Effect of Pb^{II} on the Secondary Structure and Biological Activity of Trypsin

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The effects of Pb^{II} on the secondary structure and biological activity of trypsin have been examined by monitoring changes in its conductivity and IR and circular dichroism (CD) spectra. The results show that Pb^{II} reacts with trypsin, and that the binding sites might be –OH and –NH groups in pepsin. The CD spectra indicate that interaction with Pb^{II} significantly affects the secondary structure of trypsin, the β-sheet-structure content being increased by about 42%, whilst those of α-helix and β-turn structures are decreased by 13% and 21%, respectively. The results clearly demonstrate that Pb^{II} affects the biological activity of trypsin by modifying its secondary structure. Most interesting is that Pb^{II} up-regulates the activity of trypsin at low concentrations while down-regulating it at high concentrations.

Introduction

Lead and its derivatives are some of the most serious sources of environmental pollution. Once inhaled or otherwise ingested, they are accumulated in vivo and are toxic to the reproductive, nervous, immune, skeletal, and hematopoietic systems.[1] Symptoms of chronic lead poisoning include learning disorders, IQ reduction, hyperactive behavior, ataxia, and convulsions.[2–4] Lead poisoning is of concern to the public, and extensive research work has been carried out in this field. Wei and Ru explored the interaction between lead ions and metallothionein,[5] followed by Struzynska et al., who reported the astroglial reaction during the early phase of acute lead toxicity in the adult rat brain.[6] Experiments on the toxicity of lead and lead–porphyrin derivatives in *Trypanosoma brucei* suggested that the lead porphyrins were effective in inhibiting the growth of parasites.[7] Bouton et al. employed cDNA microarrays to analyze the effects of acute lead exposure on large-scale gene-expression patterns in immortalized rat astrocytes and found that many previously reported genes were differentially regulated by lead exposure.[8] Investigations have so far focused mainly on the symptoms of lead poisoning observed in humans, animals, and plants.[9–12] However, the mechanism of lead toxicity at the molecular level is poorly understood at present. An understanding of lead poisoning must begin with the fundamental question of how lead interacts with proteins. The goal of this work was therefore specifically to study the effects of Pb^{II} on the secondary structure and biological activity of trypsin.

Results and Discussion

Determination of conductivity

To study the interaction between Pb^{II} and trypsin, the conductivities of aqueous solutions of Pb^{II} (control) and Pb^{II}/trypsin were determined. As shown in Table 1, the conductivities of the control solutions increased from 0.002 to 0.385 mS cm^{-1} as the Pb^{II} concentration was gradually increased to 1.5×10^{-6} mol L^{-1}, whereas those of the Pb^{II}/trypsin solutions increased from 0.002 to 0.326 mS cm^{-1}. The poor conductivity of the Pb^{II}/trypsin solution relative to the corresponding control solution suggested that there was an interaction (bonding) between Pb^{II} and trypsin that resulted in a reduction in the ionic activity and the transference velocity of Pb^{II} ions.

Table 1. Conductivities of Pb^{II} solutions and Pb^{II}/trypsin reaction systems.

Pb^{II} [mol L^{-1}]	0	5×10^{-8}	5×10^{-7}	1×10^{-6}	1.5×10^{-6}
$S^{[a]}$ [ms cm^{-1}]	0.002	0.035	0.132	0.257	0.385
$S^{[b]}$ [ms cm^{-1}]	0.002	0.013	0.115	0.215	0.326

[a] $Pb(NO_3)_2$ solutions. [b] $Pb(NO_3)_2$/trypsin reaction systems. The concentration of trypsin was 1.0×10^{-5} mol L^{-1} in all of the systems.

FTIR

To examine the possible effect of Pb^{II} ion on trypsin, the IR spectra of pure trypsin (control) and of various reaction systems were determined (Figure 1). The bands at 3375–3427, 1631–1635, and 1389 cm^{-1} represented the –OH group, amide I bands, and NO_3^- ion, respectively. The main vibrational frequencies of trypsin are shown in Table 2. The IR spectra of the five reaction systems differed markedly from that of pure trypsin, the IR frequencies of the trypsin hydroxy groups in the reaction systems being shifted by 16 to 52 cm^{-1} (relative to pure trypsin) with increasing Pb^{II} ion concentration (Table 2). In contrast, the amide I bands were shifted by 6 to 22 cm^{-1}.

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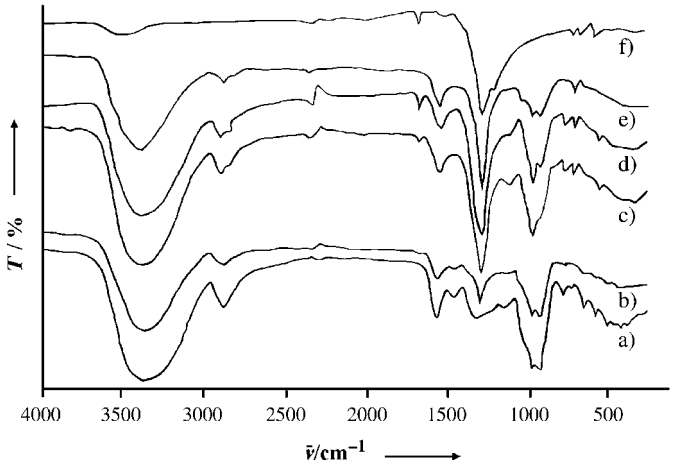

Figure 1. Infrared spectra of pure trypsin and Pb^{II}/trypsin reaction systems with different concentration of Pb^{II} at 25 °C, pH 5. The concentration of trypsin is 1.0×10^{-5} mol L^{-1} in all of the systems. a) Pure trypsin solution, [trypsin] = 1.0×10^{-5} mol L^{-1}. b) System 1, [Pb^{II}] = 1.0×10^{-8} mol L^{-1}. c) System 2, [Pb^{II}] = 1.0×10^{-7} mol L^{-1}. d) System 3, [Pb^{II}] = 1.5×10^{-6} mol L^{-1}. e) System 4, [Pb^{II}] = 1.0×10^{-5} mol L^{-1}. f) $Pb(NO_3)_2$.

Table 2. The main vibrational frequencies [cm^{-1}][a] in the FTIR spectra of pure trypsin, Pb^{II}/trypsin reaction systems with different concentration of Pb^{II}, and $Pb(NO_3)_2$ at 25 °C, pH 5.

Assignment	–OH [cm^{-1}]	Amide I [cm^{-1}]	NO_3^- [cm^{-1}]
Pure trypsin	3375	1653	–
System 1	3391	1647	1389
System 2	3396	1636	1389
System 3	3407	1636	1389
System 4	3427	1631	1389
Pure $Pb(NO_3)_2$	–	–	1389

[a] The concentration of trypsin was 1.0×10^{-5} mol L^{-1} in all systems; the concentrations of Pb^{II} in Systems 1–4 were 5×10^{-8}, 5×10^{-7}, 1.5×10^{-6}, and 1.0×10^{-5} mol L^{-1}, respectively.

Amide I bands are characteristic of proteins, and arise from the coupling of the stretching vibrations of C=O bonds, the bending vibrations of N–H bonds, and the stretching vibrations of C–N bonds. The exact locations of amide I bands are subject to the natures of the hydrogen bonds between the C=O and NH groups,[13–15] and the IR frequencies of the amide I bands indicate an interaction between the Pb^{II} ions and the NH and OH groups in trypsin, resulting in impairment and even rupture of the intramolecular hydrogen bond.

IR spectroscopy is a well established technique for studying the secondary structures of proteins. The secondary structure of trypsin includes four different types: α-helix, β-sheet, β-turn, and random, and the shapes of amide I bands depend on the percentages of these types. Curve-fitting was performed by the method previously described by Seba et al.[14] Typical absorption regions of the four types are as follows: 1646–1661 cm^{-1} (α-helix), 1615–1637 and 1682–1698 cm^{-1} (β-sheet), 1661–1681 cm^{-1} (β-turn), and 1631–1645 cm^{-1} (random).[14] The curve-fitting results for the amide I bands for pure trypsin (as an example) are shown in Figure 2. The calculated percentages, based on the curve-fitting band area, of each secondary structure in pure trypsin and in the different reaction systems are shown in Table 3. The percentage of β-sheet in pure trypsin amounted to 16.0%, while in the Pb^{II}/trypsin solution ([Pb^{II}] = 1×10^{-5} mol L^{-1}) it had increased to 61.0%. In contrast, the proportion of α-helix in pure trypsin was only 22.0%, but had decreased to 5% in Pb^{II}/trypsin solution ([Pb^{II}] = 1×10^{-5} mol L^{-1}). Similarly, the percentage of β-turn also decreased proportionally as the Pb^{II} concentration increased. In the α-helix structure of a protein, the hydrogen bonds formed between the oxygen atom of the *i*th carboxyl group and the hydrogen atom of the (*i*+4)th amino group are a key stabilizing factor. There are 3.6 amino acid residues in every turn of an α-helical segment. The β-sheet structure can be visualized as a helix comprised of two amino acid residues per turn by stretching an α-helix. In the Pb^{II}/trypsin solutions, the percentage of α-helix decreased while that of β-sheet increased (Table 3); this indicated that Pb^{II} ions might combine with trypsin, weakening or breaking the hydrogen bond. Hence, some α-helix was stretched and transformed into β-sheet. It was found that the proportion of β-sheet was evidently increased at lower concentrations of Pb^{II} (System 1: [Pb^{II}] = 1×10^{-8} mol L^{-1} and [trypsin] = 1×10^{-5} mol L^{-1}). When the Pb^{II} concentration was 5×10^{-6} mol L^{-1} or above, the proportions of the secondary structure remained unchanged; this indicated that the trypsin had been denatured.

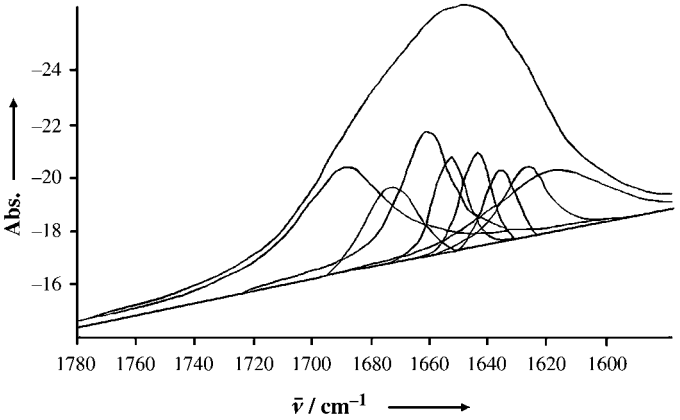

Figure 2. Curve-fitting results for the IR spectral amide I band of pure trypsin.

Table 3. The percentages of the four types of trypsin secondary structure in pure trypsin and in Pb^{II}/trypsin reaction systems [%] as determined from their IR spectra at 25 °C, pH 5.[a]

Assignment	α-helix	β-sheet	β-turn	random	RMSD
Pure trypsin	22	16	40	22	0.098
System 1	16	29	36	19	0.110
System 2	11	45	26	17	0.089
System 3	6	60	19	15	0.093
System 4	5	61	18	14	0.112

[a] The concentration of trypsin was 1×10^{-5} mol L^{-1} in all solutions. System 1: [Pb^{II}] = 5×10^{-8} mol L^{-1}. System 2: [Pb^{II}] = 5×10^{-7} mol L^{-1}. System 3: [Pb^{II}] = 1.5×10^{-6} mol L^{-1}. System 4, [Pb^{II}] = 1×10^{-5} mol L^{-1}.

Circular dichroism (CD) spectra

Although IR spectroscopy has many advantages for studying the secondary structures of proteins, uncertainties might arise from the curve-fitting technique based on the method normally performed for proteins. CD spectroscopy is also frequently used to investigate secondary structures of proteins because of its high sensitivity. This study also utilized CD spectroscopy to corroborate the changes in the secondary structure of trypsin. The CD spectra of pure trypsin and of the other four Pb^{II}/trypsin solutions were characterized by two broad negative bands at about 203 and 222 nm and a positive band at about 195 nm, attributable to the presence of mixtures of α-helix and β-structures (Figure 3).[16,17] As the Pb^{II} concentration increased,

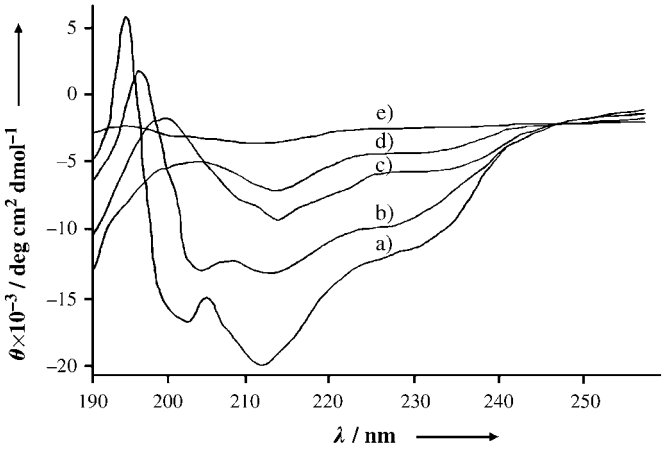

Figure 3. CD spectra of trypsin solution and Pb^{II}/trypsin reaction systems at 25 °C, pH 5. The concentration of trypsin was 1.0×10^{-5} mol L^{-1} in all of the systems. θ expresses the CD intensity. a) Pure trypsin solution, [trypsin] = 1.0×10^{-5} mol L^{-1}. b) System 1, $[Pb^{II}]=1.0\times10^{-8}$ mol L^{-1}. c) System 2, $[Pb^{II}]=1.0\times10^{-7}$ mol L^{-1}. d) System 3, $[Pb^{II}]=1.5\times10^{-6}$ mol L^{-1}. e) System 4, $Pb^{II}=1.0\times10^{-5}$ mol L^{-1}.

the β-sheet structure was again formed, as indicated by the disappearance of the negative double minima at 203 and 222 nm and positive maximum at 195 nm (Table 4). The β-sheet content increased significantly, from 10% for pure trypsin to 53% for Pb^{II}/trypsin solution 3 ($[Pb^{II}]=1.5\times10^{-6}$ mol L^{-1}), while α-helix and β-turn decreased under the same conditions,

Table 4. The percentages of the four types of trypsin secondary structure in pure trypsin and in Pb^{II}/trypsin reaction systems [%] as determined from their CD spectra at 25 °C, pH 5.[a]

Assignment	α-helix	β-sheet	β-turn	random	RMSD
Pure trypsin	17	10	46	27	0.157
System 1	12	24	38	26	0.134
System 2	7	37	33	23	0.115
System 3	3	53	25	19	0.151
System 4	3	54	24	19	0.087

[a] The concentration of trypsin was 1×10^{-5} mol L^{-1} in all solutions. System 1: $[Pb^{II}]=5\times10^{-8}$ mol L^{-1}, 2: $[Pb^{II}]=5\times10^{-7}$ mol L^{-1}, 3: $[Pb^{II}]=1.5\times10^{-6}$ mol L^{-1}, 4: $[Pb^{II}]=1\times10^{-5}$ mol L^{-1}.

with the former being changed from 17% to 3% and the latter from 46% to 25%. The β-sheet and α-helix content did not change any further when the Pb^{II} concentration was raised to 1.5×10^{-6} mol L^{-1} or above. Simultaneously, the content of random structure also fell when the Pb^{II} concentration was increased. The results obtained from the CD spectra are in good agreement with these from the IR spectra. It is speculated that Pb^{II} ions interact with –OH and –NH groups in trypsin, resulting in the change in the secondary structure.

Biological activity of trypsin

The function of trypsin is to hydrolyze arginine and tyrosine residues in proteins selectively. Interaction of Pb^{II} and trypsin was characterized by the up-regulation of trypsin activity when the concentration of Pb^{II} was between 0 and 5×10^{-8} mol L^{-1}, followed by inhibition when $[Pb^{II}]$ was $>5\times10^{-8}$ mol L^{-1} (Figure 4). This suggests that Pb^{II} is favorable for the active-site

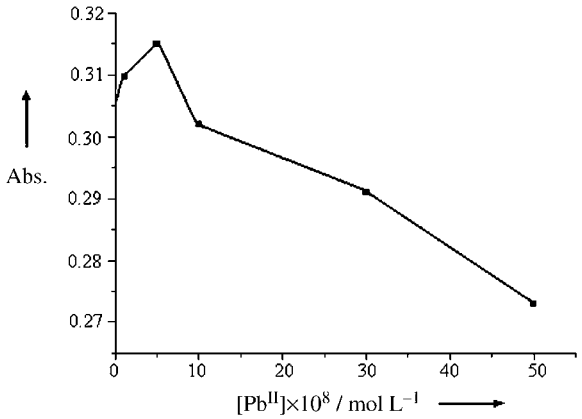

Figure 4. The effect of $[Pb^{II}]$ on the activity of trypsin measured by visible spectrophotometry at 30 °C, pH 8. Casein was used as a substrate and a mixture of phosphate–tungstic acid and phosphomolybdic acid was used as reactive reagent. The greater the absorbance (A), the stronger the activity of trypsin was.

conformation of trypsin at low concentrations and destructive at high concentrations. The mechanism of activation of trypsin at the low Pb^{II} concentration remains unknown. Perhaps Pb^{II} bonded to the active site as the metal center, but when the Pb^{II} concentration increased further, Pb^{II} bonded not only to the active site but also to other trypsin sites, resulting in a significant change in the second structure and a sharp reduction in its activity.

Conclusion

The hazards of lead pollution are well documented. Tissue damage caused by lead is slow and progressive. This study has used trypsin as an example protein to investigate how Pb^{II} would interact with –OH and –NH groups in a protein and affect its biological activity. The results have clearly demonstrated that the secondary structure of trypsin changed significantly in the presence of Pb^{II}. If the data could be extrapolated

to in vivo situations, lead toxicity would be associated with modification of the secondary structures of various functional proteins in living cells.

Experimental Section

Materials and apparatus: Anhydrous lead nitrate of analytical purity and trypsin with a molecular weight of 23300 were obtained from Sigma. The instruments used in this study included a DDS-11A digital conductimeter; Bio-Rad FTS-40 spectra were obtained on a Fourier transform infrared spectrograph and Jasco J-810 spectropolarimeters. The water used for all the experiments was doubly distilled.

Establishment of reaction systems: Aqueous $Pb(NO_3)_2$ (100 mL, 1.007×10^{-1} mol L^{-1}) was prepared by dissolving $Pb(NO_3)_2$ (3.3121 g) in doubly distilled water (100 mL). The solution was diluted to produce a series of solutions (5×10^{-5} mol L^{-1}, 5×10^{-4} mol L^{-1}, 1×10^{-3} mol L^{-1}, 1.5×10^{-3} mol L^{-1}). Trypsin in aqueous solution (100 mL, 1.0×10^{-5} mol L^{-1}) was prepared with the pH value being adjusted to 5 with dilute HCl solution (1 mol L^{-1}). Aqueous trypsin solution (10 mL) was placed in four round-bottomed flasks (25 mL), followed by addition of one of the aqueous $Pb(NO_3)_2$ solutions (10 μL). The four resulting solutions were named System 1, System 2, System 3, and System 4, respectively, and the concentrations of Pb^{II} in the four systems were 5×10^{-8} mol L^{-1}, 5×10^{-7} mol L^{-1}, 1.5×10^{-6} mol L^{-1}, and 1.0×10^{-5} mol L^{-1}, respectively. Three parallel experiments were performed simultaneously for each reaction system. The reactive solutions were stirred for 2 days at room temperature. The conductivities of the reaction systems and of the aqueous solutions of $Pb(NO_3)_2$ with the same concentration of Pb^{II} were determined. This was followed by recording of the IR and CD spectra of pure trypsin and of the reaction systems.

FTIR: The $Pb(NO_3)_2$/trypsin reaction solutions were dried for 48 h under vacuum at 35 °C to form solid films. FTIR spectra between 4000 cm^{-1} and 400 cm^{-1} were measured 16 times in a KBr flake. In order to obtain detailed information about the changes in the trypsin secondary structure, the shapes of the amide I bands of the pure trypsin and of the reaction systems were analyzed by derivatization, deconvolution, and curve fitting techniques.[18] The percentages of the α-helices, β-sheets, β-turns, and random structure were calculated by addition of the areas of all bands assigned to each of the structures and expression of the sum as a fraction of the total amide I band area.[19–23] The analytical program used during the derivatization, deconvolution, and curve fitting in the experiment was WIN-IR 4.0. A number of studies have analyzed a wide range of water-soluble proteins and compared quantitative estimates based upon infrared spectra with the available X-ray diffraction data.[24] Good agreement between the two techniques has been reported. The FTIR technology has been widely used to characterize the secondary structures of proteins.

Far-UV CD spectra: Far-UV spectra (190—260 nm) of the trypsin were recorded on a Jasco J-810 spectropolarimeter. This instrument had previously been calibrated for wavelength with benzene vapor and for optical rotation with D-10-camphorsulfonic acid. A cell with a pathlength of 1 cm was used. A thermostatically controlled cell holder and a Thermo NESLAB (Portsmouth, NH, USA) RET-111M temperature controller were used to maintain the desired temperature. The parameters were as follows: bandwidth, 1 nm; step resolution, 0.1 nm; scan speed, 50 nm min^{-1}; response time, 0.25 s. Each spectrum was obtained by averaging four to six scans. Quantitative estimations of the secondary structure content were made with the aid of the CDPro software package, which includes the programs CDSSTR, CONTIN, and SELCON3 (http://lamar.colostate.edu/~sreeram/CDPro).[25] We used these three programs to analyze our CD spectra. The α-helical fractions derived from the CDPro programs are in a good agreement with those calculated based on empirical methods from ellipticities at either 208 or 222 nm.[16,17]

Determination of biological activity of trypsin: Trypsin can selectively hydrolyze arginine and tyrosine residues in a protein. In this study, casein was used as a substrate and the hydrolysis products were treated with a mixture of phosphate–tungstic acid and phosphomolybdic acid; this resulted in an absorption band at 680 nm. The greater the absorbance, the stronger the activity of trypsin was (the hydrolytic activity of trypsin was determined in a 722-Visible spectrophotometer).[12] Pb^{II} solutions (10 μL) of different concentrations as described above were added to trypsin solution (10^{-5} mol L^{-1}, 10 mL). After 2 days, the reaction solution (1 mL) was sampled into a test tube, the pH was adjusted to 8 with boric acid buffer solution, and casein solution (2 mL) was added. The mixed solutions were then thoroughly stirred and allowed to react for 15 minutes at 30 °C, and the mixed solution of phosphate–tungstic acid and phosphomolybdic acid (3 mL) was then added. The absorbance at 680 nm was measured.

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