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Interaction of Cu(II) 3,5-diisopropylsalicylate with human serum albumin – an evaluation of spectroscopic data

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The copper(II) complex of 3,5-diisopropylsalicylate is a lipophilic water-insoluble binuclear complex, $Cu(II)_2(3,5\text{-}DIPS)_4$, that has attracted interest because of a wide range of pharmacological activities. This study was undertaken to examine bonding interactions between the complex and human serum albumin (HSA) to help elucidate the mode of transport of the complex *in vivo*. Electron paramagnetic resonance, numerical magnetic resonance and UV-visible absorption spectroscopic studies were performed using 200 μM aqueous solutions (pH 7.5) of HSA to which had been added up to three molar equivalents of $CuCl_2$, $CuSO_4$, or $Cu(II)_2(3,5\text{-}DIPS)_4$. Both EPR and UV-visible spectra demonstrated the presence of more than one copper bonding site on HSA, and proton NMR spectra showed that the 3,5-DIPS ligand is also bonded to HSA. These results indicate that there is no observable direct coordination of the ligand to copper in the presence of HSA, and that the majority of the copper and 3,5-DIPS bond to HSA at separate sites. Addition of solid $Cu(II)_2(3,5\text{-}DIPS)_4$ to HSA at pH 7.5 similarly resulted in spectra that suggest that there are no ternary Cu(II)(3,5-DIPS), $Cu(II)(3,5\text{-}DIPS)_2$, or $Cu(II)_2(3,5\text{-}DIPS)_4$ complexes formed with HSA. It is concluded that any ternary complexes formed in the presence of HSA are below the spectroscopic detection limits and represent less than 5% of the total copper.

Keywords: copper(II) 3,5-diisopropylsalicylate, EPR, human serum albumin, NMR

Introduction

The copper(II) complex of 3,5-diisopropylsalicylic acid, $Cu(II)_2(3,5\text{-}DIPS)_4$, has attracted much interest because of its radioprotectant, radiorecovery, anti-inflammatory, antiulcer, antineoplastic, antimutagenic, anticarcinogenic, anticonvulsant, antidiabetic, and analgesic activities as well as its reduction of ischemia-reperfusion injury produced with nontoxic nanomole doses in animal models of these disease states (Sorenson 1989, Sorenson *et al*. 1993, Baquial & Sorenson, 1995). The tan solid, which is the form of this complex used to formulate administered doses in most pharmacological tests, has been shown to be an aquated binuclear carboxylate-bridged complex, $Cu(II)_2(3,5\text{-}DIPS)_4(H_2O)_2$ (Greenaway *et al*. 1988). This extremely lipophilic water-insoluble complex is normally administered subcutaneously or orally in the form of a suspension in a non-ionic vehicle, Tween-80 or propylene glycol and 1.4% polyvinylalcohol in saline. A green binuclear form, $Cu(II)_2(3,5\text{-}DIPS)_4(solvent)_2$, is also known. This has two solvent molecules bonded apically, one to each copper. In polar organic solvents, the tan and green binuclear complexes dissociate into a mononuclear complex, $Cu(II)(3,5\text{-}DIPS)_2(solvent)_2$, in which the copper is coordinated to two bidentate 3,5-DIPS ligands through their carboxylic and phenolic oxygen atoms, and two solvent molecules (Greenaway *et al*. 1988). While a great deal of mechanistic data, including down-regulation of nitric oxide synthase

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(Baquial & Sorenson 1995), accounting for the pharmacological activities of this complex have been published (Sorenson 1989, Sorenson *et al.* 1993, Baquial & Sorenson, 1995), the form or forms of this complex undergoing tissue distribution *in vivo* remain unknown and the important questions of stability and fate of the administered complex *in vivo* remain unanswered.

Human serum albumin, HSA, is an abundant blood plasma protein known to enter into bonding interactions with drugs following absorption and to assist in transporting them to all tissues. Concentrations of HSA in plasma range between 3.5 and 5.0 g/100 mL (0.5 to 0.8 mM). This protein contains a large number of acidic and basic amino acids, 117 and 99 respectively, which imparts a large net negative charge of –18 at physiological pH (7.4). In addition to these functional groups HSA contains 1 thiol, 17 disulfides (cystinyl), and 62 aromatic amino acids including 15 histidines. These functional groups offer many potential bonding sites for copper, 3,5-DIPS, and copper-3,5-DIPS compounds. Absorption of lipophilic drugs to hydrophobic bonding sites on HSA and the water solubility of HSA at physiological pH account for the observation that many water-insoluble drugs 'dissolve' in plasma (Rowland & Tozer 1995), as does $Cu(II)_2(3,5\text{-}DIPS)_4$ in aqueous solutions of HSA (Shuff *et al.* 1992). A large portion of drug transport in plasma can be accounted for by the observation that most drugs actually bond to HSA and the pharmacological efficacy of some drugs is dependent on drug-albumin bonding interactions that enable the drug to be distributed to all tissues. A consequence of this broad distribution is that most of an administered drug, which may be greater than 99%, is lost due to distribution to tissues that are not affected by a disease process, chemical transformations in plasma, and/or metabolism in the liver to inactive forms of drug, or excretion (Rowland & Tozer 1995). While very little of an administered drug actually reaches cells of disease-affected tissue, this small percentage represents many molecules of drug.

Since therapeutic effects of $Cu(II)_2(3,5\text{-}DIPS)_4$ exceed those of Cu(I) and Cu(II) salts or 3,5-DIPS alone, which in some models may be devoid of any therapeutic effect (Sorenson 1989, Sorenson *et al.* 1993, Baquial & Sorenson, 1995), it has been suggested that a ternary complex of Cu(II), 3,5-DIPS, and HSA may account for distribution to affected tissues via blood (Shuff *et al.* 1992). Electon paramagnetic resonance (EPR) studies of pyruvaldehyde bis(N^4-methyl-thiosemicarbazonato) copper(II), Cu PTSM, which showed that the complex remains intact when bonded to HSA (Yuan *et al.* 1996), provide a precedent for such a suggestion. Thus, it seemed reasonable to suggest that a Cu-3,5-DIPS compound, such as Cu(3,5-DIPS), $Cu(II)(3,5\text{-}DIPS)_2$ and/or $Cu(II)_2(3,5\text{-}DIPS)_4$, is transported as an albumin-bonded Cu-3,5-DIPS-HSA ternary complex and that the small fraction of the intact drug ultimately reaching the site of pharmacological action accounts for its effects. UV-visible spectrophotometric (Shuff *et al.* 1992), circular dichroism (CD) and numerical magnetic resonance (NMR) (Bligh *et al.* 1992) studies confirmed that 3,5-DIPS is bonded to albumin in plasma, but neither study showed whether the copper remains bonded to the 3,5-DIPS ligand in the presence of HSA. The present study investigated the ternary formation hypothesis using EPR to probe the copper ligand environment, UV-visible spectrophotometry to monitor the Cu(II) d-d and the aromatic π-π^* transitions of the ligand, and NMR to search for free 3,5-DIPS ligand, approaches that are more structure- and species-specific than previous absorption spectrophotometric work (Shuff *et al.* 1992) utilizing electronic transitions of the organic ligand that are only slightly perturbed by bonding to Cu(II).

Materials and methods

Experimental procedures have been previously detailed (Shuff *et al.* 1992). A stock 1 mM HSA solution (pH 7.5) was prepared as described therein. Solutions were typically 0.2 mM in HSA and between 0.02 and 0.6 mM in Cu(II). Aqueous 1 mM solutions of $CuSO_4$ or $CuCl_2$, prepared with deionized water, were used to make additions of inorganic copper to aqueous HSA solutions. Because of its low solubility in water, aqueous 20% ethanol solutions of $Cu(II)(3,5\text{-}DIPS)_2$ were prepared by first dissolving solid $Cu(II)_2(3,5\text{-}DIPS)_4$ in ethanol and then adding the ethanol solution to four volumes of deionized water to achieve a final concentration of 1 mM. No buffer was used. The pH was adjusted using small amounts of HCl(aq) or NaOH(aq).

EPR spectra were acquired using a Varian E-9 spectrometer operating at 9.1 GHz with 100 kHz modulation, and interfaced to a personal computer by means of an interface and data acquisition program obtained from Scientific Software Services, (Bloomington, IN). A flat spin-free quartz cell was used to obtain spectra of aqueous solutions at 295 K and a spin-free quartz tube was used to obtain spectra of frozen solutions at 110 K. The frequency was measured using a Hewlett-Packard frequency counter and the magnetic field was calibrated using a Magnion gaussmeter. Absorption spectra were measured using a Hewlett-Packard Model 8452A diode array spectrophotometer. Proton NMR spectra were obtained with a Bruker AC 200 spectrometer operating at 200 MHz.

Results

EPR spectroscopy confirmed that Cu(II)$_2$(3,5-DIPS)$_4$ has low solubility ($< 2 \times 10^{-4}$ M) in aqueous solution (pH 7.5) and that the small amount that does dissolve dissociates into a monomeric species with $g_\parallel = 2.366$ and $A_\parallel = 0.0146$ cm^{-1} that has previously been identified as Cu(II)(3,5-DIPS)$_2$(H$_2$O)$_2$ (Greenaway *et al.* 1988).

When $CuCl_2$(aq) or $CuSO_4$(aq) was added to solutions of HSA at pH 7.5, more than one copper species was observed by EPR spectroscopy. The major species, observed when up to one molar equivalent of copper was added, has $g_\parallel = 2.17$ and $A_\parallel = 0.022$ cm^{-1}. Both these parameters and the substantial nitrogen superhyperfine structure on the $g_\perp$ peak, indicate a tetragonally-distorted octahedral geometry with a primarily nitrogen ligand environment for the copper (Peisach & Blumberg 1974, Sawada *et al.* 1996). When more than one molar equivalent of Cu(II) was added to HSA, two additional species, one with $g_\parallel = 2.29$ and $A_\parallel = 0.017$ cm^{-1} and one with $g_\parallel = 2.31$ and $A_\parallel = 0.015$ cm^{-1}, were also present.

Identical EPR spectra were obtained when Cu(II)$_2$(3,5-DIPS)$_4$ dissolved in ethanol/water was the source of copper instead of $CuSO_4$ or $CuCl_2$. To ensure that the result was not an artifact due to the presence of ethanol, 0.2 mg of Cu(II)$_2$(3,5-DIPS)$_4$ solid was vortex stirred with 2 mL of 0.2 mM HSA (pH 7.5) to give a solution equimolar in copper and HSA, whereupon the solid slowly dissolved once again resulting in an identical EPR spectrum. Although solid Cu(II)$_2$(3,5-DIPS)$_4$ dissolves only slowly in a solution of HSA at pH 7.5, when it does so most of it must rapidly dissociate since we were unable to detect any intermediate species. There were no differences between EPR spectra of samples in liquid solution at 25 °C or in frozen solution at 110 K (at pH 7.5), except for a small increase in linewidth at the higher temperature attributed to a change in the spin-lattice relaxation rate.

These results indicate that the primary copper bonding sites on HSA are independent of the source of copper and involves only ligand atoms from HSA. At least 95% of the Cu(II) from Cu(II)$_2$(3,5-DIPS)$_4$ is strongly bonded to the HSA and not simultaneously to 3,5-DIPS. This observation is consistent with the reported formation constant of copper with the primary bonding site on HSA being significantly larger than with 3,5-DIPS (Peters & Blumenstock 1967, Jackson *et al.* 1978, Masuoka *et al.* 1993, Masuoka & Saltman 1994).

Visible absorption spectra were consistent with these results. When either Cu(II)(aq) or a solution of Cu(II)$_2$(3,5-DIPS)$_4$ was added to HSA at pH 7.5, an absorption peak was observed at 525 nm for solutions containing up to one molar equivalent of Cu(II). At higher Cu:HSA ratios, the peak was indistinct, consistent with the bonding of Cu(II) to additional non-specific sites on HSA. Similarly, when solid Cu(II)$_2$(3,5-DIPS)$_4$, which is very insoluble in water, was added to a stirred solution of HSA (pH 7.5), the solid dissolved slowly and a peak appeared at 525 nm. Solutions of Cu(II)$_2$(3,5-DIPS)$_4$ (without HSA) have a peak near 620 nm at pH 7.5 (Greenaway *et al.* 1988). Kinetic studies were performed by adding an ethanol/water solution of Cu(II)$_2$(3,5-DIPS)$_4$ to the HSA solution, and monitoring the absorption spectrum at 25 °C. A peak appeared at 525 nm and maximum absorbance was attained within three min.

Proton NMR spectra were obtained of HSA before and after addition of either 3,5-DIPS or Cu(II)$_2$(3,5-DIPS)$_4$. Results were similar in both cases, with the normally prominent resonances at 2.2 p.p.m. due to the methyl groups of the free 3,5-DIPS ligand being very broad, even when excess 3,5-DIPS or its copper complex were added. The breadth of the resonances is consistent with bonding of the 3,5-DIPS ligand to HSA (Bligh *et al.* 1992). Bligh *et al.* have previously shown that free and HSA-bonded 3,5-DIPS are not in fast exchange, thus this result indicates that there is little free 3,5-DIPS in solution.

Discussion

HSA is known to have one strong-bonding site for Cu(II) (Sarkar & Wigfield 1968, Appleton & Sarkar 1971, Lau & Sarkar 1971 1975, Laussac & Sarkar 1980, Rakhit & Sarkar 1981, Sarkar *et al.* 1983, Zgirski & Frieden 1990, Masuoka *et al.* 1993, Sadler *et al.* 1994) that, between pH 6.5 and 11, has EPR parameters of $g_\parallel = 2.169$ and $A_\parallel = 0.0216$ cm^{-1}, consistent with coordination by four nitrogen ligand atoms (Peisach & Blumberg 1974, Rakhit & Sarkar 1981, Sawada *et al.* 1996). The coordinating ligands have been ascribed to the α-amino nitrogen of the amino-terminal aspartate, two intervening peptide nitrogens, and the imidazole ring of the histidine residue in the third position (Peters & Blumenstock 1967) with an axial carboxylate from an aspartate residue (Sarkar & Wigfield 1968, Laussac & Sarkar 1980, Rakhit & Sarkar 1981, Sarkar *et al.* 1983) or an axial amine from a lysine residue (Sadler *et al.* 1994). Our EPR results agree with these data for the major species produced upon addition of up to

one molar equivalent of copper for both simple Cu(II) salts and $Cu(II)_2(3,5\text{-DIPS})_4$. Upon addition of more than one equivalent of Cu(II), we observe additional Cu(II)-HSA species that are not affected by the presence of 3,5-DIPS and in which the copper is coordinated through a combination of nitrogen and oxygen ligand atoms from the protein (Peisach & Blumberg 1974). Similar weaker bonding sites have been previously observed on bovine serum albumin (Peters & Blumenstock 1967, Zgirski & Frieden 1990). The EPR spectrum of the binuclear $Cu(II)_2(3,5\text{-DIPS})_4$ complex, which is very different from that of mononuclear forms of Cu(II) (Greenaway *et al*. 1988), was not observed in the presence of HSA, indicating that at least 99% had dissociated into mononuclear forms of Cu(II).

Previous studies have shown a 525 nm absorption band due to the strong copper-bonding site in HSA (Peters & Blumenstock 1967, Appleton & Sarkar 1971, Lau & Sarkar 1975). We also observed this band regardless of the origin of the copper. In addition, when the copper:HSA ratio exceeded 1:1, we observed a band due to other, weaker Cu(II)-bonding sites analogous to the 650–700 nm band reported for bovine serum albumin (Masuoka & Saltman 1994), and consistent with our observation of EPR signals from additional copper species at these ratios.

Both the EPR and the absorption spectroscopic results show that the copper bonded to HSA is spectroscopically unaffected by the presence of 3,5-DIPS. It is of interest to determine how the stability of CuHSA and its redox potential are affected by the HSA-bonded 3,5-DIPS. While it is difficult to experimentally obtain accurate stability constants for copper-protein complexes, it has been shown that the stability constants have a high correlation with $g_\parallel$ (Sawada *et al*. 1996) for CuN_4 metal-ligand environments such as our EPR data indicate exist for the Cu-3,5-DIPS-HSA system. The fact that we observed identical $g_\parallel$ values for CuHSA in the presence and absence of 3,5-DIPS thus indicates that 3,5-DIPS has no effect on the CuHSA stability constant, which can be estimated from the EPR data as log $K_\beta = 28$ (Sawada *et al*. 1996), where $K_\beta = [\text{CuHSA}]/[Cu^{2+}][HSA_{dep}]$ and HSA_{dep} indicates HSA that is already deprotonated. Previous workers have found log $K'_\beta = 10$–12 (Zgirski and Frieden 1990, Masuoka and Saltman 1994) where K'_β differs from K_β by the protonation constants for the copper-coordinating peptide nitrogens of HSA, which are not accurately known. In addition, similar correlations between reduction potential (E^o) and $g_\parallel$ indicate an extremely negative redox potential of –800 mV relative to the standard hydrogen electrode for CuHSA in the presence or absence of 3,5-DIPS. Such a potential suggests that the Cu(II)HSA complex is very difficult to reduce to Cu(I)HSA. HSA is generally thought to be involved in transport of the copper 3,5-DIPS complex and not in the mechanism of action of the drug (which may involve a redox mechanism). The extremely negative potential of Cu(II) when bonded to HSA suggests that the mechanism of release of Cu(II) from HSA does not involve a redox reaction, at least in the absence of a pH change or a tetrahedral distortion of the copper-ligand geometry (Yokoi and Addison 1977), which are both factors that may alter reduction potentials and stability constants.

Computer modelling studies based on stability constants for Cu(II)/3,5-DIPS species have indicated that almost complete dissociation of $Cu(II)_2(3,5\text{-DIPS})_4$ is to be expected in serum albumin (Jackson *et al*. 1978, Fiabane & Williams 1978). It has been suggested that various Cu(II) complexes bond to albumin in the form of a ternary complex at the primary bonding site on albumin (Rakhit & Sarkar 1981, Zgirski & Frieden 1990), although there is little evidence for this and recent results have not supported this hypothesis, at least for copper histidinate (Masuoka & Saltman 1994). Our results have utilized EPR parameters and the position of the copper d–d absorption band, which are both very sensitive to the copper ligands, and we conclude that there is no spectroscopic evidence for ligation of 3,5-DIPS to the copper in the presence of HSA. However, since EPR parameters and positions of the d–d bands of tetragonally-elongated copper compounds are not sensitive to the axial ligands, which are farther away due to a Jahn-Teller distortion, our results do not exclude the possibility that 3,5-DIPS may be weakly coordinated to the Cu(II), although significant bonding as a unidentate ligand is unlikely for 3,5-DIPS. The limits of discrimination between EPR and electronic spectra for two copper(II) species also do not allow us to eliminate the possibility that as much as 5% of the $Cu(II)(3,5\text{-DIPS})_2$ or 1% of the $Cu(II)_2(3,5\text{-DIPS})_4$ may remain intact either in solution or bonded to HSA.

Serum albumin is known to bond strongly to small negatively-charged hydrophobic organic ligands, including 3,5-DIPS, at two sites that have been identified by crystallographic studies (Carter & He 1990, 1992) as being within subdomains IIA and IIIA, and not near the copper-bonding N-terminus.

To investigate whether HSA resulted in the release of 3,5-DIPS from $Cu(II)_2(3,5\text{- DIPS})_4$, we performed NMR studies of this system. 3,5-DIPS

ligand has a strong methyl resonance at 1.2 p.p.m. Previously, Bligh *et al*. (1992) had performed similar studies with human blood plasma and Cu(II)$_2$(3,5-DIPS)$_4$, and found that the 3,5-DIPS methyl resonances were too broad to be observed in the presence of plasma, indicating bonding of the ligand to HSA. We obtained the same result for both Cu(II)$_2$(3,5-DIPS)$_4$ and for 3,5-DIPS ligand in the presence of HSA, indicating that not more than a small and non-NMR-detectable amount of 3,5-DIPS is free in solution. Although we conclude that the bonding of 3,5-DIPS does not affect copper bonded to HSA, we were unable to determine whether the bonding of 3,5-DIPS to HSA was affected by the presence of copper.

Our investigation has addressed the question of whether the copper compound that is administered in pharmacological studies remains (a) fully intact, (b) partially dissociated but with the copper still bonded to the organic ligand in some possibly new way, or (3) fully dissociated where the copper and the organic ligand are no longer bonded to one another. Our results show no evidence for a ternary Cu(II)-3,5-DIPS-HSA complex where the three components are all bonded to one another. Copper (II) and 3,5-DIPS both bond to HSA but apparently at different sites. Our conclusion is that at least 95% of the copper and 3,5-DIPS of Cu(II)$_2$(3,5-DIPS)$_4$ bond separately at different sites on HSA. The results contrast with those from a similar EPR study with pyruvaldehyde bis(N^4-methyl-thiosemicarbazonato) copper(II), CuPTSM, which was found to remain intact when bonded to HSA (Yuan *et al*. 1996).

The apparent dissociation of Cu(II)$_2$(3,5-DIPS)$_4$ in the presence of HSA raises questions about why the complex has been found to be more active in a variety of therapeutic model systems than copper or 3,5-DIPS alone. While the presence of a long-lived hydrophobic Cu(II)$_2$(3,5-DIPS)$_4$ species that decomposes only slowly would provide a possible explanation for the activity of the solid administered in non-solvating media (because of enhanced transport or absorption), the observation of therapeutic effects when copper/3,5-DIPS is administered by means of an ethanolic solution where decomposition to mononuclear species, Cu(II)(3,5-DIPS)$_2$, has already occurred suggests that the therapeutic effect may not be due to the binuclear Cu(II)$_2$(3,5-DIPS)$_4$ species.

Furthermore, our results indicate that most of the mononuclear species Cu(II)(3,5-DIPS)$_2$ also rapidly dissociates in the presence of HSA. The possibility remains that the reported therapeutic actions of Cu(II)$_2$(3,5-DIPS)$_4$ may be due to a small proportion of the mononuclear or binuclear complex that can bond intact to HSA and be transported to the site of action. That is, an effective antiinflammatory dose is 1 μmol per kg of body mass, which represents 10^{19} molecules per 70 kg adult. If only 0.001% of this dose were distributed throughout the body, which has an estimated 10^{12} cells, there would be 100 molecules for every cell in the adult body. Since there is always vasodilation at the site of disease-affected cells and greater perfusion of the affected cells by plasma filtrate containing HSA, the concentration of drug in the environment of disease-affected cells would actually be greater than this, so that even smaller daily doses ranging down to 0.7 μmol/adult could provide 10 molecules per cell. It is also possible that either HSA delivers copper and 3,5-DIPS to the same cellular site or that the administration of a complexed form of copper is only important in facilitating absorption and that other chelates or complexes formed with ligands in plasma or within cells, such as Cu-thionein (Hartmann *et al*. 1993), copper-dependent enzymes (Sorenson 1989), and copper glutathione (Freedman & Peisach 1989, Corazza *et al*. 1996) following ligand exchange, account for the pharmacological effects of the administered complex.

Our work provides no evidence in support of the hypothesis of a ternary Cu-3,5-DIPS-HSA complex as the active therapeutic transporting agent, but neither are we able to completely disprove the hypothesis. It is, however, clear that future investigations of the hypothesis will require detection of very low-levels of ternary complexes in the presence of substantially higher concentrations of their components.

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