Probing of the Interaction Between Human Serum Albumin and A New Synthesized Pd(II) Complex Using Spectroscopic Methods

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Abstract

Human serum albumin (HSA) is an abundant, multifunctional and nonglycosylated negatively charged plasma protein. HSA ascribed ligand-binding and transport properties, antioxidant functions and enzymatic activities. In the present study, the interaction and side effects of a new designed anti-cancer compound (1,10-phenanthroline butyl dithiocarbamato palladium(II) nitrate) on HSA have been investigated by different spectroscopic (UV-visible, fluorescence and circular dichroism (CD)) techniques at two temperatures of 25 and 37°C. By the analysis of fluorescence spectra, it was observed that this complex has an ability to quench the intrinsic fluorescence of HSA through a static quenching procedure. The number of binding sites and the association binding constants of Pd(II) complex were calculated at 25 and 37°C. Also, the negative $\Delta H^0$ and positive $\Delta S^0$ values for the interaction calculated by the vant’s Hoff equation showed that the electrostatic interaction has a major role in the binding process. The quantitative analysis of CD spectra represented that Pd(II) complex induces significantly alterations in the secondary structure of HSA via decreasing in the content of $\alpha$ helical structure of the protein. Our results suggest that the new synthesized Pd(II) complex can bind to the blood carrier protein of HSA and change the tertiary and secondary structure of the protein, which may be considered as side effects of this new synthesized drug.

Keywords: HAS; Pd(II) complex; Side effect; Fluorescence; Circular dichroism

Introduction

Human serum albumin (HSA) is an abundant, multifunctional and nonglycosylated, negatively charged plasma protein [1]. In normal individuals, albumin is present in concentrations of 42 mg/ml [2].

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Due to a high concentration of HSA in plasma (0.6 mM), it is responsible for about 80% of the colloid osmotic pressure of that fluid [3]. In addition, HSA possesses enzymatic properties and serves as a transport protein for numerous compounds [3].

HSA consists of a single nonglycosylated all-α chain of 586 amino acids organized in three similar domains (I, II and III), each of which contains two subdomains (IA, IB, etc) [4, 5]. Each domain is made up by two separate helical subdomains (named A and B), connected by random coils [4].

HSA has two important structurally selective drug binding pockets in subdomains IIA and IIIA, which correspond to sites I and II proposed by Sudlow [4, 5]. Bulky heterocyclic anions bind preferentially to Sudlow’s site I, whereas Sudlow’s site II is preferred by aromatic carboxylates with an extended conformation. Remarkably, warfarin and ibuprofen are the prototypical ligands of Sudlow’s site I and II, respectively [6-8].

Drug–protein interactions are determinative factors in pharmacodynamics and pharmacokinetics of drugs. The unbound drug is considered to diffuse from the blood to the extravascular site of drug action and to exhibit the pharmacological activity and/or side effect. In order to adjust the optimum therapeutic dose of a drug, it is necessary to know the extent of drug–protein binding. In addition, the displacement from HSA due to the competitive binding of simultaneously administered drugs may lead to increase levels of free drug, and thus potentially side effects. So the effect of the displacement of drug caused by simultaneously administered exogenous compounds should be known to avoid unwanted side effects for the patient [9].

The importance of metal compounds in medicine is undisputed, as can be judged by the use of compounds of antimony (anti-protozoal), bismuth (anti-ulcer), gold (anti-arthritic), iron (anti-malarial), silver (anti-microbial) and platinum (anti-cancer) in the treatment of various diseases. In terms of anti-tumor activity, a wide range of compounds of both transition metals and main group elements have been investigated for efficacy [10]. The earliest reports on the therapeutic use of metals or metal-containing compounds in cancer and leukemia date from the sixteenth century [10].

There is steadily a growing interest in investigations of transition metal complexes other than the traditional platinum-based compounds for use as chemotherapeutic agents against cancer. Current metal-based drugs research is moving towards the development of the new agents able to improve effectiveness and reduce the severe side effects of cis-diaminedichloroplatinum(II) (cisplatin) and its analogues that are still the most widely used anticancer therapeutics [11].

The success of cisplatin, oxaliplatin and other Pt(II) complexes in the treatment of testicular, ovarian, head and neck, esophageal and non-small cell lung cancers, and the similarity of the properties of Pt(II) and its congener Pd(II) has led to a large effort in the search to find Pd(II) antitumor drugs that are effective against Pt(II)-resistant therapies that have fewer side effects. An important difference between two metal ions is that the reaction of Pd(II) is 10³ times faster than that of Pt(II), which can lead to the hydrolysis of Pd complexes before they reach to their target DNA [12-13].

The present study focuses on biophysical interactions of a new designed Pd(II) complex (1,10-phenanthroline butyl dithiocarbamato palladium(II) nitrate) with HSA that plays an important role in the drug transport and storage in vertebrates. The molecular interactions are monitored by different spectroscopic techniques, which they are sensitive and relatively easy to use. Drugs administered during multi-drug therapy may alter each other binding properties to the transporting protein. So it is necessary to determine both the location of the binding sites and the possible interactions for each individual drug.

Materials and Methods

Materials

Human serum albumin (HSA) was purchased from Sigma. Pd(II) complex was synthesized in our laboratory using previous procedures [15]. All other materials and reagents were of analytical grade. All solutions were made in double-distilled water. NaCl solution, 5 mM, was used as a solvent. Since Pd(II) complexes do not dissolve in any buffers with pH 7, then we have to dissolve them in NaCl (5 mM). Concentration of HSA was determined spectrophotometrically using a molecular absorption coefficient of ε¹⁰⁶ at 278 nm=5.3 M⁻¹cm⁻¹ [16].

Fluorescence Measurements

Fluorescence intensity measurements were carried out using a Cary Spectrofluorimeter. The excitation wavelength was adjusted at 290 nm, and the emission spectra were recorded for all of samples at different temperatures (25 and 37 °C) in the range of 300-500 nm. Measurements were made by applying a 1 cm path length fluorescence cuvette and slit width of 5 nm. The fluorescence intensities of the Pd(II) complex at the highest denaturant concentration at excitation 280 nm has been checked. The magnitudes of emissions for this compound were very small and negligible. The
concentration of the protein (HSA) in fluorescence studies was 4.5 µM.

The binding of a hydrophobic fluorescent probe, ANS, to HSA was monitored by exciting the ANS (200 µM) at 350 nm and recording the emission spectra in the range of 400 - 600 nm. The extrinsic fluorescence spectra of the HSA (4.5 µM) were measured in the absence and presence of 0, 40, 70, and 120 µM of the Pd(II) complex at both temperatures of 25 and 37 °C.

**CD Measurements**

CD spectra were recorded on an Aviv Spectropolarimeter model 215. Changes in the secondary structures of HSA were monitored in the far UV region (200 -260 nm) using 0.1 cm path length cells, with a resolution of 0.2 nm, scan speed of 20 nm min⁻¹, time constant of 2.0 s, 10 nm bandwidth, and sensitivity of 20 A°. The protein concentration in the experiments for the far UV region was 4.5 µM. The samples were prepared beforehand, and then added into a 0.1 cm quartz cell to be determined. Alterations in the secondary structure of the protein were investigated in the absence and presence of different concentrations of Pd(II) complexes (0, 40, 70, and 120 µM). The results were expressed in molar ellipticity [θ] (deg cm²dmol⁻¹) based on a mean amino acid residue weight of 113.7 (MRW). The molar ellipticity was determined as [θ] = (100 × MRW × θobs/c), where θobs is the observed ellipticity in degrees at a given wavelength; c is the protein concentration in milligrams per milliliter; and l is the length of the light path in millimeters. The CD software (CDNN) was used to predict the secondary structure of the protein according to the statistical method.

**Results and Discussion**

Proteins are considered to have intrinsic fluorescence due to the presence of amino acids, mainly tryptophan, tyrosine and phenylalanine. Intrinsic fluorescence of HSA mainly results from the sole tryptophan residue (Trp-214) in the hydrophobic cavity of subdomain IIA. The fluorescence of Trp-214 may change when HSA interacts with other molecule, which is a common response to conformational transition, subunit association, or substrate binding [17]. Figure 1 shows changes in fluorescence emission spectra of HSA upon addition of different concentrations of Pd(II) complex at 37 °C. It is observed that increasing the concentration of the complex leads to a reduction in the fluorescence signal of Trp-214. This gives a primary indication of interaction of Pd(II) complex with HSA. However, the maximum emission wavelength does not change for a fixed concentration of HSA indicating binding of complex to HSA quenches the intrinsic fluorescence of the protein without altering the local dielectric environment.

![Figure 1: Fluorescence titration curve of HSA (4.5 µM) with the But Pd(II) complex in 5 mM NaCl solution, pH 7.4, at 37°C.](image1.png)

![Figure 2a: Stern-Volmer plot for quenching Pd(II) complex to HSA in 5 mM NaCl solution at 25 (●) and 37°C (○).](image2a.png)

![Figure 2b: Best linear plot of F/F₀ versus 1/[Q] according to equation 2 at 25 (●) and 37°C (○).](image2b.png)
A quenching process can be usually induced by a dynamic or a static quenching mechanism. Both mechanisms can be distinguished by their differing dependence on temperature. The quenching rate constants are expected to decrease with increasing temperature for static quenching. In contrast, the reversed effect was observed for a dynamic quenching. Higher temperature leads to the dissociation of weakly bound complexes owing to a faster molecular diffusion. As a result, larger amounts of molecules are collisionally quenched and the quenching rate constant will increase with raising temperature [18].

In order to confirm the quenching mechanism, the fluorescence quenching data are analyzed by the Stern-Volmer equation:

\[
\frac{F_0}{F} = 1 + K_{SV} [Q]
\]

where \(F_0\) and \(F\) are the fluorescence intensities in the absence and presence of quencher (Pd(II) complex), respectively. \(K_{SV}\) is the Stern-Volmer quenching constant, and \([Q]\) is the concentration of the quencher. The plots of \(F_0/F\) versus \([Q]\) show a positive deviation (Figure 2a). The non-linear of the Stern-Volmer plot is either the result of a combination of static plus dynamical quenching or due to the higher concentration of the ligands around the fluorophor.

To estimate the values of Stern-Volmer quenching constants at both temperatures, the Modified Stern-Volmer equation was considered:

\[
\frac{F_0}{F_0 - F} = \frac{1}{f_a K_{SV} [Q]} + \frac{1}{f_a}
\]

where \(f_a\) is the fraction of the initial fluorescence, which is accessible to the quencher. The value of \(f_a\) refers to the fraction of fluorescence accessible to quenching, which need not be the same as the fraction of tryptophan residue accessible to quenching.

The dependence of \(F_0/F\) on the reciprocal value of the quencher concentration (1/[Q]) is a linear plot. The values of \(f_a\) and \(K_{SV}\) were obtained from the values of intercept and slope, respectively. The values of \(f_a\) for HSA in the presence of the complex were found 1.16 at 25°C and 1.22 at 37°C. It means that 98.84% (at 25°C) and 98.78% (at 37°C) of fluophores of protein are affected by Pd(II) complex (Figure 2b). The Stern Volmer quenching constant was calculated and listed in Table 1. As seen in Table 1, values of Stern Volmer quenching constants \(K_{SV}\) are explicitly dependent on the temperature. On the other hand, by increasing the temperatures from 25 to 37°C the value of \(K_{SV}\) is decreased indicating the highest contribution of static mechanism of quenching.

Table 1. Binding parameters of Pd(II) complex to HSA at two temperatures of 25 and 37 °C

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>n</th>
<th>(K_a) (M(^{-1}))</th>
<th>(K_{SV}) (μM(^{-1}))</th>
<th>(f_a)</th>
<th>(\Delta G^\circ) (kJ/mol)</th>
<th>(\Delta H^\circ) (kJ/mol)</th>
<th>(\Delta S^\circ) (J/mol. K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1.74</td>
<td>208449088</td>
<td>0.06</td>
<td>1.12</td>
<td>-47.4</td>
<td>-40.28</td>
<td>+24.23</td>
</tr>
<tr>
<td>37</td>
<td>1.70</td>
<td>110917482</td>
<td>0.04</td>
<td>1.19</td>
<td>-47.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3a. Best linear plot of \(\log F_0/F\) versus \(\log [Pd]\) according to equation3 at 25 (●) and 37°C (○). Values of \(K_a\) and n of binding of Pd complex to HSA can be obtained from the slope and the vertical intercepts, respectively.

Figure 3b. ANS Fluorescence curve of HSA with Pd(II) complex of Butyl in the presence of ANS (200 μM) at 25°C.
**Analysis of Binding Equilibria**

Fluorescence quenching data were analyzed to obtain various binding parameters for the interaction of Pd(II) complex and HSA. The apparent association constant \( K_a \) and number of binding site \( n \) were calculated using the equation [19]:

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K_a + n \log [Q] \tag{3}
\]

The plot of log \([(F_0 - F)/F]\) versus log \([Q]\) gave a straight line. The values of \( K_a \) and \( n \) were thus obtained from the intercept on the Y-axis and the slope, respectively (Figure 3a). The calculated values of \( K_a \) and \( n \) for Pd(II) complex at two temperatures were listed in Table 1. As shown in Table 1, the value of \( n \) at different temperatures of 25 and 37 °C was obtained approximately equal to 2, which indicated that there are two binding sites for this complex on HSA. Moreover, a decrease in binding constants with increasing the temperature suggests the involvement of weak interacting forces during complexation process taking place between the Pd(II) complex and HSA, which become even weak with increasing the temperature [20]. The association constant calculated for the complex-HSA suggests a high affinity Pd(II) complex-HSA binding, compared to the other strong ligand protein complex [21].

**Binding Modes**

In view of the dependence of binding constant on the temperature, thermodynamic parameters at different temperatures were analyzed to characterize the acting forces dominating the interaction. Generally the molecular forces contributing to the proteins interactions with small molecular substrates may be hydrogen bond, Vander Waals, electrostatic interaction and hydrophobic interaction [22]. Thermodynamic parameters, enthalpy change \( \Delta H^0 \) and entropy change \( \Delta S^0 \) of the binding reaction are the main evidences for confirming binding modes. The enthalpy \( \Delta H^0 \), free energy \( \Delta G^0 \), and entropy \( \Delta S^0 \) changes are calculated according to the following equation [23]:

\[
\ln K = \frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \tag{4}
\]

\[
\Delta G^0 = \Delta H^0 - T \Delta S^0 = -RT \ln K \tag{5}
\]

where \( R \) is the gas constant.

The plot of \( \ln K \) versus \( 1/T \) gave a straight line. The calculated \( \Delta H^0 \), \( \Delta S^0 \) and \( \Delta G^0 \) were listed in Table 1.

Ross and Subramanian [24] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes. For typical hydrophobic interactions, both \( \Delta H^0 \) and \( \Delta S^0 \) are positive, while these are negative for Vander Waals forces and hydrogen bond formation in a low dielectric media [25]. Moreover, the specific electrostatic interaction between ionic species in an aqueous solution is characterized by a positive \( \Delta S^0 \) value and a negative \( \Delta H^0 \) value (very small, almost zero) [25].

The negative value of \( \Delta G^0 \) indicates that the interaction of Pd(II) complex with HSA is a spontaneous process, and the negative \( \Delta H^0 \) and positive \( \Delta S^0 \) indicate that the binding is exothermic and entropy driven, and are indicative of an electrostatic interaction with partial immobilization of the protein and the ligand. This occurs in an initial step involving hydrophobic association that results in a positive \( \Delta S^0 \) value [26].

**ANS Fluorescence Studies**

ANS is an extensively utilized fluorescent probe for the characterization of protein hydrophobic pockets.

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![Figure 4](image-url)

**Table 2.** Changes in secondary structure of HSA in the absence and presence of various concentrations of palladium complex at 25°C

<table>
<thead>
<tr>
<th>[palladium] (μM)</th>
<th>%α-helix</th>
<th>%β-sheet</th>
<th>%random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55.3</td>
<td>22.8</td>
<td>21.8</td>
</tr>
<tr>
<td>40</td>
<td>46.7</td>
<td>26.8</td>
<td>26.5</td>
</tr>
<tr>
<td>70</td>
<td>35.3</td>
<td>31.5</td>
<td>33.1</td>
</tr>
<tr>
<td>120</td>
<td>34.3</td>
<td>32.6</td>
<td>33.8</td>
</tr>
</tbody>
</table>
ANS binds in the hydrophobic subdomains (II₉ and III₇) of HSA [27]. ANS fluorescence studies were carried out at two temperatures of 25 and 37 °C. In this study, at first, we incubated different concentrations of complex and HSA for three minutes, and then ANS was added. After five minutes, the emission of ANS was recorded and represented in Figure 3b. Results of ANS fluorescence studies showed that interaction of the complex decreases the ANS fluorescence emission, meaning that complex causes conformational changes that lead to the decrease of nonpolar or hydrophobic surfaces of the protein at both temperatures.

**CD Studies**

CD spectra can sensitively monitor conformation changes in the protein upon interaction with ligand [28]. As shown in Fig. 5, HSA exhibits two negative bands in the ultraviolet region at 208 nm (π→π*) and 222 nm (→π*), which are characteristic of the α-helical structure of proteins. The most abundant content of HSA is α-helical (almost about 60%) and there is no β-sheet content [29].

The far-UV CD spectrum of HSA in the absence and presence of different concentrations of Pd(II) complex (0, 40, 70 and 120 μM) at temperature of 25 °C is shown in Figure 4. As an evident from Fig. 4, the band intensities of HSA at 208 and 222 nm decrease with negative cotton effect by the binding of Pd(II) complex without causing any significant shift of the peaks, which indicate considerable changes in the protein secondary structure. The α-helical content of HSA decreases while we can see a significant increasing in the content of disorder structure and β-sheet contents of the protein at both temperatures (see Table 2).

Fluorescence intensity studies showed that Pd(II) complex has a strong ability to quench the intrinsic fluorescence of HSA. The number of binding sites and the association binding constants of Pd(II) complex were calculated at 25 and 37 °C. Also, the negative Δ𝐻 and positive Δ𝑆 values for the interaction between Pd(II) complex and has, using vant’s Hoff equation, showed that the electrostatic interaction has a major role in the binding process and the negative value of Δ𝐺 indicated that the interaction of Pd(II) complex with HSA is a spontaneous process. The quantitative analysis of CD spectra represented that Pd(II) complex induces significantly alterations in the secondary structure of protein via decreasing in the content of α helical structure of the protein. From the above results, it can be concluded that the binding of newly designed drug (Pd(II) complex) to the blood carrier protein of HSA resulted in significant changes on the structure and conformation of the protein, which may be considered as side effects of this new synthesized drug. HSA is a flexible molecule and binding of a drug often affects the simultaneous binding of other drugs. Consequently, the study of the interaction between HSA and drugs is imperative and can provide much information on the drug action, which was investigated in the present study.

**Acknowledgements**

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**References**


