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Structural changes of human serum albumin in response to a low concentration of heavy ions

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Abstract

Lead ions in solution interact strongly with human serum albumin and modify the properties and function of albumin molecules. In the present study, we used optical spectroscopic techniques to explore the binding sites of lead, present in albumin. Structural and chemical analysis of albumin molecules using fluorescence and Raman spectroscopy, predicted the modification of two major amino acids in albumin due to lead binding. No secondary structural changes are observed in the protein molecule, which is further confirmed using circular dichroism absorption measurements. The results indicate that loss of charge from the binding site of albumin by the charged lead ions, give rise to dipole interaction which acts as the major contributor to promote protein agglomeration.

Stepwise formation of a protein cluster in the presence of a lead ion.

Keywords

albumin; lead; fluorescence; Raman; circular dichroism spectroscopy

1. Introduction

Heavy metals, such as lead, mercury, cadmium, and arsenic, present a major threat to human health and society. In particular, lead deserves special attention since this metal is the most toxic biochemical agent responsible for the world's most common environmental caused disease, lead poisoning [1]. Although last several decades of an extensive research on lead poisoning has helped in significant reduction of lead exposure in the biosphere but still 300 million tons of this metal is circulating in soil and groundwater [2]. While effect of high dosages of this toxic metal in adults are quite evident, the effect of low concentration of lead remains mostly unrecognized [3]. In most cases, children affected with lead poisoning

although showed no clinical sign of toxicity, but are left with long term deficit with IQ scores, learning disabilities and behavioral disorders [3]. Thus, lead poisoning, presents an open challenge to the scientific community to develop sensors with high precision and study explicitly the affect of lead ion on body's physiology. Although the Environmental Protection Agency (EPA) limits the allowable level of lead in drinking water to 15 ppb (73 nM), recent studies show that there is no threshold for the adverse effect of lead. This universal toxic metal, tends to substitute biological reactions mediated by calcium, iron, and other metal ions and enzymes [3–4] and leads to serious medical conditions such as improper brain development and delayed neural development [4].

Various techniques have been proposed in response to the increasing demand for detection and study of adverse effect of lead ions in our body. This includes methods like atomic absorption spectrometry, inductively coupled plasma (ICP) atomic emission spectroscopy, and ICP mass spectrometry [5]. Recently catalytic DNAs have been demonstrated as a unique class of biosensors for metal ion sensing [6]. However, most of these techniques are inefficient in detecting lead ions below micromolar concentration or are outrageously expensive. It is also worth noting that, since the range of symptoms of lead poisoning is so broad, the exact molecular mechanism of lead poisoning is still unknown. A zinc enzyme called aminolevulinic acid dehydratase (ALAD) is one of the most documented targets of lead ions, whose activity is also correlated with blood lead level [7–10]. But inhibition of ALAD does not account for the behavioral problem seen in children with low blood lead level [11]. Several other studies suggest that transcription factors containing Zn binding sites are promising targets for lead [12–18]. However, the exact binding site of lead is still a mystery and remains a high priority for pediatric health community.

To find an alternative method to detect the heavy lead ions in solution, we evaluated the natural transport path of those ions in a human body. The most common media for transporting heavy metal ions is through binding to serum albumin, which is one of the most abundant proteins in blood serum, and is also responsible for transport function [19, 20]. This heavy protein molecule consisting of 585 amino acids has three structurally similar domains which are stabilized by 17 disulfide bridges [21]. These domains have numerous binding sites for fatty acids, glycerol and metal ions. These binding sites enable the molecule to regulate intercellular metabolic fluxes and interactions with pharmaceuticals. Thus, the ability of serum albumins to bind lead ions is well known, and has been used to detect micromolar concentrations of lead ions in solution [22, 23]. In this report we illustrate a method which not only increases the sensitivity of detection by several orders of magnitude but also demonstrates the physiological changes in albumin due to the interaction with lead.

We used a battery of optical spectroscopic techniques for this purpose. Optical methods offer a significant advantage with respect to other methods because of their non-invasiveness and ability to perform *in situ* measurements in a real-time [24, 25]. A powerful combination of fluorescent spectroscopy, non-resonant Raman scattering and circular-dichroism absorption spectroscopy are used to figure out the binding site of lead ion in albumin molecule. An advanced fluorescent technique known as excitation–emission matrix method is used here to sense picomolar lead concentration and to evaluate the effect of lead ion on fluorescent properties of albumin. Difference Raman spectra are analyzed to investigate the binding sites of lead in albumin molecule. In order to understand the structural transformations of albumin molecule the circular dichroism absorption measurements are studied. We also proposed a possible physical model which leads to the formation of protein clusters due to the presence of heavy metal ion in the solution.

2. Experimental

2.1 Sample preparation

Pure defatted human serum albumin (HSA) used for these studies was purchased from ICN Biomedical, Inc. (Aurora, OH, USA). Inorganic compounds such as disodium hydro phosphate (Na_2HPO_4), sodium dihydro phosphate (NaH_2PO_4), lead nitrate ($\text{Pb}(\text{NO}_3)_2$) and sodium chloride (NaCl) were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI, USA). The buffer solution prepared in accordance with Ref. [26] by adding Na_2HPO_4 , NaH_2PO_4 and NaCl to a de-ionized, double distilled water, was maintained at the $\text{pH} = 7.0$. The concentration of albumin in the solution was 40 mg/ml. The buffer solution of albumin was prepared and kept for 24 hrs in a dark and dry place at the temperature of about 4 °C before taking all the described measurements. This concentration of albumin was used for Raman spectroscopy measurements. For all other experiments, the solution was diluted with the buffer solution to the concentration of 1 mg/ml. The lead salt solution was prepared by dissolving an appropriate amount of lead nitrate ($\text{Pb}(\text{NO}_3)_2$) (Sigma-Aldrich, Inc.) in de-ionized, double distilled water. To ensure that albumin itself is not undergoing any spontaneous agglomeration we performed the experiments 24 hrs after preparing the solution.

2.2 Experimental methods

Fluorescence is a powerful tool to study the binding sites of large biological molecules. Earlier, methods based on fluorescence quenching were successfully applied to study the binding sites of HSA. In this report we take advantage of the excitation-emission matrix (EEM) method for fluorescence detection which has been proven a valuable method for multi-component analysis. We used a commercially available Fluorolog-3 (Jobin-Yvon, Inc.) for collecting fluorescence spectra. The fluorescence signal from a solution, placed in a quartz cell, was collected in a back-reflection geometry, which minimizes the effect of light propagation inside the absorbing medium. An experimentally recorded EEM consists of 31 individual emission spectra (in a spectral region from 260 nm to 600 nm) for a range of the excitation wavelength from 250 to 400 nm with sequential increments of 5 nm. Fluorescence spectra were recorded with an integration time of 15 s using excitation and emission slit-widths of 2 nm. To ensure the stability of the solution, the experiment was repeated several times after the scan was completed. The spectra were not normalized to instrument response function since all the data were compared to each other and no absolute measurements were needed.

For non-resonant Raman measurements we employed a home-built confocal Raman microscope system equipped with a intracavity doubled Nd:YVO₄ laser excitation source ($\lambda_{\text{ex}} = 532$ nm). For all the described measurements, we used a high numerical aperture objective lens (Nikon, Inc.; 60X water immersion, fluorite aberration corrected objective lens with a numerical aperture N.A. = 1.0) to maximize the collection efficiency of the signal. The Raman signal was collected in a back-scattering geometry and was directed to a high-throughput spectrometer, where the spectrally resolved signal was detected with a liquid-nitrogen-cooled CCD camera. Typical spectral resolution in the spectral region of interest was 3 cm^{-1} , and the reproducibility of the Raman peaks' position was within 0.5 cm^{-1} .

Circular dichroism (CD) absorption measurements indicate the changes of the secondary structure of proteins. We used Jasco-710A (Jasco, Inc) CD-absorption spectrometer to measure the secondary structure of albumin in solution for different concentrations of lead ions. We used a 100-micron-thick quartz cell and separately measured the CD-absorption spectra of the buffer solution and the solution containing lead ions. While both of these

solutions provided essentially zero background to our measurements, very strong absorption of lead salt solution at the wavelengths shorter than 240 nm prevented us from measuring CD-absorptions spectra for the concentrations of lead ions exceeding 0.01 mM.

3. Results and discussion

Figure 1(a) shows the semilogarithmic fluorescence intensity plot of EEM data of HSA in buffer solution. Pure lead is not making any contribution to the fluorescence signal which was verified by taking fluorescence spectrum of lead solution (Data not shown). Although Raman spectrum of water is visible in those plots, it still appears several orders of magnitude weaker than the fluorescence intensity of HSA. It is important that the shape of the EEM plot and the absolute fluorescence intensity were not affected by the change in albumin and/or Na^+ concentrations. Figure 1(b) shows the EEM of albumin treated with a 10 pM solution of lead ions.

The right side window in Figure 1(a) and (b) shows an emission spectrum at a particular excitation wavelength of 350 nm for comparison. The top window in Figure 1(a) and (b) shows an excitation spectrum for a particular emission wavelength of 450 nm. For a pure albumin solution, a strong fluorescence signal is visible for the excitation wavelength from 275 nm to 295 nm. This strong fluorescence signal mostly comes from two aromatic residues of tyrosine (Tyr) and tryptophan (Trp) located in subdomain IIIA and 214th residue of subdomain IIA, respectively [22]. The only other feature of this spectrum is due to a weak Raman signal from water molecules at around 3400 cm^{-1} with respect to the excitation wavelength. Next, the albumin solution was treated with solutions containing lead ions starting from 1 pM (10^{-12} M) concentration. No significant changes were observed in the shape of the EEM spectra for the lowest concentration (10^{-12} M); however, starting with as little as 10 pM concentration of lead ions, a fluorescence peak at around 430 nm, when excited at around 350 nm, becomes more and more noticeable (Figure 1(b)). The increase in fluorescent spectra excited by the 350 nm radiation is shown in Figure 2(a) for increasing concentration of lead ions. The amplitude of the fluorescence signal changes dramatically. On the other hand, the fluorescence emission excited by the 275 nm radiation shows a gradual decrease with increase in concentration of lead (Figure 2(b)). No significant changes were observed in the fluorescence emission excited by the 295 nm excitation. At the same time, the fluorescence signal, excited by the 350 nm radiation, shows a remarkable sensitivity to the presence of lead ions in solution.

To attain a more detailed information about the interacting sites of albumin due to the effect of lead ions, we collected Raman spectra from pure albumin in buffer solution and albumin treated with different concentrations of lead ions. A typical Raman spectrum of pure albumin in buffer solution is shown in Figure 3. This spectrum was processed using a Matlab routine which eliminates the autofluorescence background due to the protein molecule.

Figure 3 shows the raw data, the background fluorescence and the actual Raman spectrum after background subtraction. A broad band with a center peak around 1653 cm^{-1} corresponds to Amide I band, which is a characteristic feature of α -helical conformation of the polypeptide backbone originating mainly from peptide $\text{C}=\text{O}$ stretching vibration [27]. Human serum albumin is predominantly an alpha helical molecule (67%) where each of the subdomains (A & B) of three domains I, II and III share a common helical motif. These helices are formed through intramolecular hydrogen bonding between carbonyl oxygen of every fourth peptide bond and hydrogen atom of the same molecule. Thus, any change in this band would reflect a modification in the secondary structure of the albumin molecule. Vibrational bands at 1340 cm^{-1} and 880 cm^{-1} correspond to vibrations of the aromatic

amino acids in HSA containing a single Trp residue [28, 29], while a shoulder at 850 cm^{-1} and 830 cm^{-1} on the broad peak at 880 cm^{-1} are assigned to the Tyr residues [30]. Extensive Raman studies suggest that this closely spaced doublet is due to a Fermi resonance between ring-breathing vibration and overtone of an out of plane ring bending vibration of para- substituted aromatic moieties [30]. The intensity ratio of these two bands varies due to the change in frequency of the two vibrations. The breathing vibration of a benzene ring of Phenylalanine (Phe) is detected at 1004 cm^{-1} [31] which is also known to overlap with Trp frequency. There are total of 31 Phe residues present in albumin reflecting the strength of this line.

Difference Raman spectra of albumin for different concentrations of lead ions in solution are exhibited in Figure 4 to highlight the changes in the different bands. No significant variations in position or intensity of the bands at 1340 cm^{-1} and 880 cm^{-1} are observed as a result of the lead-ion treatment, which indicates that there is no observable change in Trp residue which is considered as one of the major binding site of albumin for other heavy ions.

Major changes of Raman spectra at around 1004 cm^{-1} and 1314 cm^{-1} are clearly evident from this figure. The vibration line at 1004 cm^{-1} , although shows no significant change in intensity for low concentration of lead ($10^{-11} - 10^{-9}\text{ M}$), displays a substantial change for a high lead concentration. Both Trp and Phe can contribute to this line; however, our fluorescent measurements, as well as the absence of any significant changes in the other Trp Raman bands, manifest that there are, most probably, no significant changes occurring at Trp site due to albumin interaction with lead ions. Thus, we can conclude that Phe is the dominate site, which is affected due to a relatively high concentration ($10^{-7} - 10^{-5}\text{ M}$) of lead ions. Apparently, Phe fluorescence is weak because of its low absorption and low quantum yield [32], thus, making direct fluorescence measurements of Phe rather difficult. The change of intensity of the 1314 cm^{-1} peak reflects the change in the C-H vibration, which may come from side chains of any amino acid. To extract the most information about Tyr residue, we performed multipeak fit of a relatively broad band at 880 cm^{-1} which contains the doublet $850/830\text{ cm}^{-1}$ band of Tyr residue as weak shoulder. Figure 5 displays the deconvolution of Raman peak at 880 cm^{-1} , fitted with Gaussian peaks at three wavenumbers 830 cm^{-1} , 850 cm^{-1} and 880 cm^{-1} .

There is no major change in the position of the peaks; however, the amplitude ratio of the two peaks at 850 cm^{-1} and 830 cm^{-1} shows a gradual decrease with the increase in concentration of lead ions. This change in ratio of $850/830\text{ cm}^{-1}$ peaks' intensity as a function of the lead concentration is shown in Figure 6.

No variation of the Amide I band at 1653 cm^{-1} was observed, which confirms that albumin is, most likely, not going through any secondary structural alteration. The later was independently verified by performing circular dichroism measurements, which provide an accurate assessment of the secondary structural modifications of protein molecule (see Fig. 7). As it is evident from Figure 7, there are no changes in the secondary structure of HSA up to $10\text{ }\mu\text{M}$ concentration of lead ions in solution.

The two main dominant site of albumin for binding of lead are Tyr and Phe. Our previous study on albumin and lead shows that the preferential site of lead binding is Tyr 84 which is present close to Cys 34 [33]. The change in the other amino acid Phe which was not evident from the fluorescent data is observed by analyzing the Raman difference spectroscopy. Clear evidence of change in Tyr residue is present in Raman spectroscopic measurements where the doublet at 850 cm^{-1} and 830 cm^{-1} , responsible for Tyr, changes their ratio with increase in lead concentration. No other structural changes are observed from Raman and Circular

dichroism measurements. Circular dichroism measurement confirms that the secondary alpha helix structure is not going through any alteration due to lead binding.

The electrostatic interaction in the solution is the main physics acting behind the interaction of protein molecule with heavy ions in the solution. Albumin molecules have a negative surface charge, which under normal *pH* conditions introduces repulsive interaction between protein molecules. When a large positive heavy ion like lead is introduced into the solution, it interacts with a protein, forming a Coulomb complex with a common hydration shell (as it is illustrated in the Figure 8).

The surface charge of protein molecule is now compensated due to binding of the lead ions and thus the protein molecules, which were experiencing a Coulomb repulsion initially, now starts experiencing dipole-dipole interaction. It creates favorable conditions to form macromolecular complexes (agglomerates) composed of albumin molecules. Those complexes can be the source of the observed visible fluorescence. The exact origin of fluorescence at around 430 nm is still unclear. Other reports suggest that aggregates of albumin in the solution, caused by prolonged storage of albumin in solution, can also produce a fairly strong fluorescence emission in this spectral range [34]. Presence of agglomerates in the protein solution is also verified by light scattering measurements. We used a light scattering apparatus (Zetasizer microvolt; Malvern Instruments, Inc.) to quantitatively characterize the size of agglomerates in solutions. Zetasizer enables determination of the particles' size distribution in a sample ranging from 0.6 nm to 6 μm [35]. An increase in the particles' size, or the mean diameter, which is denoted by Z_{Ave} , was observed starting at 10^{-9} M concentration of lead ions in solution. The intensity distribution dominated by particles having $Z_{\text{Ave}} 4 \pm 1$ nm was observed for pure albumin. However, for 10^{-9} M concentration of lead the intensity distribution showed a sudden increase in peak intensity from particles having Z_{Ave} or average diameter of $85 \text{ nm} \pm 39 \text{ nm}$. The sensitivity of the detection did not allow the detection of agglomeration at any lower concentration of lead ions.

4. Conclusion

The present study, combined with our previous work describing HSA-Lead interaction shows that even pM concentration of lead is capable of affecting the functional properties of albumin. A powerful combination of fluorescence and Raman spectroscopic techniques identified the amino acids, Tyr and Phe which are mainly affected due to interaction of lead with albumin. No secondary structure transformation is observed in the albumin molecule which is confirmed by using circular dichroism measurements. We can conclude from the results that the positively charged lead ion compensates the surface charge of the protein molecule due to binding, after which the albumin molecules start experiencing dipole-dipole interaction making a favorable conditions to form macromolecular complexes (agglomerates). We believe that UV resonant Raman scattering spectroscopy in conjunction with fast mixing experiments can shed some light on the detailed structure and dynamics of albumin transformation in solution caused by a presence of heavy ions.

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Biographies

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Figure 1.

(a) (online color at: www.biophotonics-journal.org) Semilogarithmic plot of the excitation-emission matrix of albumin in a buffer solution, (b) treated with lead (10 pM).

Figure 2.

(a) Fluorescence intensity of albumin solution as a function of lead concentration. Excitation wavelength – 350 nm, (b) – 275 nm.

Figure 3.
(online color at: www.biophotonics-journal.org) Raman spectrum of pure albumin in a buffer solution (black line), background fluorescence (red line), and Raman spectrum after background correction (blue line).

Figure 4.
(online color at: www.biophotonics-journal.org) Difference Raman spectra of pure albumin solution and albumin solution treated with lead concentrations **(a)** 10^{-11} , **(b)** 10^{-9} , **(c)** 10^{-7} , and **(d)** 10^{-5} M.

Figure 5. (online color at: www.biophotonics-journal.org) Raman peak at 880 cm^{-1} with doublet at 850 cm^{-1} and 830 cm^{-1} . Experimental data (solid black line) fitted with Gaussian functions (green line with solid circle) shown with reconstituted deconvoluted spectra (red triangle).

Figure 6.
Ratio of peaks' intensities at 850 cm^{-1} and 830 cm^{-1} as a function of lead.

Figure 7.
(online color at: www.biophotonics-journal.org) Circular dichroism absorption spectra of pure albumin solution and solutions of albumin treated with different concentrations of lead ions.

Figure 8.
(online color at: www.biophotonics-journal.org) Schematic cartoon diagram of a protein cluster formation.