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Title: Zinc Binding with L-Dopa Peptides

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Abstract

The combination of zinc dyshomeostasis and oxidative damage has been linked to a number of human disease pathologies. A common pathway of oxidative damage centers on tyrosine with the generation of 3,4-dihydroxyphenylalanine (L-dopa). Once formed this catecholic moiety can be involved in metal binding. Herein, an L-dopa residue is incorporated into a peptide designed to adopt a β -hairpin configuration. Variation of the cross strand partner to L-dopa introduces an aromatic pair to enhance structure. Mass spectrometry indicates successful zinc binding, consistent with a 1:1 peptide:zinc complex. NMR and spectrophotometric investigations reveal the L-dopa as the binding locus with association energies ranging between 4.3 and 5.2 kcalmol⁻¹.

Keywords: zinc complexes; zinc chelation; β -hairpin peptide; peptide bound L-dopa; peptide binding

Introduction

Zinc is an essential biological cofactor and plays a critical role in the central nervous system as it is involved in apoptosis, oxidative stress, and immune defense [1]. The concentration of intracellular zinc typically ranges between 180 and 250 μM in eukaryotic cells [2]. However, very little zinc exists as ‘free’ ions within the cell, usually only pico- to low nanomolar concentrations [3]. Zinc dyshomeostasis has been implicated as a pathological feature of Alzheimer’s disease [4,5], depression [6], Parkinson’s disease [7], amyotrophic lateral sclerosis (ALS) [8], and epilepsy [9].

Alzheimer’s disease (AD) displays a progressive neurological decline affecting tens of millions worldwide. Its pathological characterization involves the presence of neurofibrillary tangles, from the Tau protein, and amyloid plaques, resulting from the deposit of amyloid beta peptide ($\text{A}\beta$) [10]. A genetic risk factor for familial AD is the $\epsilon 4$ apolipoprotein E gene, which is associated with elevated serum levels of zinc, copper and insulin [11]. The excess levels of zinc were determined to be a primary risk factor and not $\epsilon 4$ apolipoprotein E itself. Further, zinc has been shown to bind to amyloid precursor protein (APP) in the extracellular E2 domain [12]. An examination of $\text{A}\beta$ senile plaques from the amygdala of AD patients found elevated levels of zinc in both the rim and core regions [13]. Finally, it has been observed that APP has ferroxidase activity, which appears to be inhibited by zinc accumulated in fibrils [14]. As such, zinc binding may

provide a possible link to subsequent oxidative damage in concert with iron dyshomeostasis and A β imbalance [15].

A summative hypothesis of AD pathogenicity is the “two-hit hypothesis” where both oxidative stress and mitogenic dysregulation are needed for disease progression [16]. A β itself has been shown to bind to first row transition metals, which can accelerate aggregation and generate reactive oxygen species [17]. Indeed, the generation of H₂O₂ has been implicated as a defining element of A β toxicity [18]. The most vulnerable sites of metal mediated oxidative damage in A β are the His and Tyr residues [19]. Density functional theory calculations have implicated Tyr10 as a pivotal residue for H₂O₂ production given the stability of tyrosyl radicals. This yields the posttranslational modification of tyrosine to diTyr and hydroxytyrosine (L-dopa) [20]. DiTyr results from the cross-linking of two A β peptides; protein bound L-dopa can undergo redox activity to generate cross-linked Michael type products [21]. These covalent bridging products of A β peptides may contribute to A β toxicity and fibrilization [22]. Despite the interplay of protein metal binding and structure there have been only limited studies on peptide bound L-dopa, which often interrogate only unstructured peptides [23, 24, 25].

Herein, a series of peptides incorporating the noncanonical L-dopa amino acid are studied for their potential to form metal:peptide complexes with zinc. A series of peptides is synthesized in a β -hairpin configuration with differing cross-strand partners to the L-dopa residue. This yields a different local environment surrounding the L-dopa to determine the underlying factors involved in complex formation.

Materials and Methods

Materials

Resin, rink amide PS, and N-9-fluorenylmethyloxycarbonyl (fmoc) amino acids, fmoc-Trp, fmoc-Tyr(boc), fmoc-Phe, fmoc-1Nal, fmoc-Gly, fmoc-dPro, fmoc-Val, fmoc-Orn(boc), and fmoc-Dopa, were purchased from Anaspec. Coupling reagents, including 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydrobenzotriazole (HOBt), were obtained from Anaspec. Solvents, including N,N-dimethylformamide (DMF), methanol, acetonitrile and isopropyl alcohol, were obtained from Pharmco. Water used for the high-performance liquid chromatography (HPLC) was from a Milli Q A10 purifier. Deuterated water and 10% DCl in deuterated water were purchased from Cambridge Isotope Laboratories. Deuterated sodium acetate, deuterium chloride in D₂O, N,N-diisopropylethyl amine and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) were obtained from Fisher Scientific

Peptide Synthesis and Purification

Peptide synthesis used Fmoc chemistry on a Rink amide PS resin. Tyrosine and ornithine were side chain protected with the Boc functionality. Residue coupling was effected with HBTU and HOBt in DMF. Peptides were cleaved from the resin using a mixture of TFA (95%) in water (5%). Purification of the peptides was performed on an Agilent 1100 HPLC with a Higgins Analytical C18 silica column (TS-2510-C185). Eluent was a gradient of A and B (A, 95% H₂O/ 5% ACN with 0.1% TFA; B, 95% ACN/ 5% H₂O with 0.1% TFA). Detection with spectrophotometry at 220 and 280 nm gave

purified peptide at less than 20% B. Following purification peptides were lyophilized to powder for characterization [26].

Mass Spectrometry

Matrix-assisted laser desorption ionization mass spectra were collected on a Bruker Microflex MALDI-TOF mass spectrometer. Calibration was performed with Bruker peptide calibration standard containing angiotensin II, angiotensin I, substance P, bombesin, ACTH clip1-17, ACTH clip 18-39 and somatostatin 28. The matrix for all samples was α -cyano-4-hydroxycinnamic acid. All analysis used reflection collection with an ion source voltage of 20.09 KV. Relative laser power was between 10-40% [27]. Samples were made from stocks to deliver equimolar amounts of peptide and zinc chloride. Reported spectra are summed from a minimum of 200 shots to give representative mass collections.

NMR Spectroscopy

NMR samples were taken on a Varian INOVA 500 MHz instrument. Peptides were dissolved in buffered D₂O to a final concentration of 1 – 4 mM. Deuterated buffer was made from 50 mM d₃-NaOAc and 100 μ M DSS pH adjusted with 10% DCl in D₂O to a pH of 5.4 (uncorrected). One-dimensional NMR spectra were collected by using 32K data points and between 8 and 512 scans using a 1.5 sec pre-saturation. All two-dimensional NMR experiments used pulse sequences from the chempack software, including Total Correlation Spectroscopy (TOCSY), gradient Correlation Spectroscopy (gCOSY) and Nuclear Overhauser Effect Spectroscopy (NOESY). Two-dimensional NMR scans were taken with 8 to 64 scans in the first dimension and 128 to 512 in the

second dimension with standard processing. TOCSY spectra were recorded with 80-msec spin-lock. Assignments were made using standard methods as described by Wüthrich [28].

Spectrophotometric Measurements

Spectrophotometric measurements were taken either on a Cary 100 spectrometer or fluorescence measurements were taken on a Horiba Scientific Fluoromax 4 spectrofluorometer. Metal contamination was minimized by washing cuvettes with concentrated nitric acid and rinsing with deionized water prior to use. Solutions were prepared in a 50:50 DMSO:Tris buffer solvent system. The Tris was adjusted to a pH of 7.5 at a 10 mM final concentration. The titrant solutions were made fresh for each experiment, and were composed of 25 mM zinc nitrate, from $\text{ZnNO}_3 \cdot 6 \text{H}_2\text{O}$, and 50 μM peptide. Peptide solutions were prepared to a final concentration of 50 μM . Peptide concentrations were confirmed using an by UV-Vis. Zinc/peptide solutions were titrated into peptide solutions reaching up to 20 equivalents. Each addition was mixed and allowed to equilibrate for 30 minutes prior to each scan. Fitting of the UV-Vis data was analyzed by standard methods to determine binding constants [29].

Results and Discussion

A central feature of the peptide design is the incorporation of a strong reverse turn. The d-Pro-Gly diad has been shown to be a strong promoter of a reverse turn due to the constrained proline [30]. Furthermore, when flanked at the *i*-1 and *i*+2 positions with Val and Orn, an optimized turn sequence develops a reverse turn to nucleate a highly

stabilized β -hairpin structure [31]. The series of **DopaXxx** peptides used in this study leverage this design to generate a folded structure, Figure 1.

Figure 1. The sequence for all peptides is (Dopa)VdPGOX-NH₂. Proline is in the D-stereochemistry, O is the unnatural amino acid ornithine and X is varied as indicated. Peptides are identified with abbreviations, in bold.

Chemical shift deviations (CSD) provide an indication of local structure in small peptides. Formation of a β -hairpin results in a shift of the H α resonances. Downfield shifting is correlated to β -strand formation and upfield shifting with either α -helix or turn geometries [32, 33]. The **DopaXxx** peptides show a consistent pattern for the Val-Gly region of strand and turn, Figure 2.A. Also, the Gly methylene in the turn is diastereotopic. With geometric restriction these chemically equivalent protons become magnetically inequivalent. Further, the designed type II' turn from the dPro-Gly sequence should provide a cross strand hydrogen bond between Val and Orn. The Val amide CSD is most significant for all peptides up to 0.5 ppm from random coil, Figure 2.B. The Orn CSD is most perturbed in **DopaGly** and becomes increasingly shielded with

larger aromatic residues in the C-terminal position. The coupling constants of the amides resonances are between 7 – 9 Hz consistent with an extended conformation. The ring current effects from the aromatic residues appear to be influencing the H α and amide CSDs. Close approach of the L-dopa side chain to the cross strand aromatic ring yields an anisotropic shielding, Figure 2.C (Rajagopal 2012). All **DopaXxx** peptides show a small to moderate shift of the L-dopa aryl resonances except for **DopaGly**, which has only a methylene as the cross-strand partner. Of all aromatic resonances the H₂ and H₆ positions of L-dopa are most perturbed. The observed CSD are as much as 0.3 ppm, which is comparable to other peptides with a lateral aromatic pairing [34]. There is only negligible shifting observed (<0.05 ppm) on the non-L-dopa aromatic partner. This suggests an edge-to-face (EtF) geometry of the local aromatic structure [35]. Overall, the structure of the peptides is consistent with that shown in Figure 1.

Figure 2. Chemical shift deviation (CSD) of **DopaXxx** A) H α , B) amide and C) aromatic L-dopa resonances.

The binding event between a catechol and metal has been explored with a wide range of metals, which has shown the potential for a diverse stoichiometry. Perhaps the most well studied are interactions with iron, such as those observed with the byssal attachment of mussels, a marine bioadhesive [36]. Depending on the environment discrete species have been observed containing up to three ligands. However, interactions between flavonoids and zinc commonly yield 1:1 or 2:1 type binding [37, 38]. Mass spectroscopy is used to explore the binding stoichiometry of the peptide:zinc complex.

Treating the **DopaXxx** peptides gave unambiguous evidence for peptide:zinc complex

formation from the mass spectrometry data, Table 1. Figure 3 shows a typical mass spectrum of **DopaGly** in the absence and presence of zinc. With no zinc there are three significant masses corresponding to **DopaGly** + H⁺, **DopaGly** + Na⁺, and **DopaGly** + K⁺. In the presence of zinc a unique mass cluster appears at 683.197 amu. This mass is consistent with the addition of zinc and elimination of two protons, **DopaGly** + Zn²⁺ – 2H⁺. Theoretical studies on dihydroxyflavone binding to zinc implicate the doubly deprotonated catechol as the specific locus of binding [39]. Deprotonation yields a hard bidentate oxygen ligand capable of strong metal ligation [40]. The observed masses herein provide excellent support for the predicted binding model, Figure 4 [41]. Indeed, all investigated peptides in the presence of zinc contain a new mass cluster with an additional mass of 61.9 amu. The unique isotopic distribution of zinc gives further evidence that the observed mass is a peptide:zinc complex, Table 2. While it is clear that a peptide:zinc complex is formed, no bispeptide:zinc complex was observed. While flavonoids have been shown to assemble in 1:1 and 2:1 complexes with zinc using electrospray detection, the ligand size and MALDI-TOF conditions used herein appear to favor a single peptide complexed with metal.

Figure 3. MALDI-TOF mass spectrum of A) **DopaGly** and B) **DopaGly** in the presence of zinc. Labeled masses are in a.m.u.

Figure 4. Proposed binding interaction between L-dopa and zinc.

Absorption spectroscopy has proven to be a useful technique in studying metal-catecholic interactions. A small, unstructured peptide based on the mussel byssus was examined with a variety of transition metals [23]. Further studies on naturally occurring polyphenolic, small molecules have been widely investigated for metal binding, including catechols, flavones, flavonoids and catechins [42]. The formation of a ligand to metal charge transfer manifests as a novel absorbance. Zinc binding to flavonoids has been shown to result in a narrowing of the HOMO-LUMO separation resulting in

spectrophotometric bathochromatic shifting [43, 44].

The combination of **DopaXxx** peptides and zinc gives rise to similar spectrophotometric changes. Neither the L-dopa containing peptides nor zinc solutions have significant absorbance above 300 nm. **DopaGly**, Figure 5, has only one aromatic ring, L-dopa, with λ_{max} at 283 nm. The titration of zinc yields an enhancement of the $\pi-\pi^*$ transition with red shifting. Notably, a new absorbance between 300 and 350 nm appears with increasing zinc. The absorbance that develops near 330 nm is consistent with changes to the L-dopa aromatic ring upon metal binding [45]. Upon their mixing all investigated **DopaXxx** peptides develop similar absorbance bands between 300 - 370 nm, Figure 6, and experience a mild $\pi-\pi^*$ enhancement with modest changes to the λ_{max} between 278 and 283 nm. This behavior is consistent with complex formation as observed with zinc binding to catechol containing natural products such as quercetin and epicatechin [46, 47].

Figure 5. A) Spectrophotometric titration of **DopaGly** with Zn^{2+} , insert shows the absorbance at 325 nm. B) Fluorescence emission with zinc addition from 0 to 1.4 equivalents of **DopaGly**.

Figure 6. Spectrophotometric absorbance of A) **DopaPhe**, B) **DopaTyr**, C) **DopaTrp** and D) **Dopa1Nal** with increasing concentration of zinc.

Metal:catechol binding can have varied effects on fluorescence with copper quenching, but enhancement with zinc complexation [48]. An investigation of **DopaPhe** demonstrates this effectively, Figure 5.B. In the absence of zinc the peptide has little absorbance at 320 nm. However, in the presence of zinc there is increasing absorbitivity between 300 and 350 nm. Consequently, excitation at 320 nm with increasing amounts of zinc yields significant enhancement of fluorescence emission, maximally at 400 nm. The observed fluorescence response is dose dependent and directly correlated to the increasing absorbance at 320 nm.

It is notable that catechol-iron binding yields multiple colorimetric changes. These changes are consistent with the formation of different iron species, each of which has a signature color. The single transition observed when the **DopaXxx** peptides undergo zinc titration suggest the formation of a single peptide:zinc complex, consistent with the observed masses.

Fitting of the spectrophotometric titration data with a 1:1 binding stoichiometry gave binding constants for the peptide:zinc complex, Table 3. All **DopaXxx** peptides effectively bind to zinc and are consistent with other catechol based binding agents [48, 49]. Notably, **DopaGly** has the weakest observed association; all peptides with an aromatic cross strand partner bind zinc more tightly. The successive deprotonation of metal free catechol has pK_a values of 9.3 and 13.1, but can be dramatically altered to become more acidic in the presence of metal [50]. The chemical shifts of the L-dopa

residue implicate a close association with a cross strand aromatic ring, resulting in an EtF interaction. This may destabilize the unbound state favoring zinc binding.

The combination of catechol and zinc yields changes to the NMR spectrum. Typically, an increasing concentration of zinc results in a broadening and/or shielding of the aromatic resonances proximal to the binding locus [38]. This behavior can be seen in Figure 7.A, where the catechol, L-3,4-dihydroxyphenylalanine methyl ester, is treated with an increasing amount of zinc. The three aromatic L-dopa resonances, H₂, H₅ and H₆, display chemical shifts up to ~0.1 ppm and significant line broadening. Titration of **Dopa1Nal** with zinc shows related behavior, Figure 7.B. The L-dopa H₂ and H₅ resonances are shielded, albeit mildly, by 0.04 and 0.01 ppm. Interestingly, position H₆ is deshielded. This may result from a change in the local aromatic interaction with the cross strand 1Nal residue [51].

Figure 7. NMR of A) 3,4-dihydroxyphenylalanine methyl ester without (lower) and with zinc (top). B) NMR Titration of **Dopa1Nal** with increasing amounts of zinc as labeled.

Conclusion

Inclusion of the unnatural amino acid L-dopa can successfully generate a folded peptide with a metal binding locus. The peptide series studied herein adopts a β -hairpin configuration which displayed zinc complexation. The strength of this interaction was consistent across all investigated peptides. There may be a mild impact from cross strand electronic effects. Further, metal acquisition may cause local geometric changes. It seems clear that oxidatively damaged L-dopa type amino acids resulting from oxidative damage

can participate in metal binding. This may lead to further reactive oxygen species generation, metal chelation complexation, or redox induced cross linking. Further study is needed to clearly delineate the role of neighboring residues on L-dopa's chelating behavior.

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Table 1. Calculated and observed isotopic masses for the L-dopa peptide species.

Peptide	Peptide + H ⁺		Peptide + Na ⁺		Peptide + Zn ²⁺ - H ⁺	
	Calculated ^a	Observed	Calculated	Observed	Calculated	Observed
DopaGly	621.3355	621.294	643.3174	643.259	683.249	683.197
DopaPhe	711.3824	711.435	733.3644	733.381	773.2959	773.344
DopaTyr	727.3774	727.231	749.3593	749.229	789.2908	789.129
DopaTrp	750.3933	750.410	772.3753	772.376	812.3068	812.313
Dopa1Nal	761.3981	761.424	783.3800	783.383	823.3116	823.319

^a All masses are reported in a.m.u.

Table 2. Observed and calculated isotope distributions for peptide:zinc complexes.

Isotope Number	DopaGly		DopaPhe		DopaTyr		DopaTrp		Dopa1Nal	
	Obs ^a	Calc	Obs	Calc	Obs	Calc	Obs	Calc	Obs	Calc
0	99	100	99	100	100	100	99	100	90	100
1	35	35	44	43	45	43	43	46	50	48
2	68	65	59	68	67	68	71	69	71	70
3	27	30	34	35	32	35	38	37	39	38
4	48	46	56	49	52	49	54	50	51	50
5	15	15	24	19	17	19	18	20	24	21
6	6	4			6	6	13	6		

^a Individual isotopic integration values are determined from Bruker Flex Analysis.

Table 3. Binding constants for peptide:zinc complex formation.

Peptide	Binding Constant	ΔG^a
DopaGly	1500	-4.3 (0.2)
DopaPhe	5300	-5.1 (0.2)
Dopa1Nal	3900	-4.9 (0.2)
DopaTrp	6700	-5.2 (0.2)
DopaTyr	6100	-5.2 (0.2)

^a Binding is determined from a non-linear fit of spectrophotometric absorbance. Binding constant units are M^{-1} and ΔG is given in units of $kcal\ mole^{-1}$. Error, in parentheses, reflects the determined 95% confidence integral.

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