Structural Characterization of a Gold/Serum Albumin Complex

Alessandro Pratesi,[†] Damiano Cirri,[†] Dolores Fregona,[‡] Giarita Ferraro,[§] Anna Giorgio,[§] Antonello Merlino[§,*] and Luigi Messori[†,*]

[†]Laboratory of Metals in Medicine (MetMed), Department of Chemistry "U. Schiff", University of Florence, via della Lastruccia 3, 50019 Sesto Fiorentino, Italy.

[‡]Department of Chemical Sciences, University of Padova, Via Marzolo 1, 35131 Padova, Italy.

[§]Department of Chemical Sciences, University of Naples Federico II, via Cinthia, 80126 Napoli, Italy.

Supporting Information Placeholder

ABSTRACT: The medicinal gold(III) dithiocarbamato complex AuL12 forms a stable adduct with bovine serum albumin. The crystal structure reveals that a single gold(I) center is bound to Cys34, 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 is the major and most abundant serum protein performing crucial roles in the binding and blood transport of a variety of exogenous substances and drugs.[1,2] Notably, serum albumin has been reported to be the carrier of several metal ions and metal-containing species in the blood stream.[3,4] Though extensive studies have been carried out to characterize the interactions of serum albumin with several metal-based drugs, until now very few structures have been reported for serum albumin/metal drugs adducts.[5,6] In particular, no structure has ever been reported for serum albumin adducts with gold compounds.

AuL12 (Figure 1) is a mononuclear gold(III) complex developed in the group of Dolores Fregona in Padua that has revealed very encouraging cytotoxic and anticancer properties both in vitro and in vivo.[7,8]

This compound consists of a square planar gold(III) centre with a bidentate dithiocarbamato ligand and two bromide ligands.[9] Notably, AuL12 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 AuL12 with proteins - also with serum albumin - 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 AuL12 with bovine serum albumin and have 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/ESI-MS protocol recently developed in our laboratories to elucidate protein metalation processes.[5,10,11]

First, the reaction of AuL12 with serum albumin was analyzed spectroscopically according to classical methods and clear evidence was gained for the occurrence of a redox process whereby the gold(III) centre undergoes reduction to gold(I) and the resulting gold(I) ion tightly binds the protein

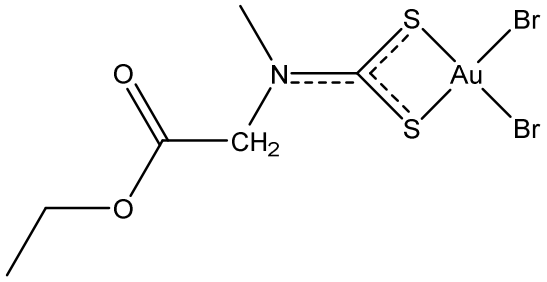

Figure 1. AuL12 structure.

(Figure S1).[8] Accordingly, the band at 320 nm characteristic of the gold(III) center in AuL12 progressively disappears and a new band builds up at 380 nm. Further independent evidences for the occurrence of an interaction between AuL12 and BSA have been obtained with the aid of other biophysical methods such as circular dichroism (see supporting info).

Afterward, the interaction of AuL12 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] the other with 3,5-diiodosalicylic acid[15] and there are no structures of adducts with metallodrugs. The crystals of AuL12/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 AuL12. Root mean square deviations between carbon alpha atoms of 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 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 centre 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 Au to Cys34, we compared the electron density of Cys34 in the AuL12/BSA adduct with that of BSA in the absence of any ligands. No electron density was observed around the side chain of Cys34 in the structure of BSA in the

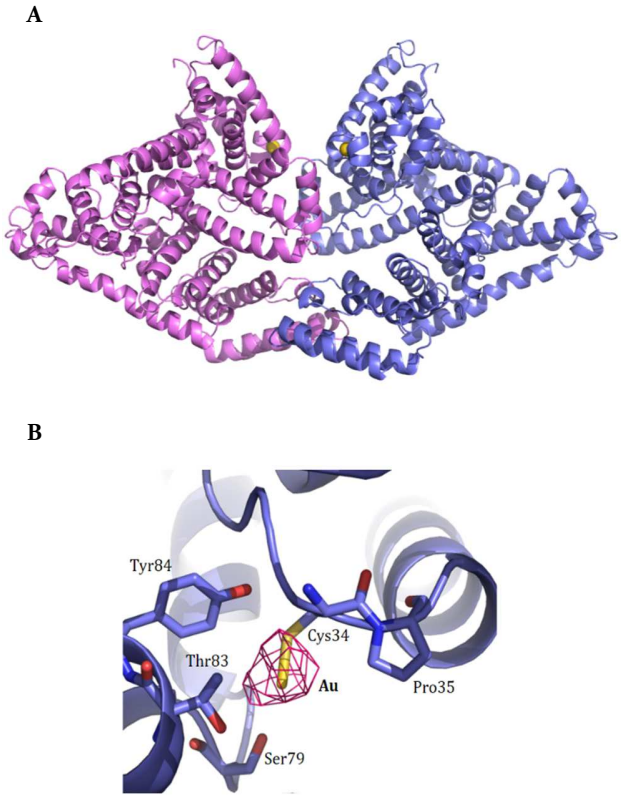

Figure 2. (**A**) Asymmetric unit content of crystals of AuL12/BSA adduct. Gold atom is indicated as a yellow sphere. (**B**) Detail of the Au binding site in one of the molecules of the adduct in the a.u. Anomalous difference electron density map corresponding to the gold centre is reported at 3.5 sigma value. The same map is reported in the SI for the other molecule of the adduct in the a.u., together with omit F_{obs}-F_{calc} electron density map of this region (Figure S2).

absence of the gold compound, further confirming our assignment.

Close to the gold centre, there is no any electron density corresponding to the dithiocarbamato ligand; however, due to the low resolution of the structure of the adduct, we cannot exclude that the absence of the electron density for the ligand could be due to conformational disorder.

Thus, to gain additional and independent data on this system, high-resolution ESI mass spectra on the AuL12/BSA system 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 mass spectrum of BSA dissolved in 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_2H) and a signal at 66593.622 Da belonging to a glycated form of serum albumin.[16,20]

Subsequently, an aliquot of AuL12 stock solution in DMSO (maximum DMSO final concentration 5% v/v) was added in

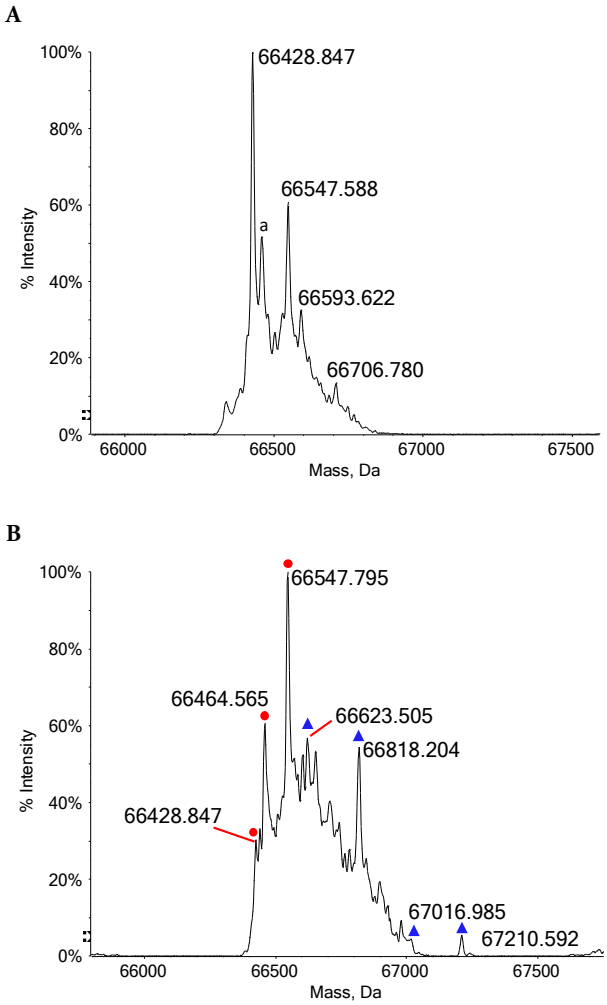

Figure 3. (**A**) Deconvoluted ESI mass spectrum of BSA (10^{-5} M) in 20 mM ammonium acetate buffer, pH 6.8. (a = 66461.80 Da). (**B**) Deconvoluted ESI mass spectrum of the AuL12/BSA mixture. AuL12 (stock solution in DMSO) was incubated for 3 h with BSA (10^{-4} M) in 1:1 metal to protein molar ratio at 37 °C in 20 mM ammonium acetate buffer, pH 6.8.

1:1 protein-to-metal molar ratio to a solution of BSA dissolved in 20 mM ammonium acetate buffer at pH 6.8. The obtained sample was incubated at 37 °C and the ESI mass 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 mass 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 3 and 4 gold ions, respectively. Moreover, the spectrum also shows a signal at 66428.847 Da, corresponding to a small residual amount of unreacted BSA and other two 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 AuL12 equivalents; however, upon protein binding, AuL12 undergoes invariantly reduction to gold(I) and loses all its ligands so that only

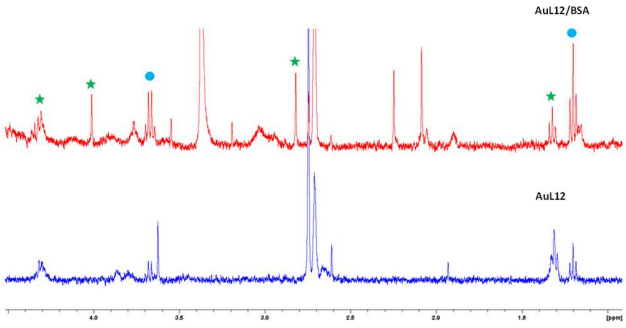

Figure 4. ^{1}H NMR spectrum of 0.45 mM AuL12 in D_2O with 3% of DMSO-d_6 (blue); ^{1}H NMR spectrum of 0.45 mM AuL12 incubated with 0.45 mM BSA in D_2O with 3% of DMSO-d_6 (red). The acquisitions were performed using cmpgpr1d pulse sequence.

naked gold(I) ions are eventually associated with the protein. This behavior toward serum proteins under physiological-like conditions is common for Au(III) complexes and is well documented in the literature (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 Au(I) compounds in BSA.[16] The reaction of gold compounds with BSA gives rise to mono- or di-gold 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 AuL12, it is evident that BSA can bind up to four gold atoms. To explain this behavior, we have hypothesized that AuL12 undergoes reduction in aqueous solution, leading to the formation of reactive Au(I) species and to the release of the dithiocarbamato ligand.[22,23] The presence in aqueous solution of the gold(III) complex can cause the oxidative opening of some disulphide bonds in BSA,[24] with the subsequent ligation of the reduced gold(I) specie.[25] To verify this hypothesis, AuL12 was reacted with an aqueous solution of cystine and an ESI mass spectrum recorded. The spectrum shows a principal peak at 508.9936 Da (Fig. S3-S5) that corresponds to a single Au(III) atom coordinated to one dithiocarbamato moiety and one cysteine residue (See 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 the coordination is completed with a cysteinato residue deriving from the cleavage of the cystine disulphide bond, stabilizing the gold center in the +3 oxidation state.[25]

Therefore, 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. To further support this picture some ^{1}HNMR measurements were also carried out. ^{1}H-NMR spectra were acquired using a mono-dimensional CPMG pulse sequence with solvent presaturation (cpmgpr1d). This experimental procedure permits to remove from the ^{1}H-NMR spectra all broad signals originating from nuclei with short T2, usually those belonging to large macromolecules (*i.e.* BSA and BSA-AuL12 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 AuL12/BSA system is shown in Figure 4; the spectrum of AuL12 alone recorded under the same solution conditions is also reported for comparison purposes. The low signal-to-noise ratio of these ^{1}H-NMR spectra is strictly related to the fact that optimal BSA concentration (hence also AuL12 concentration) turned out to be 0.45 mM. Indeed, larger BSA concentrations produce a drastic increase in viscosity accompanied by a relevant broadening of NMR signals. Notably, the ^{1}H-NMR spectrum of BSA treated with AuL12 reveals several and 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 figure SI). In figure 4, a relatively weak peaks are observed for the signals assigned to AuL12; in contrast, a few more intense signals are observed that could be assigned to ethyl sarcosinate (δ: 4.30, 3.99, 2.79, 1.29; marked with green stars) and ethanol (δ: 3.64, 1.17; marked with cyan circles), that originate most likely from the decomposition of the dithiocarbamato ligand. Some additional signals refer to dimethylsulfoxide residual peak (δ: 2.74; 2.71; see supporting information), to BSA impurities (δ: 3.34; see supporting information) or to ^{13}C couplings (satellite signals at δ: 3.52; 3.16). These results support the concept that AuL12 breaks down to a large extent upon interaction with BSA and releases its dithiocarbamato moiety; the latter in turn may undergo further degradation processes.

In conclusion, we have succeeded in solving the crystal structure of the AuL12/BSA adduct. The structure reveals some important features; a single gold centre 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 centre has lost its original dithiocarbamato ligand. This concept is strongly supported by independent ESI-MS showing that a number of naked gold(I) centres are bound to the protein. Independent ^{1}H-NMR measurements point out that the signals of AuL12 greatly reduce their intensity in the presence of BSA; in addition, signals of chemical species deriving from the 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 AuL12/BSA adduct. It is worth reminding that this is the first crystal structure for a gold/serum albumin adduct. This structure may be of particular interest and relevance in relation to those processes of gold nanoparticle formation and assembly assisted by serum albumin, that have attracted a lot of attention in recent years.[27]

ASSOCIATED CONTENT

Supporting Information

Electronic Supplementary Information (ESI) available: experimental procedures, additional ESI-MS spectra, UV-Vis spectrum, CD spectra and crystallographic data refinement. This material is available free of charge via the Internet at http://pubs.acs.org

Accession Code

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/

AUTHOR INFORMATION

Corresponding Authors

*E-mail: luigi.messori@unifi.it
*E-mail: antonello.merlino@unina.it

ORCID

Alessandro Pratesi: 0000-0002-9553-9943
Damiano Cirri: 0000-0001-9175-9562
Dolores Fregona: 0000-0002-8101-1101
Giarita Ferraro: 0000-0001-9385-2429
Anna Giorgio: 0000-0001-5099-6967.
Antonello Merlino: 0000-0002-1045-7720
Luigi Messori: 0000-0002-9490-8014

Notes

The authors declare no competing financial interest.

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Table of Contents Graphic and Synopsis

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We described the first X-ray structure for a gold adduct of serum albumin obtained upon protein reaction with the medicinal gold(III) dithiocarbamato complex AuL12.