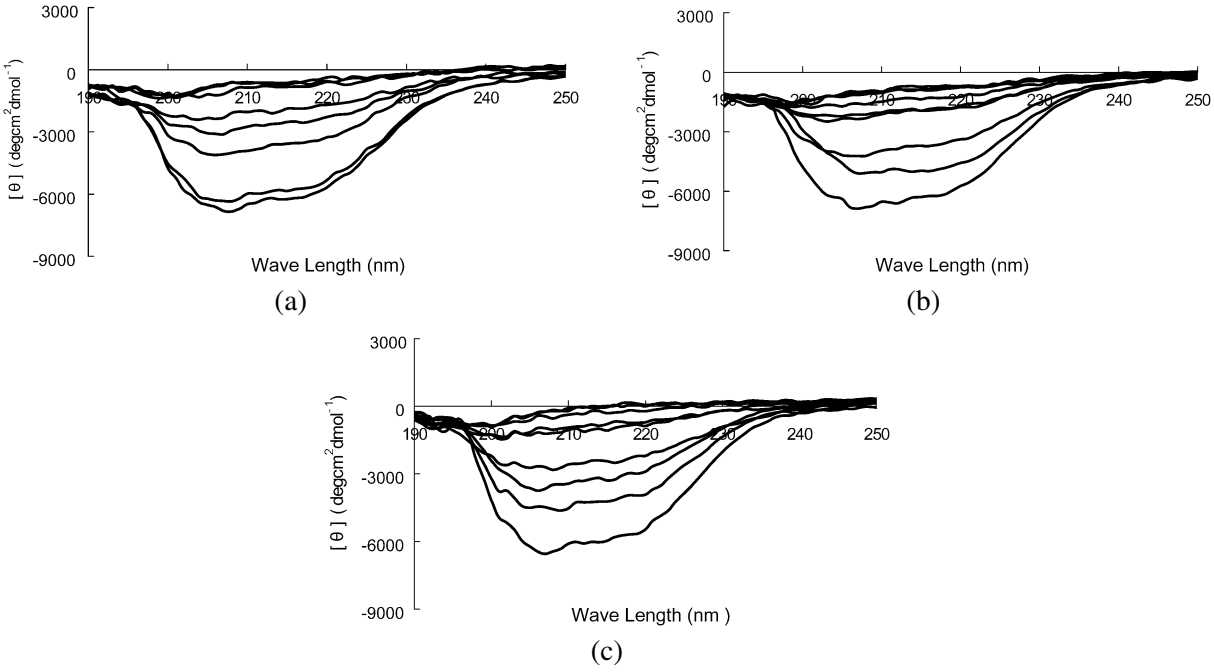

Fig. 2. Far-UV CD spectra of GIP: The titration of 30 μM GIP with increasing concentration of Pb^{2+} (a), Cu^{2+} (b), Zn^{2+} (c). For each figure, the plots correspond to (top to bottom) 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.4, 3.0 μM of corresponding metal ions in 30 μM protein sample.

was measured as a function of the added metal concentration (Fig. 3). Addition of all these three metal ions to GIP results in a moderate but consistent decrease in fluorescence intensity (Fig. 3). It is clear from Fig. 3 that even at high concentrations of metal ions, quenching is not saturated. The lack of saturation in the fluorescence quenching even when the protein is saturated with the ligand, indicates that only

Table 1
Deconvolution of CD data of Pb^{2+} titration. Deconvolution was carried out using CDPro

Concentration of Pb^{2+} (μM)	% Helix	% Sheet	% Turn	% Random coil
0	10.2	32.6	22.7	34.5
0.3	9.0	33.6	22.8	34.5
0.6	5.7	37.2	22.5	34.6
0.9	4.6	39.0	22.3	34.0
1.2	4.5	40.1	22.0	33.4
1.5	4.2	41.3	21.4	33.2
1.8	3.9	41.5	21.5	33.1
2.4	4.4	40.4	21.8	33.4
3.0	3.4	41.5	21.0	34.1

Table 2
Deconvolution of CD data of Cu^{2+} titration. Deconvolution was carried out using CDPro

Concentration of Pb^{2+} (μM)	% Helix	% Sheet	% Turn	% Random coil
0	10.2	31.6	23.0	35.2
0.3	9.5	35.1	22.3	34.0
0.6	6.4	36.9	22.6	34.2
0.9	4.6	39.9	22.0	33.5
1.2	4.3	40.1	21.8	33.8
1.5	4.3	40.3	21.7	33.5
1.8	4.2	40.7	21.5	33.6
2.4	4.2	40.8	21.6	33.4
3.0	4.1	40.8	21.7	33.5

Table 3
Deconvolution of CD data of Zn^{2+} titration. Deconvolution was carried out using CDPro

Concentration of Pb^{2+} (μM)	% Helix	% Sheet	% Turn	% Random coil
0	10.1	32.6	22.8	34.5
0.3	5.9	37.0	22.4	34.6
0.6	5.0	38.5	22.0	34.6
0.9	4.8	39.5	22.0	33.7
1.2	3.8	41.4	21.2	33.6
1.5	4.1	41.2	21.6	33.2
1.8	3.5	41.3	21.4	33.8
2.4	3.5	41.1	21.4	34.1
3.0	3.5	41.5	21.4	33.7

a fraction of the metal ion quenches the fluorescence. For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by equation [6,14, 28]: $\log(F_0-F)/F = \log K + n \log[Q]$, where K is the binding constant, $[Q]$ is the ligand concentration and n is the number of binding sites per GIP. The GIP–Pb^{2+} titration yielded a dissociation constant $K_{\mathrm{D}} = 1.4$ μM, and the number of binding sites per protein is $n = 0.65$ (Fig. 3(b)). The GIP–Cu^{2+} titration yielded a dissociation constant $K_{\mathrm{D}} = 2.38$ μM, and the number of binding sites per protein is $n = 0.58$ (Fig. 3(d)). The GIP–Zn^{2+} titration yielded a dissociation constant $K_{\mathrm{D}} = 2.85$ μM, and the

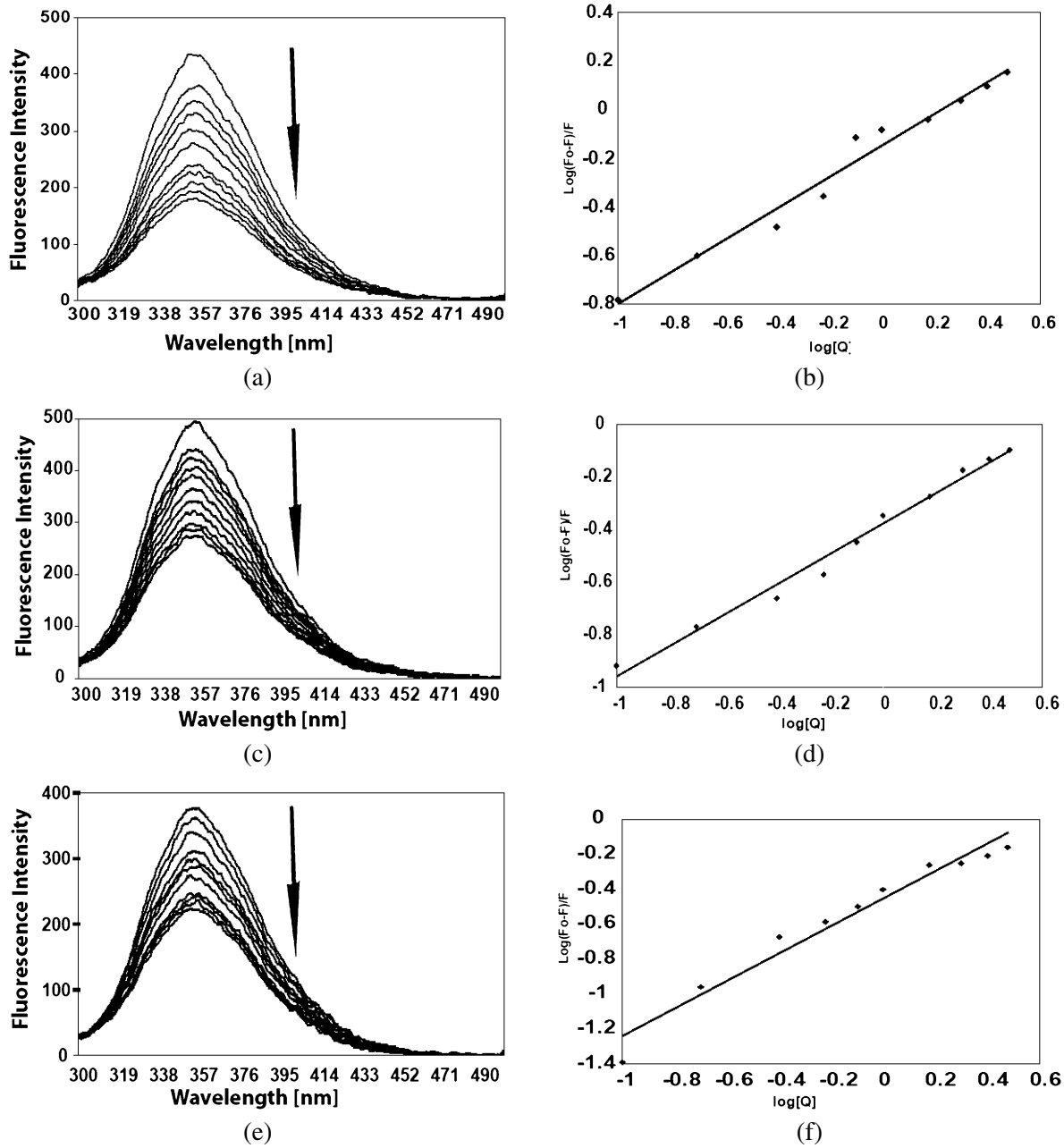

Fig. 3. Interaction of GIP with Pb^{2+} (a), Cu^{2+} (c) and Zn^{2+} (e). Fluorescence emission spectra of GIP upon addition of all three metal ions. The plots correspond to (top to bottom) 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2, 2.5 μM of corresponding metal ions in 1 μM protein sample. Double-log plot of quenching of GIP fluorescence by Pb^{2+} (b), Cu^{2+} (d) and Zn^{2+} (f).

number of binding sites per protein is $n = 0.78$ (Fig. 3(e)). The binding affinity of these three metal ions to GIP is: $Pb^{2+} > Cu^{2+} \approx Zn^{2+}$. Low K_D values suggest that there are strong binding between GIP and all these three metal ions.

4. Discussion

Metal ions, particularly divalent metal cations, perform a number of essential functions in a wide range of biological processes. There is large body of evidence implicating Pb^{2+}, Cu^{2+} and Zn^{2+} in the etiology of a wide range of disorders including Parkinson's disease, ALS, and Alzheimer's disease. The glutamate signaling system is tightly associated with those diseases. Since GIP plays a significant role in glutamate metabolism, we initiated this study to investigate the effects of the above metals on the physiological state of GIP.

The allowed level of lead concentration in blood correlates within the range where our studies have been carried out, thus the concentration of lead acetate used in this study is of biological relevance. It is clear from our interaction studies that all the three metal ions can interact strongly with GIP ($K_D < 3$ μM), and significantly change its conformation even at very low concentrations. Metal ions generally coordinate up to four or six ligand coordinating groups. Functional groups of several amino acids take part in metal ion coordination, particularly the side chains of His, Cys, Asp and Glu. Side chain of several other amino acids such as Tyr, Arg, Lys, Met, Asn, Gln, Ser and Thr have also been reported to be involved in metal coordination [8]. GIP does not contain any Cys residue; however, there are 2 His residues (His19 and His90), 7 Asp residues and 7 Glu residues. Since most Asp and Glu residues are either located in loops or far away from each other in the 3-dimensional model, making it unlikely to coordinate with metal ions and affect the overall conformation of GIP, the only plausible binding site can be located on the second α-helix of GIP (α-2), where His90, Asp91 and Arg94 appear to be in proper position for coordination with a metal ion (Fig. 4). In general, the metal binding site typically has

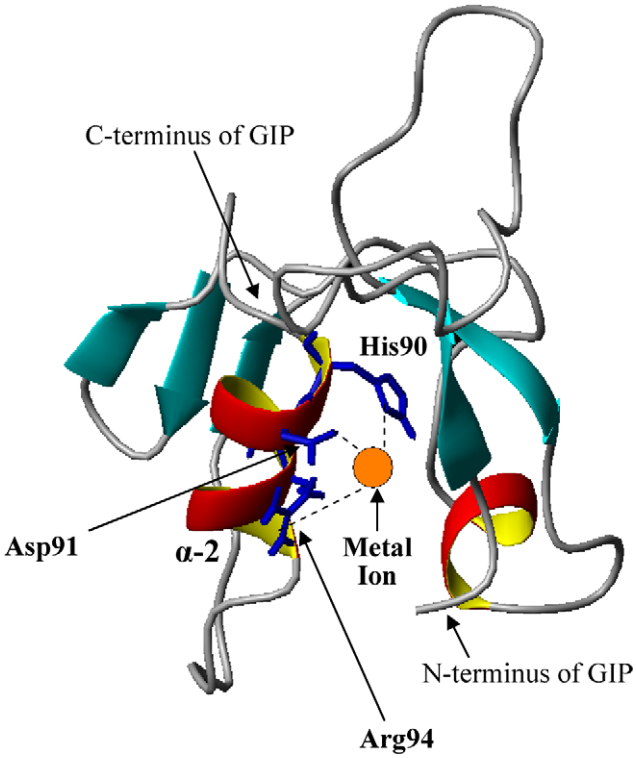

Fig. 4. Proposed mechanism of metal ions binding to GIP. The residues His90, Asp91 and Arg94 coordinating the metal ion are shown. The figure was prepared with the program MOLMOL.

tetrahedral coordination geometry, being coordinated to the amino acid side chains that fulfill the steric and electronic constraints of metal ions [17]. In the case of GIP, it is possible that the His90, Asp91, Arg94, and a water molecule coordinate with the metal ion. Such metal binding arrangements are common in metalloproteins [23]. We propose that, upon binding to GIP, the metal ions significantly change the overall protein conformation by disrupting the critical Asp91–Arg94 salt bridge, which plays a vital role in organization and stabilization of PDZ domain structure [26]. Moreover, our CD deconvolution data indicates a dramatic drop of the percentage of α-helix content in GIP upon metal ions binding. It is likely that the metal ions eliminate the salt bridge upon binding to GIP, causing a conformational change with a reduction of α-helix content in GIP.

Our finding implies that GIP may have a potential role in brain diseases including Parkinson's disease, ALS and Alzheimer's disease. A hypothetical interaction pathway can be postulated with metal ions (Pb^{2+}, Cu^{2+} or Zn^{2+}) first interacting with GIP, resulting in the dysfunction of glutamate signaling, thus leading to the neurodegenerative symptoms observed in patients. Whereas this research is still in its early stages, future experiments should provide a much greater understanding of these issues and assist in the development of novel therapeutic strategies.

5. Conclusion

Although metal poisoning especially that of lead has been implicated in a variety of physiological and psychological dysfunctions such as low IQ, learning disabilities, premature birth, low birth weight, etc. especially in children [20], the molecular details of the chemistry of metal toxicity is poorly understood. Complete understanding of the chemistry of metal poisoning requires the combination of many approaches. In present studies, CD and fluorescence spectroscopy identified a conformational change of the human brain protein GIP in the presence of metal ions (Pb^{2+}, Cu^{2+}, Zn^{2+}), which provides a framework for future investigation and analysis of the effect of toxic metals on different proteins involved in glutamate signaling. This, in turn, will have a far-reaching impact not only on the study of GIP and metal ions (Pb^{2+}, Cu^{2+}, Zn^{2+}), but a clearer understanding of the effect of metal ions on critical signaling proteins in the brain, which will help in the understanding of the neurotoxic effects of metal poisoning.

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