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The sub-picomolar Cu²⁺ affinity of human serum albumin

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Dedication ((optional))

Abstract: The apparent affinity of human serum albumin (HSA) for divalent copper has long been the subject of great interest, due to its presumed role as the major Cu²⁺ binding ligand in blood and cerebrospinal fluid. Using a combination of electronic absorption, circular dichroism and room temperature electron paramagnetic resonance spectroscopies, together with potentiometric titrations, we competed the tripeptide GGH against HSA to reveal a conditional binding constant of $\log^{\circ}K_{\text{Cu(HSA)}}^{\text{Cu}} = 13.02 \pm 0.05$ at pH 7.4. This rigorously determined value of the Cu²⁺ affinity has important implications for the extracellular distribution of copper.

HSA is the most abundant serum protein in the blood and acts as a carrier for numerous cargo molecules including lipids, hormones, metabolites, drugs, and essential and toxic metals.^{1,2} It also contains an N-terminal site (NTS) for binding divalent copper ions, which is comprised of the amino-terminal copper and nickel (ATCUN) motif, comprising a H₂N-Xaa-Yaa-His sequence.^{3,4} Albumin is a part of an elaborate copper trafficking machinery consisting of two types of copper carriers.⁵ The first group are proteins that act as extracellular carriers in blood plasma represented by ceruloplasmin, albumin, and transcuprein. Transmembrane copper transporters such as human copper transporter 1 (hCtr1) protein make up a second group of proteins involved in shuffling copper from extracellular environment to the intracellular or to individual compartments within a cell. However, albumin is thought to be a major constituent of exchangeable pool of copper. Indeed, it has been suggested that albumin could transfer Cu(II) to an ATCUN site of hCtr1.⁶ Therefore it is of a great importance to know the exact binding constants in order to model possible interactions between HSA and other copper carriers.

A number of early estimates for the Cu²⁺ binding affinity of HSA placed the value in the range $11 < \log^{\circ}K_{\text{Cu(HSA)}}^{\text{Cu}} < 16$.⁷⁻⁹ Direct measurement of the stability constants of proteins with

very high metal binding affinity is challenging, because the concentration of free copper is extremely low at protein concentrations typically employed for measurements. Potentiometric methods represent the most accurate approach for determining stability constants; however, they are restricted to small peptides.¹⁰ Thus, the Cu²⁺ affinities of truncated N-terminal peptides such as DAH and DAHK have been studied in order to infer the corresponding affinity of HSA.^{11,12} Measurement of the binding constant of HSA itself requires a competition experiment with a well-characterized Cu²⁺ chelator.

The generally accepted conditional binding constant for HSA, using nitrilotriacetic acid (NTA) as a competing ligand, was determined to be $\log^{\circ}K_{\text{Cu(HSA)}}^{\text{Cu}} = 12.0$ at pH 7.4.⁹ However, a potential problem with that study was the possibility of ternary Cu(NTA)(N_{im}) complex formation with the numerous surface His residues of HSA, which would lower the apparent Cu²⁺ binding affinity of the ATCUN site. Here, we eliminate the possibility of confounding ternary complex formation by using H₂N-Gly-Gly-His-OH (GGH) as a competitor. Since GGH also contains an ATCUN motif, this ensures a simple equilibrium between just two tetradentate Cu²⁺ binding species, Cu(HSA) and Cu(GGH). Here, we rigorously determined the stability constants of GGH and used these to obtain the conditional Cu²⁺ binding constant for HSA in 100 mM NaCl at pH 7.4.

Despite sharing a very similar first coordination sphere, the spectra of Cu(HSA) and Cu(GGH) complexes could easily be distinguished. In the case of EPR spectra, each copper complex has a very different rate of molecular rotation, with the low molecular weight Cu(GGH) complex (330 Da) exhibiting a motionally-averaged spectrum and the heavy Cu(HSA) complex (66.5 kDa) yielding a broad spectrum approaching that typically observed from a frozen solution (Figure 1a, Figure S1). The presence of isosbestic points confirmed the presence of just two Cu²⁺ binding species. In the case of CD spectra, the presence of Cu(HSA) and Cu(GGH) could be monitored by shifts of *d-d* band intensities and extrema in the range of 490–570 nm (Figure 1b, Table S1).

Using GGH as a competitor, the binding constant for HSA can be expressed as a function of the Cu²⁺ affinity of GGH:

$$K_{\text{Cu(HSA)}}^{\text{Cu}} / K_{\text{Cu(GGH)}}^{\text{Cu}} = \frac{[\text{Cu(HSA)}] ([\text{GGH}]_{\text{T}} - [\text{Cu(GGH)}])}{[\text{Cu(GGH)}] ([\text{HSA}]_{\text{T}} - [\text{Cu(HSA)}])} \quad (1)$$

where [Cu(HSA)] and [Cu(GGH)] are measured by decomposition of experimental spectra (Figure 1c,d), and [HSA]_T and [GGH]_T are the total added concentrations of each ligand (see Supporting Information). Using equation 1 and the data in Figure 1e, the ratio of the conditional binding constants of HSA and GGH could be calculated and indicated that the affinity of HSA is higher than GGH by 0.805 ± 0.049 log units (Figure 1f).

To obtain the conditional Cu²⁺ binding affinity of HSA, we next determined the stability constants of Cu(GGH) species by potentiometric titrations, corroborated by UV-Vis and CD titrations (Figure 2). The peptide carries three protonating residues, namely the C-terminal carboxylate of His3, the imidazole side chain of His3, and the N-terminal amine.

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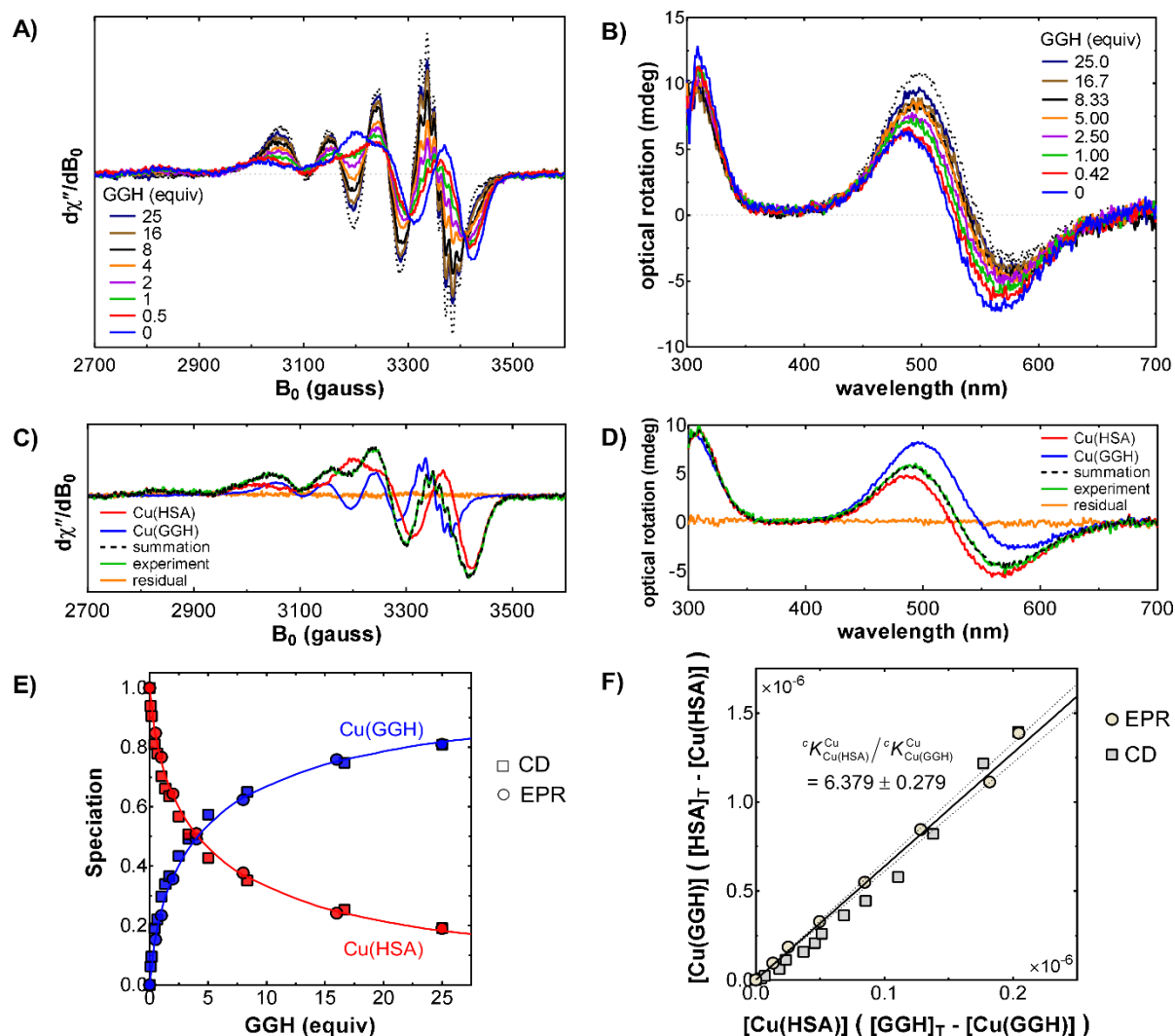


Figure 1. Determination of the relative conditional Cu^{2+} binding affinity of HSA and GGH. **(A)** EPR spectra and **(B)** CD spectra of Cu/HSA/GGH 0.83:1: n ($0 \leq n \leq 25$) in 100 mM NaCl, pH 7.4, 25°C ($[\text{HSA}]_{\text{T}} = 0.6$ mM). For clarity, only selected values of n are shown in panel B, but the complete dataset (see Figures S2 and S3) was used in panels E and F. Dotted lines in panels A and B correspond to the spectrum of Cu/GGH 0.83:1. **(C,D)** Example of the decomposition of EPR (panel C) and CD (panel D) spectra when $n = 1$. **(E)** Speciation diagram obtained by decomposition of all EPR (circles) and CD (squares) spectra (see Figures S1 and S2). The solid lines were calculated using the ratio of the binding constants derived from panel F and ${}^{\circ}K_{\text{Cu}(\text{GGH})} \gg 1/[\text{Cu}]_{\text{T}}$. **(F)** Determination of the relative Cu^{2+} binding strength of HSA and GGH. The margin of error derives from the 95% confidence interval (dashed lines).

Accordingly, potentiometric titrations yielded three protonation constants for the peptide alone, while three Cu^{2+} complex species, all with the 1:1 peptide: Cu^{2+} stoichiometry, were obtained by fitting the peptide/ Cu^{2+} titration curves. The protonation values are typical for the respective groups and nearly identical with those obtained previously for GGH under similar conditions.¹³ The $\log\beta$ values for the three complex species detected by us are within 0.2–0.4 log units from those reported earlier. We did not detect a CuL species (presumably 3N), which was also not seen by spectroscopy (see below).

The potentiometric species distribution is fully corroborated by spectroscopic titrations (Figure 2c). The existence of the minor CuHL complex is ascertained by the absorption trace at 685 nm, while the absence of the corresponding signal in CD spectra is indicative of a macroloop structure, with the Cu^{2+} ion coordinated to the N-terminal amine and an imidazole nitrogen.¹⁴ The CuH_{-1}L and CuH_{-2}L species share all spectroscopic properties, both being typical ATCUN complexes

isostructural with the N-terminal site of HSA.^{3,4,9,12,15} The pK_{a} of transition from CuH_{-1}L to CuH_{-2}L is consistent with the deprotonation of the non-bonding terminal carboxylate, elevated due to the decrease of the complex charge compared with the free peptide. The value of $\log {}^{\circ}K_{\text{Cu}(\text{GGH})}^{\text{Cu}}$ at pH 7.4 could now be easily derived from the potentiometric data using the competitiveness index (CI) method.¹⁶ The resulting value, 12.215 ± 0.005 at pH 7.4, is slightly but significantly lower than the value of 12.4 that can be derived from previously published data.¹¹

Substituting the above value of $\log {}^{\circ}K_{\text{Cu}(\text{GGH})}^{\text{Cu}}$ into equation 1, we obtain $\log {}^{\circ}K_{\text{Cu}(\text{HSA})}^{\text{Cu}} = 13.02 \pm 0.05$ at pH 7.4. This is one log unit higher than previously determined using the competitive ligand nitrilotriacetic acid (NTA).⁹ The most likely reason for this discrepancy is the omission of ternary $\text{Cu}(\text{NTA})(\text{N}_{\text{im}})$ complexes formed with surface His residues of HSA.^{17,18} The value of ${}^{\circ}K_{\text{Cu}(\text{HSA})}^{\text{Cu}}$ is also closer to the conditional constants calculated by us from potentiometric data of N-terminal peptides DAH-NH_2 ($\log {}^{\circ}K_{\text{Cu}(\text{DAH})}^{\text{Cu}} = 13.7$)¹¹ and DAH-K-NH_2 ($\log {}^{\circ}K_{\text{Cu}(\text{DAH-K})}^{\text{Cu}} = 13.8$).¹²

The MDH-am tripeptide, a model of the N-terminal site of hCtr1 binds Cu^{2+} with a conditional $\log {}^c K_{\text{Cu}(\text{MDH})}^{\text{Cu}} = 13.1$ at pH 7.4, and a longer peptide containing the first 14 residues has a comparable $\log {}^c K_{\text{Cu}(\text{Ctr1-14})}^{\text{Cu}} = 13.0$.^{6,19} The revised conditional binding constant for HSA is therefore comparable with that for hCtr1. Although this may appear to be at odds with a model in which HSA can exchange copper with hCtr1 via an affinity gradient at the extracellular surface, hCtr1 could still access around half of the Cu^{2+} bound to HSA, with Le Chatelier's principle ensuring a net Cu^{2+} flow from HSA to hCtr1 as copper is shuttled through its homotrimeric channel and released into the cytosol.

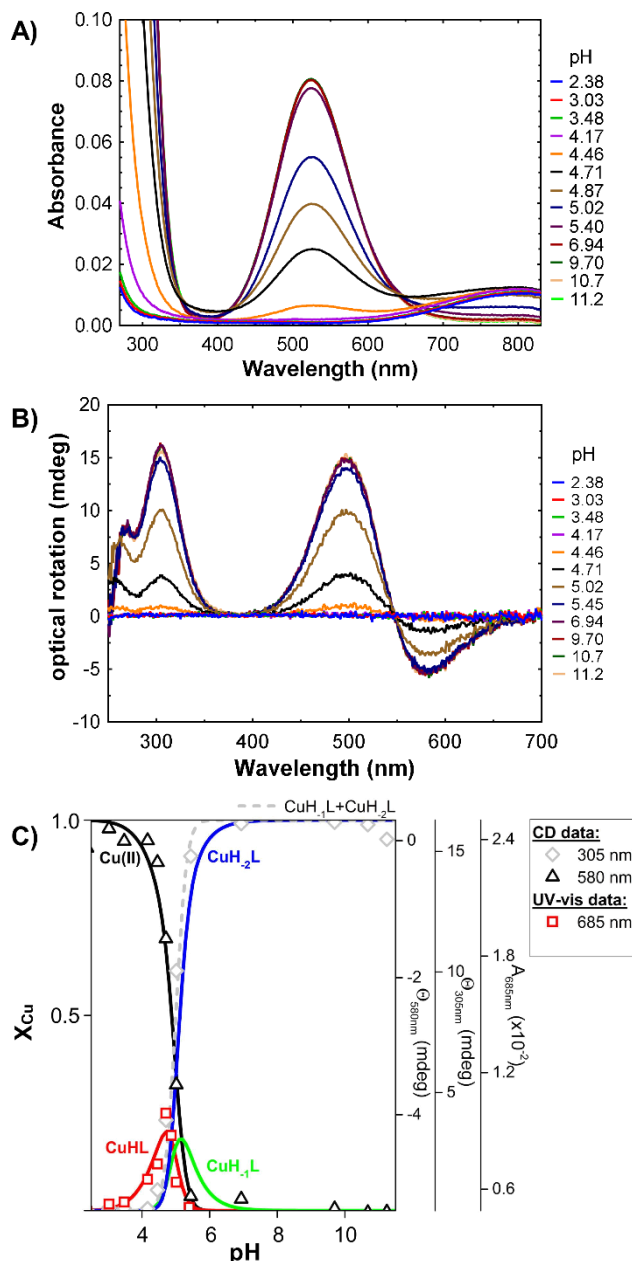


Figure 2. (A) UV-Vis, (B) CD, and (C) species distribution plot of the pH-dependent Cu^{2+} complexes formed by GGH (0.8 mM CuCl_2 , 0.95 mM GGH). The pH dependence of selected parameters from UV-Vis (685 nm) and CD (305 nm, 580 nm) is overlaid with the species distribution in panel C.

Table 1. Protonation and stability constants with their respective pK values, together with assignments of protonation events calculated from potentiometric data for Cu/GGH at 25 °C, $I = 0.1 \text{ M KNO}_3$.

	$\log \beta^{[a]}$	pK	Deprotonation event
LH ₃	17.778(3) ^[b]	2.86	COOH (C ^{term})
LH ₂	14.914(3)	6.85	N _{im} (His3)
LH	8.062(4)	8.06	NH ³⁺ (N ^{term})
CuHL	12.85(2)		2N: {N _{im} , N ^{term} }
CuL	Not detected		
CuH ₁ L	2.94(2)		4N: {N ^{term} , 2xN ⁻ , N _{im} }
CuH ₂ L	-1.746(6)	4.68	4N: {N ^{term} , 2xN ⁻ , N _{im} }; COOH (C ^{term})
Cl _{7,4} ^[c]	12.215(5)		

[a] $\beta(\text{Cu}_m\text{H}_n\text{L}) = [\text{M}_m\text{H}_n\text{L}]/([\text{M}]^m[\text{L}][\text{H}^+]^n)$

[b] Parentheses contain standard deviation of the final digit

[c] Competitivity index at pH 7.4 (equivalent to $\log {}^c K_{\text{Cu}(\text{GGH})}^{\text{Cu}}$)

The conditional Cu^+ binding constant for HSA in 50 mM HEPES buffer at pH 7.4 was previously determined to be a surprisingly high $\log {}^c K_{\text{Cu}^+(\text{HSA})}^{\text{Cu}^+} = 14.0 \pm 0.1$ by competition with colorimetric ligand BCA.²⁰ The new conditional Cu^{2+} binding constant of $\log {}^c K_{\text{Cu}^{2+}(\text{HSA})}^{\text{Cu}^{2+}} = 13.02$, as compared with 12.0, lowers the reduction potential of $\text{Cu}(\text{HSA})$ according to the Nernst equation²¹ by $0.0592 \log (\Delta {}^c K_{\text{Cu}^{2+}(\text{HSA})}^{\text{Cu}^{2+}} / {}^c K_{\text{Cu}^+(\text{HSA})}^{\text{Cu}^+}) = 60 \text{ mV}$. The reduction potential of $\text{Cu}(\text{HSA})$ remains unknown, although a correlation between the EPR spectral parameter g_{\parallel} and redox potential $E_{1/2}$ of various CuN_4 complexes has been used to infer a very low E^0 value;²² using our value of $g_{\parallel} = 2.174$ (Table S1), the ATCUN site would have $E^0 < 600 \text{ mV}$ vs SHE. On the other hand, reduction rates depend not only on redox potential but also the structure of the copper-bound protein in each oxidation state, and presently the structural reorganisation and intermediates required to achieve a Cu^+ state remain unclear. The accessibility of a Cu^+ site may determine the origin of the 'ascorbate oxidase' activity and ROS production observed for both $\text{Cu}(\text{BSA})$ ²³ and $\text{Cu}(\text{HSA})$.^{17,24} Reduction of $\text{Cu}(\text{HSA})$ by ascorbate has recently been proposed as a possible pathway for extracellular Cu^+ acquisition by hCtr1, bypassing the need for Cu^{2+} transfer to its N-terminal site prior to reduction.¹⁷ In view of the relatively low value of $\log {}^c K_{\text{Cu}^+(\text{Ctr1-14})}^{\text{Cu}^+} = 10.2 \pm 0.1$ measured for an N-terminal model peptide in 50 mM HEPES buffer at pH 7.4,²⁵ it remains unclear how the extracellular domain of hCtr1 would acquire Cu^+ from HSA.

Each of these observations highlights the importance of properly determining the copper binding constants of albumin. While ternary complex formation has previously hindered the elucidation of the true Cu^{2+} affinity of the N-terminal site of HSA, our robust value of ${}^c K_{\text{Cu}(\text{HSA})}^{\text{Cu}^{2+}}$ now provides a solid foundation to investigate the potential for biological co-ligands to modulate its apparent copper affinity *in vivo*.

Acknowledgements

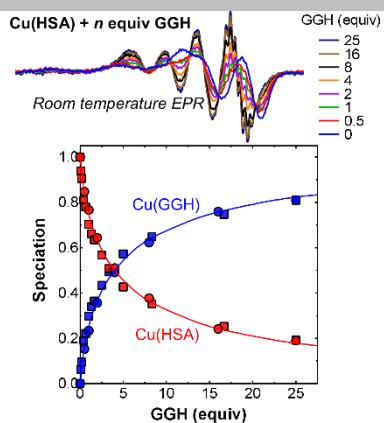
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Keywords: albumin • copper • metalloproteins • ATCUN • affinity

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COMMUNICATION

HSA is a physiological copper carrier, yet its Cu^{2+} affinity has remained uncertain. Using the tripeptide GGH as an accurate reference, we have eliminated the confounding effects of ternary complex formation with buffer molecules and other competing ligands to reveal a conditional affinity of $\log^{\epsilon} \kappa_{\text{Cu(HSA)}}^{\text{Cu}} = 13.02$ at pH 7.4.



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