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# Structural Characterization of a Gold/Serum Albumin Complex

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Supporting Information

**ABSTRACT:** The medicinal gold(III) dithiocarbamato complex $AuL_{12}$ forms a stable adduct with bovine serum albumin. The crystal structure reveals that a single gold(I) center is bound to Cys34, with the dithiocarbamato ligand being released. To the best of our knowledge, this is the first structure for a gold adduct of serum albumin.

Serum albumin (SA) is the most abundant serum protein performing crucial roles in the binding and blood transport of a variety of exogenous substances and drugs.[1,2] Notably, SA has been reported to be the carrier of several metal ions and metal-containing species in the bloodstream.[3,4] Although extensive studies have been carried out to characterize the interactions of SA with several metal-based drugs, until now very few structures have been reported for SA/metal drug adducts.[5,6] In particular, no structure has ever been reported for SA adducts with gold compounds.

$AuL_{12}$ (Figure 1) is a mononuclear gold(III) complex developed in the group of Dolores Fregona in Padua that has

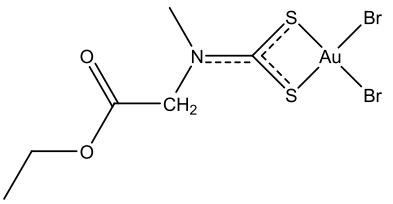


**Figure 1.** $AuL_{12}$ structure.

revealed very encouraging cytotoxic and anticancer properties both in vitro and in vivo.[7,8]

This compound consists of a square-planar gold(III) center with a bidentate dithiocarbamato ligand and two bromide ligands.[9] Notably, $AuL_{12}$ has been shown to behave as a prodrug by releasing its bromide ligands. Significant oxidizing properties of the compound were also documented.[7,8] Previous studies analyzed the interactions of $AuL_{12}$ with proteins, and also with SA, and offered a general description of the binding process that relies on a redox event.[8] Here we have explored in more detail the reaction of $AuL_{12}$ with bovine serum albumin (BSA) and attempted to characterize the products of this reaction at the molecular level. To this end, we have taken advantage of the combined X-ray diffraction (XRD)/electrospray ionization mass spectrometry (ESI-MS) protocol recently developed in our laboratories to elucidate protein metalation processes.[5,10,11]

First, the reaction of $AuL_{12}$ with BSA was analyzed spectroscopically according to classical methods, and clear evidence was gained for the occurrence of a redox process whereby the gold(III) center undergoes a reduction to gold(I) and the resulting gold(I) ion tightly binds the protein (Figure S1).[8] Accordingly, the band at 320 nm characteristic of the gold(III) center in $AuL_{12}$ progressively disappears, and a new band builds up at 380 nm. Further independent evidence for the occurrence of an interaction between $AuL_{12}$ and BSA has been obtained through other biophysical methods such as circular dichroism (CD; see the Supporting Information, SI).

Afterward, the interaction of $AuL_{12}$ with BSA was investigated by X-ray crystallography. Good-quality crystals of BSA are difficult to obtain,[12] and the first structure of the protein was solved only in 2012.[13] At the moment, in the Protein Data Bank, there are only two structures of BSA complexes, one with naproxen[14] and the other with 3,5-diiodosalicylic acid,[15] and there are no structures of adducts with metallodrugs. The crystals of $AuL_{12}$/BSA diffract X-rays to 3.2 Å resolution using a synchrotron radiation source. Two molecules of BSA are present in the asymmetric unit (a.u.) in this crystal (Figure 2A).[12] The overall structure of the protein is not affected by the reaction with $AuL_{12}$. Root-mean-square deviations between $\alpha$-carbon atoms of the adduct and native protein are within the range 0.38−0.45 Å.

Inspection of the electron density in the $F_{obs}-F_{calc}$ map allows one to easily identify the presence of a gold atom close to the side chain of Cys34 in both BSA molecules present in the a.u. The presence of the gold center was unambiguously supported by a strong peak in the anomalous difference electron density map (Figure 2B). As a negative control to confirm the binding of gold to Cys34, we compared the electron density of Cys34 in the $AuL_{12}$/BSA adduct with that of BSA in the absence of any ligands. As supposed, no electron density was observed around the side chain of Cys34 in the structure of BSA, further confirming our assignment.

Close to the gold center, there is no electron density corresponding to the dithiocarbamato ligand; however, because of the low resolution of the structure of the adduct,

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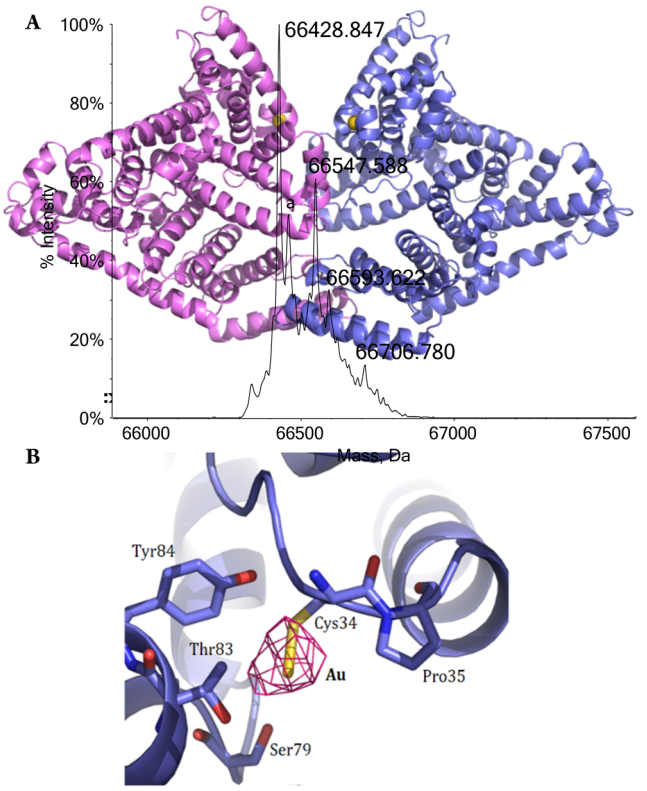


**Figure 2.** (A) a.u. content of crystals of the $AuL_{12}$/BSA adduct. The gold atom is indicated as a yellow sphere. (B) Detail of the gold binding site in one of the molecules of the adduct in the a.u. An anomalous difference electron density map corresponding to the gold center is reported at a 3.5$\sigma$ value (see also Figure S2).

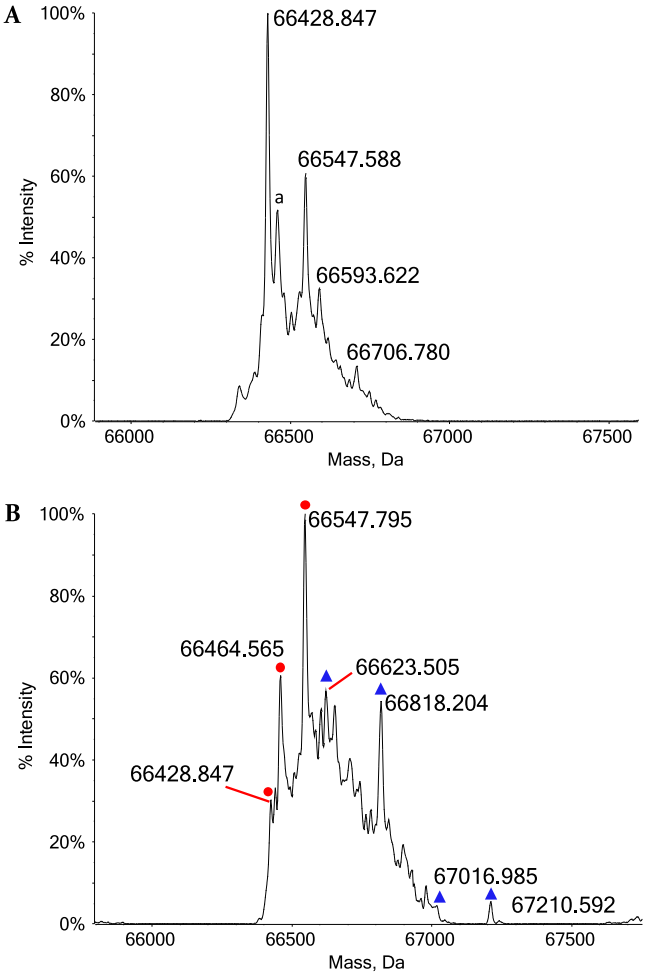


**Figure 3.** (A) Deconvoluted ESI-MS spectrum of BSA ($10^{-5}$ M) in a 20 mM ammonium acetate buffer (pH 6.8; *a* = 66461.80 Da). (B) Deconvoluted ESI-MS spectrum of the $AuL_{12}$/BSA mixture. $AuL_{12}$ was incubated for 3 h with BSA ($10^{-4}$ M) in a 1:1 metal-to-protein molar ratio at 37 °C in a 20 mM ammonium acetate buffer (pH 6.8).

we cannot dismiss that this absence could be due to conformational disorder.

Thus, to gain additional and independent data on this system, high-resolution ESI-MS spectra on the $AuL_{12}$/BSA adduct were collected. We have recently set the best conditions to analyze the intact protein and its interactions with ligands and metallodrugs.[16−19] Accordingly, the ESI-MS spectrum of BSA in a 20 mM ammonium acetate buffer (pH 6.8) was recorded (Figure 3A).

The spectrum exhibits four main peaks that are assigned to native BSA (66428.847 Da) and to its principal physiological post-translational modifications (PTMs), namely, the cysteinylation of Cys34 (66547.558 Da), an oxidized form of the same residue at 66461.80 Da (Cys-$SO_2$H), and a signal at 66593.622 Da, belonging to a glycated form of SA.[16,20]

Subsequently, an aliquot of a $AuL_{12}$ stock solution in dimethyl sulfoxide (DMSO; maximum DMSO final concentration of 5% v/v) was added in a 1:1 protein-to-metal molar ratio to a solution of BSA dissolved in a 20 mM ammonium acetate buffer (pH 6.8). The obtained sample was incubated at 37 °C, and the ESI-MS spectrum was recorded after 3 h (Figure 3B). Notably, some new signals appear at higher MWs, marked with blue triangles. In particular, the peaks with molecular masses of 66623.505 and 66818.804 Da nicely correspond to BSA adducts containing 1 or 2 nude gold ions; the peaks of smaller intensity, at 67016.985 and 67210.592 Da, belong to protein adducts containing three and four gold ions, respectively. Moreover, the spectrum also shows a signal at 66428.847 Da, corresponding to a small residual amount of unreacted BSA, and two other peaks, corresponding to the principal PTMs (e.g., sulfinylation and cysteinylation of Cys34) of BSA that remain unaltered (signals marked with red circles). This observation implies that BSA can bind an increasing amount of $AuL_{12}$; however, upon protein binding, $AuL_{12}$ undergoes an invariant reduction to gold(I) and loses all its ligands so that only naked gold(I) ions are associated with the protein. This behavior toward serum proteins under physiological-like conditions is common for gold(III) complexes and is well documented (i.e., activation by reduction).[21−23] As reported in a recent paper, the free thiol group of Cys34 is the preferential binding site for gold(I) compounds in BSA.[16] The reaction of gold compounds with BSA gives rise to mono- or digold adducts on this residue, without any evidence for the involvement of further binding sites (i.e., histidine side chains).[16] Surprisingly, in the case of $AuL_{12}$, it is evident that BSA can bind up to four gold atoms. To explain this behavior, we have hypothesized that $AuL_{12}$ undergoes reduction in aqueous solution, leading to the formation of reactive gold(I) species and to release of the dithiocarbamato ligand.[22,23] The presence in an aqueous solution of the gold(III) complex can cause the oxidative opening of some disulfide bonds in BSA,[24] with subsequent ligation of the reduced gold(I) species.[25] To verify this hypothesis, $AuL_{12}$ was


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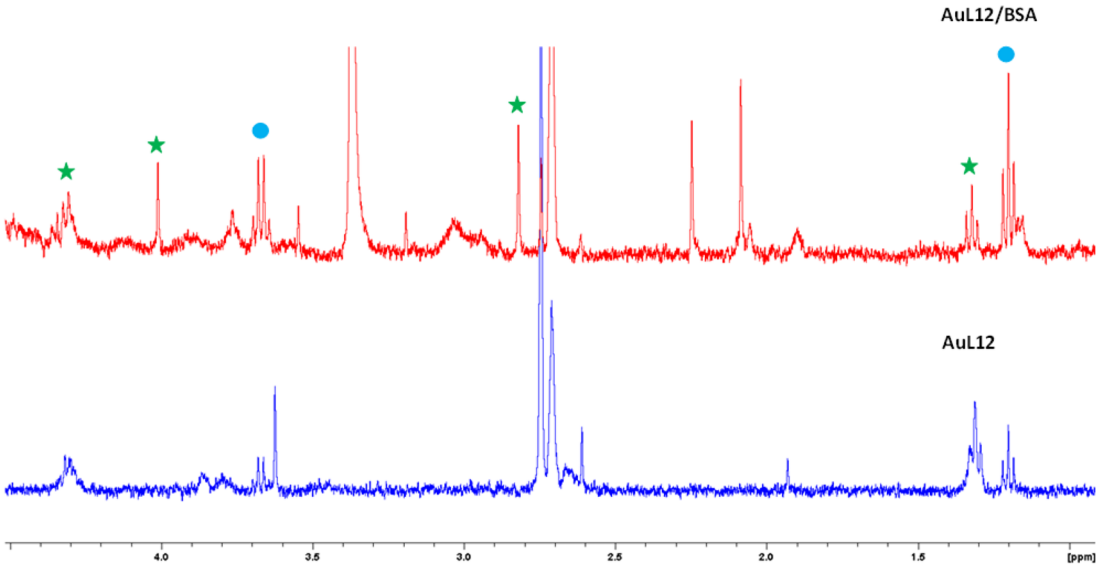


**Figure 4.** $^{1}$H NMR spectrum of 0.45 mM $AuL_{12}$ in $D_2O$ with 3% DMSO-$d_6$ (blue). $^{1}$H NMR spectrum of 0.45 mM $AuL_{12}$ incubated with 0.45 mM BSA in $D_2O$ with 3% DMSO-$d_6$ (red). The acquisitions were performed using a cmpgpr1d pulse sequence.

reacted with an aqueous solution of cystine and an ESI-MS spectrum recorded. The spectrum shows a principal peak at 508.9936 Da (Figures S3−S5) that corresponds to a single gold(III) atom coordinated to one dithiocarbamato moiety and one cysteine residue (see the SI for the isotopic pattern). This evidence perfectly fits with our hypothesis; in fact, the residual gold(III) atom maintains one bond with the dithiocarbamato moiety, while coordination is completed with a cysteinato residue deriving from cleavage of the cystine disulfide bond, stabilizing the gold center in the 3+ oxidation state.[25]

Therefore, the ESI-MS and XRD results are broadly consistent and suggest the occurrence of gold binding to the protein upon gold(III)-to-gold(I) reduction with simultaneous release of the dithiocarbamato ligand. Some $^{1}$HNMR measurements were also carried out. $^{1}$H NMR spectra were acquired using a monodimensional CPMG pulse sequence with solvent presaturation (cpmgpr1d). This experimental procedure permits one to remove from the $^{1}$H NMR spectra all broad signals originating from nuclei with short $T_2$, usually those belonging to large macromolecules (i.e., BSA and BSA/$AuL_{12}$ adduct in our cases); this typically leads to simpler $^{1}$HNMR spectra only showing the signals belonging to small molecules (or their fragments) in solution.[26]

The $^{1}$H NMR spectrum obtained for the 1:1 $AuL_{12}$/BSA system is shown in Figure 4; the spectrum of $AuL_{12}$ alone recorded under the same conditions is also reported for comparison. The low signal-to-noise ratio of these $^{1}$H NMR spectra is strictly related to the fact that the optimal BSA concentration (hence, also the $AuL_{12}$ concentration) turned out to be 0.45 mM. Indeed, larger BSA concentrations produce a drastic increase in the viscosity accompanied by a relevant broadening of the NMR signals. Notably, the $^{1}$H NMR spectrum of BSA treated with $AuL_{12}$ reveals several well-detectable signals belonging to low-molecular-weight compounds (no such signals are observed in the $^{1}$H NMR spectrum of the protein alone; see the SI). In Figure 4, relatively weak peaks are observed for the signals assigned to $AuL_{12}$; in contrast, a few more intense signals are observed that could be assigned to ethyl sarcosinate ($\delta$ 4.30, 3.99, 2.79, and 1.29; marked with green stars) and ethanol ($\delta$ 3.64 and 1.17; marked with cyan circles), which originate most likely from decomposition of the dithiocarbamato ligand. Some additional signals refer to DMSO residual peaks ($\delta$ 2.74 and 2.71; see the SI), to BSA impurities ($\delta$ 3.34; see the SI), or to $^{13}$C couplings (satellite signals at $\delta$ 3.52 and 3.16). These results support the concept that $AuL_{12}$ breaks down to a large extent upon interaction with BSA and releases its dithiocarbamato moiety; the latter, in turn, may undergo further degradation processes.

Coordinates and structure factors have been deposited in the Protein Data Bank under the accession code 6RJV. These data can be obtained free of charge via www.rcsb.org/.

In conclusion, we have succeeded in solving the crystal structure of the $AuL_{12}$/BSA adduct. The structure reveals some important features; a single gold center binds the protein at the level of the side chain of Cys34, while the overall structure of the protein is not affected. Selective modification of Cys34 by gold coordination is unequivocally demonstrated. Remarkably, when bound to the protein, the gold center has lost its original dithiocarbamato ligand. This concept is strongly supported by independent ESI-MS data showing that a number of naked gold(I) centers are bound to the protein. $^{1}$H NMR measurements point out that the signals of $AuL_{12}$ greatly reduce their intensity in the presence of BSA; in addition, the signals of chemical species deriving from degradation of the dithiocarbamato ligand are clearly seen. Thus, X-ray, ESI-MS, and NMR data nicely concur in drawing a consistent description for the $AuL_{12}$/BSA adduct. It is worth reminding that this is the first crystal structure for a gold/SA adduct. This structure may be of interest and relevance in relation to the processes of gold nanoparticle formation and assembly assisted by SA, which have attracted a lot of attention in recent years.[27]

## ■ ASSOCIATED CONTENT

### Supporting Information

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Experimental procedures, additional ESI-MS spectra, UV−vis spectrum, CD spectra, and crystallographic data refinement (PDF)

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### Notes

The authors declare no competing financial interest.

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