Bovine Serum Albumin-Gold Thiomalate Complex: ^{197}Au Mössbauer, EXAFS and XANES, Electrophoresis, ^{35}S-Radiotracer, and Fluorescent Probe Competition Studies

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Abstract: The reaction of bovine serum albumin (BSA) with sodium gold(I) thiomalate (AuSTm) and the resulting complexes were examined by a variety of physicochemical techniques. Gold and [^{35}S]thiomalate are incorporated into the resulting complex in an approximately 1:1 ratio. The amount of AuSTm which binds increases with the mercaptalbumin (Alb-SH) content of the BSA preparation. Three preparations of BSA(AuSTm)$_n$, where n = 0.44, 1.30, and 2.65, were isolated for spectroscopic characterization. The first reported ^{197}Au Mössbauer and also XANES and EXAFS spectra of gold-protein complexes demonstrate their utility for elucidating the environments of gold bound to macromolecules. The Mössbauer spectrum of BSA(AuSTm)$_{1.30}$ (corresponding to AlbS(AuSTm)$_{2.0}$) was resolved into two quadrupole doublets, IS_1 = 1.88, QS_1 = 6.68, IS_2 = 1.70, and QS_2 = 6.50 mm/s, consistent with two slightly different Au^1S_2 environments. The EXAFS/XANES spectra of BSA(AuSTm)$_{0.44}$ demonstrate that gold(I) is coordinated by two sulfur atoms at a distance of 228 ± 2 pm. In BSA(AuSTm)$_{2.65}$, all the gold atoms were in indistinguishable AuS_2 coordination environments with an average bond distance of 230 ± 2 pm. The binding of AuSTm to albumin increased its electrophoretic mobility. AuSTm did not compete with dansylamide or dansylsarcosine for anionic drug binding sites I or II. It is proposed that gold binds tightly to cys-34 to form AlbSAuSTm and that additional weaker binding occurs via bridging thiomalates (e.g., AlbS(AuSTm)$_n$ or AlbSAu$_n$(STm)$_{n-1}$) or by noncovalent interactions at remote sites on the protein [AuSTm]$_n$·AlbSAuSTm.

Chrysotherapy, the gold-based treatment of rheumatoid arthritis, is an important and increasingly used mode of therapy for this crippling disease. Gold(I) sodium thiomalate, AuSTm, and gold(I) thioglucose, AuSTg, are widely used in chrysotherapy, and a novel drug, Auranofin, (triethylphosphine)(2,3,4,6-*O*-tetraacetyl-1-thio-β-D-glucose)gold(I), is undergoing phase IV clinical testing.[1] Three recent reviews have summarized the biochemistry and inorganic chemistry of these and related gold complexes of biological interest.[1-3]

Serum albumin is the principal binding site in the blood for intramuscularly administered AuSTm and AuSTg.[1] It is a complex and enigmatic protein which binds and transports numerous drugs, metals, and endogenous ligands.[4] Depending on the dose, time after administration, and the gold complex, from 80 to 95% of the serum gold is bound to albumin in a number of species including humans.[1] Three independent thermodynamic measurements have established that strong and weak gold-binding sites exist on human and bovine albumin, but disparate estimates of the number of sites (n_i) and binding constants (K_i) were obtained:[5-7] n_1 = 0.04-1.0 and $K_1 = 6 \times 10^3$-1.5×10^6 for the strong-binding site, and n_2 = 0.3-6.6 and $K_2 = 2 \times 10^2$-1×10^5 for the weak-binding site. Both bovine and human serum albumins have been sequenced.[8,9] Each contains only a single reduced sulfhydryl group, cys-34, which has been assumed to be the strong gold-binding site.[1] Experimental examination of its role is complicated by the microheterogeneity of albumin.[10] In vivo and after isolation, it exists as a mixture of mercaptalbumin (hereafter abbreviated Alb-SH) in which cys-34 is fully reduced, oxidized forms, which include mixed disulfides of cys-34 with cysteine and glutathione, and various dimers. The percentage of mercaptalbumin in vivo is approximately 65%, but commercial preparations range from 65% to no titratable cysteine. The variation in the percentage of mercaptalbumin of a preparation will clearly alter the qualitative and quantitative aspects of its chemistry, especially in reactions with heavy metals.

The assignment of cys-34 as the primary gold-binding site is consistent with the chemistry of gold(I) thiolates. AuSTm and AuSTg are oligomers,[11,12] and, with various thiols which might serve as models for gold-protein reactions, react to form complexes which have been assigned as simple bis(thiolato)gold(I) species, $[Au(SR)_2^-]$,[1,13-15] or small clusters, $[Au_4(SR)_7^{3-}]$ or $[Au(SR)_{1.75}^{-0.75}]_n$.[2,16,17] A number of experimental studies of gold-albumin complexation are consistent with binding via cys-34, although direct structural evidence has not been previously presented. Chemical modification of cys-34 (e.g., by *N*-ethylmaleimide) reduces the affinity for gold,[5] but the extent of modification was not determined, complicating the interpretation of the data. On the basis of the lack of free thiol in solution and the ability of AuSTm to block the sulfhydryl interchange-de-

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pendent thermal denaturation of albumin, Gerber in 1964 postulated that gold bound to cys-34 with the thiomalate still coordinated.[18] Upon electrophoresis, Kamel et al. found that 10–15% of the bound AuSTm was displaced.[19]

A number of models can be proposed for the strong- and weak-binding sites in the complexes formed between albumin and AuSTm. Model **1**, linear gold(I) with cys-34 of mercaptalbumin and thiomalate as terminally coordinated ligands, is consistent with the strong tendency of gold to be two-coordinate and has been proposed previously.[1,18] Models **2** and **3** entail coordination by cys-34 and a ligand other than thiomalate. Model **2**, generated by exchange of gold for the proton of cys-34, implicitly requires water in the coordination sphere, and model **3** requires chelation of the gold by a second ligand from the protein, most likely another amino acid side chain (e.g., histidine or lysine). Models **4** and **5** require aggregation of the protein. **4** is analogous to the proposed mercury(II)-bridged "dimer" of albumin.[20] **5** is a sterically improbable oligomer with bridging cys-34 ligands to create a structure analogous to those of $[AuSTm]_n$, $[AuSTg]_n$, etc.[11,12] Structures **1–5** account for only one gold per albumin and must be models for the strong binding site. Models **2–5** would be consistent with Jellum and Munthe's observation that thiomalate is displaced from gold in vivo.[21]

AlbSAuSTm **1** AlbSAu$(OH_2)_?$ **2** AlbSAuX– **3**

AlbSAuSAlb **4** $[AlbSAu]_n$ **5** $AlbSAu_n(STm)_m$, $n < m$ **6**

AlbSAuSTm$[AuSTm]_n$ **7** $AlbSAu_n(STm)_{n-1}$ **8**

$[AuSTm]_n$AlbSH **9**

Models **6–9** can accommodate more than one gold per albumin and may be applicable to the weak- or strong-binding sites. Model **6** is an adaption of Sadler's cluster model for the product of gold thiomalate reactions with thiols.[16,17] Copper analogues with stoichiometries $Cu_5(SR)_7^{2-}$ and $Cu_4(SR)_7^{3-}$ have two- and three-coordinate copper(I) ions.[22,23] Models **7** and **8** are linear and cyclic extensions of model **1** which accommodate additional AuSTm units via bridging thiolates to account for the existence of the thermodynamically weak-binding sites. Model **8** can also be generated by the substitution of albumin, acting as a thiolate through cys-34, for one thiomalate of an $[AuSTm]_n$ oligomer or via cyclization of **7** with loss of a thiomalate ligand. Finally, binding of intact gold thiomalate clusters via noncovalent interactions (e.g., electrostatic) might be important for the weak-binding site. One possibility is electrostatic binding of AuSTm oligomers at the previously characterized anionic drug binding sites I and II, which can be identified by their affinities for dansylsarcosine (DS) and dansylamide (DA).[23,24]

Gold is a spectroscopically quiet element. The principal oxidation states in solution, Au(I) and Au(III), have d^{10} and d^8 electronic configurations and hence are not EPR active. The only stable isotope, ^{197}Au ($I = {}^3/_2$; 100% abundant), has not generated chemically useful NMR spectra. The UV–visible spectra of gold complexes lack generally distinct bands and, in particular, cannot be reliably measured and interpreted against the strong background of protein absorptions. Methods which show considerable promise as probes of gold structure and coordination geometry are ^{197}Au Mössbauer spectroscopy[12,26,27] and the related techniques, extended X-ray absorption fine structure (EXAFS) spectroscopy and X-ray absorption near edge spectroscopy (XANES).[11] From consideration of the isomers shifts (IS) and quadrupole splittings (QS) of the ^{197}Au Mössbauer spectra of complexes with known oxidation state and coordination geometry, it is possible to assign the oxidation state and coordination environment of gold in other complexes. Plots of QS and IS for linear two-coordinate gold(I) and square-planar gold(III) complexes fall on two distinct lines:[12,26] QS = 1.11IS + 4.55 for Au^IL_2 and QS = 1.74IS – 0.22 for $Au^{III}L_4$. Three-coordinate gold(I) complexes tend to have similar QS values, but lower IS values than corresponding two-coordinate complexes,[28] and therefore fall well above the line for two-coordinate complexes. From the magnitudes of the IS, the nature of the ligating atoms can often be deduced. XANES spectroscopy using the gold L_{III} absorption edge can readily distinguish gold(III) from gold(I) and gold(0), because the former experiences at 11.923 keV a bound-state transition of a 2p electron into the vacant 5d orbital; gold(I) and gold(0) have completely filled 5d orbitals and lack the transition, but can be distinguished because gold(0) has unique peaks at 11.945 and 11.967 keV.[11] By appropriate calculations using the data in the EXAFS region (i.e., beyond the absorption edge), information about the nature of th arest-neighbor atoms and the approximate number of eacl d can be obtained. Sulfur ligands can be clearly distinguis from oxygen or nitrogen donors by this method.

In this paper we demonstrate for the first time th ^7Au Mössbauer spectroscopy and XANES/EXAFS can be ılly applied to gold–protein complexes, and in particular, t he coordination geometry and oxidation state of gold in th u-min–gold thiomalate complex can be assigned unambiguou y the combination of these two techniques. To further te e various binding models discussed above, a number of sta protein chemistry techniques including electrophoresis, radiot studies of the thiomalate ligand, gel-exclusion chromatogra and competitive-binding studies with the fluorescent dyes d sylsarcosine (DS) and dansylamide (DA)[24,25] were also us Bovine serum albumin (BSA) was selected instead of human albumin (HSA) because the commercially available preparations of the former have more reproducible chemical properties, especially the level of fully reduced cys-34 side chains. AuSTm was selected as the gold complex to avoid problems associated with the presence of oxidized impurities in AuSTg.[13]

Experimental Section

Reagents. Reagents were obtained from the following sources: Sigma Chemical Co. (St. Louis, MO) bovine serum albumin (fatty acid free, lot 10F-9371; fraction V, crystallized, lots 41F-9300 and 12F-9365), gold thioglucose, dansylsarcosine (lot 87C-0377), dansylamide (lot 38C-0353), Trizma base, glutathione (reduced form); Aldrich Chemical Co. (Milwaukee, WI) mercaptosuccinimic acid (TmSH) and gold sodium thiomalate.

[^{35}S]AuSTm with an initial specific activity of 1.8 mCi/mmol was custom synthesized by New England Nuclear Corp. (Boston, MA). The purity was checked by comparing it to authentic AuSTm by using thin-layer and ion-exchange chromatography. Approximately 90% of the activity was [^{35}S]AuSTm; the major impurity was the disulfide and/or sulfide of thiomalate (TmSSTm or TmSTm).

^{197}Au Mössbauer Spectra. A previously described Mössbauer spectrometer and cryostat were used to record spectra at 4.2 K with a conventional constant acceleration drive, calibrated with a Michelson interferometer.[26] The integrating counting technique[27] was used so that the spectrum of a sample containing 50–100 mg of gold was typically obtained in 30 min. The voluminous lyophilized BSA–gold complex, $BSA(AuSTm)_{1.30}$, was fashioned into a pellet containing ca. 10 mg of gold by applying a pressure not exceeding 1000 kg/cm^2. The (cysteinato)gold(I), AuSCy, and (glutathionato)gold(I), AuSGt, samples were made into 12-mm diameter discs. Isomer shifts were measured and are reported relative to the ^{197}Pt source (1.22 mm/s vs. gold foil), which was obtained by irradiating 99.7% enriched ^{196}Pt in a high-neutron flux for 24 h. The quadrupole-split AuSCy and AuSGt spectra were fit ac-

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ceptably by a single pair of Lorentzians in each case (Figure 1a). The broader spectrum of the BSA–gold complex was fit to two pairs of Lorentzian lines (Figure 6). During the fit one set was constrained to IS = 1.70 and QS = 6.50 mm/s. These values have been reported for AuSTm by Hill et al.[12]

XANES/EXAFS Spectra. The X-ray absorption spectra were obtained at the Stanford Synchrotron Radiation Laboratory, on Beam line VII-3. A Si 2,2,0 double-crystal monochromator was used with nitrogen-filled ionization chambers to detect the radiation. The monochromatized radiation first passed through the incident beam ionization chamber, monitoring I_0. Then the beam impinged on the sample (enclosed in a helium chamber), and the transmitted radiation was measured in a second ionization chamber. Fluorescence measurements were simultaneously collected with a fluorescence ionization chamber detector of F. Lytle's design purchased from the EXAFS Co., Seattle, WA. The fluorescence detector utilizes an X-ray filter assembly of the Stern and Heald type.[29] The axis of the fluorescence detector is placed 90° to the incident and transmitted beams. Two germanium filters (generously provided by Dr. John Keem, Energy Conversion Devices, Troy, MI) were used to absorb scatter in the fluorescence detector. The germanium filters consist of two 4-in.^{2} pieces of 2.0 mil-thick Kapton film with depositions of 29 and 20 μm of germanium. The energy range for data collection was 11.5–12.9 keV, spanning the L_{III} absorption edge of Au at 11.9212 keV. The lyophilized protein samples were packed into sample cells of path length 5 mm. The spectra of the model compounds $Na_3[Au(S_2O_3)_2]\cdot 2H_2O$, $[(etu)_2Au]Cl\cdot H_2O$ (bis(ethylenethiourea)gold(I)-chloride hydrate), $[Au(NH_3)_4](NO_3)_3$, and $KAuCl_4$ were recorded on powdered solid samples diluted with Li_2CO_3 and of path length 1 mm. A gold foil spectrum was measured periodically for calibration purposes. All spectra were recorded at room temperature.

The EXAFS was extracted from the absorption spectrum following the technique of Hodgson[30-32] using programs modified for compatibility with an Amdahl 470/V7A computer and TSO IBM software in use at the University of Cincinnati.

The EXAFS data can be Fourier transformed from frequency space to yield a radial distribution-like function in distance space. The major peaks in the transform (such as that shown in Figure 3) correspond to absorber–scatterer distances phase shifted to smaller R. Fourier-filtered EXAFS data, obtained by the retransformation of a segment of the Fourier transform containing the peak(s) of interest, can then be fit to parameterized EXAFS expression: $\chi(k) = C_1 e^{C_2 k}[\sin(C_4 + C_5 k + C_6 k^2)]/k^{C_3}$. For model compounds all six parameters, C_1–C_6, are varied to give the best fit. These parameters are then transferred to fit the data for an unknown compound with only C_1 and C_5 varied. The unknown absorber–scatterer distance is calculated from the model by

$$R(\text{unk}) = R(\text{mod}) + [C_5(\text{unk}) - C_5(\text{mod})]/2$$

and the number of scatterers is given by

$$N(\text{unk}) = N(\text{mod})[C_1(\text{unk})R^2(\text{unk})]/[C_1(\text{mod})R^2(\text{mod})]$$

Parameters for Au–S, Au–N, and Au–Cl were obtained by using the model compounds $Na_3[Au(S_2O_3)_2]\cdot 2H_2O$, $[Au(etu)_2]Cl\cdot H_2O$, $[Au(NH_3)_4](NO_3)_3$, and $K[AuCl_4]$, where etu is ethylenethiourea.

Electrophoresis. BSA (0.6 mM) was incubated with AuSTm (0.72 or 7.2 mM) for 12–15 h. Three-microliter samples were applied to cellulose polyacetate strips in a Gelman Electrophoresis Chamber using Gelman Tris/barbital buffer, pH 8.6. A current of 1 mA/strip was applied for 1 h. All samples were run in duplicate or triplicate. Strips were stained in Ponceau S dye (0.5 g/100 mL of 7% acetic acid), cleared by treatment with methanol and 10% HOAc/methanol, and then dried at 100 °C for 10 min. The cleared strips were scanned and recorded on a Helena Laboratories Quick-scan densitometer.

Gel-Exclusion Chromatography. BSA (0.1 mM) was incubated with [^{35}S]Na_2AuSTm (0.1 mM, 0.166 μCi/mmol) in 40 mM Tris/0.15 M KCl buffer, pH 8.6, and fractionated at periodic intervals over 24 h by gel-exclusion chromatography on a Sephadex G-75 column (1 × 19 cm) eluted with the same buffer at a flow rate of 50 mL/h. Twenty fractions of 1.7 mL each contained the protein and unbound gold. Each fraction was analyzed for gold by atomic absorption spectroscopy, for ^{35}S by scintillation counting, and for protein by measurement of A_{279}. Protein recovery was 97–100%, gold 70–90%, and radiolabel 50–80%. Variable losses of radiolabel occurred in the presence and absence of albumin, due to an apparently irreversible binding of the label to the gel. It could not be reversed by treatment with dithiothreitol, thiomalate, unlabeled gold sodium thiomalate, or albumin.

Fluorescence of Albumin–Gold Complex and Dansyl Dye Reactions. BSA (0.500 g) was dissolved in 6 mL of 40 mM Tris–HCl/150 mM KCl buffer, pH 8.6, which also contained 2.0 mM AuSTm. After overnight incubation to ensure complete reaction, the solution was fractionated on a Sephadex G-75 column (1.5 × 40 cm) using Tris–HCl/KCl buffer. The albumin containing fractions were collected, pooled, and passed through an 8-μm Millipore filter to remove any suspended particulates. An aliquot of the filtrate was diluted with buffer to produce 32 mL of 19.0 μM albumin solution. To three separate 10-mL aliquots were added 25 μL of buffer, 25 μL of 7.79 mM DS (final concentration 19.5 μM), or 60 μL of 3.33 mM DA (final concentration 20.0 μM). The albumin and DA or DS concentrations provided approximately 50% bound albumin and fluorophore. Before the dye was added and immediately and 0.5, 1.0, 2.0, 4.75, and 24 h after the solution was mixed, the fluorescence of the bound dye was measured at 485 nm by using 355-nm excitation. Samples were counted for 5 s at 23.0 ± 0.5 °C on an SLM Model 8000S fluorescence spectrometer equipped with digital electronics. The dark count was 15 cps; 20 μM albumin produced 2.4×10^3 cps and the DA or DS/BSA complex 1.11×10^5 cps; AuSTm did not fluoresce under these conditions.

In the reactions where large excesses of AuSTm were used, 10 mM AuSTm was added to the protein and the mixture was incubated 20 h. After incubation the dye was added and the solution was brought up to 10 mL total volume, giving concentrations of 23.3 μM DA. The AuSTm concentrations were 0, 2.3, and 11.4 mM.

Fluorescence of Dansyl Dye–Albumin Complex + AuSTm Reactions. Ten-milliliter aliquots of ca. 20 μM BSA in 40 mM Tris–HCl/150 mM KCl buffer, pH 8.6, which had been Millipore filtered to remove particulates were incubated at least 2 h with DA or DS. After the initial fluorescence of the solutions was checked, sufficient 2.2 mM AuSTm and buffer were added to prduce final concentrations of 19.4 μM albumin, 18.9 μM DS, or 19.4 μM DA, and 0, 10.7, 21.3, or 32.1 μM AuSTm. In the case of the reactions involving large excesses of AuSTm, 10 mM AuSTm was added to the protein–dye complex and the mixture was subsequently brought up to 10.0 mL total volume, giving concentrations of 23.3 μM BSA and 20.4 μM DS or 22.3 μM BSA and 20.5 μM DA. The AuSTm concentrations were 0, 2.3, and 11.4 mM. The fluorescence of the mixture and control was measured over 27 h.

Scintillation Counting. ^{35}S was counted by β-scintillation counting using standard conditions for ^{14}C/^{35}S on a Nuclear Chicago Unilux III counter. Samples (0.2 mL) of filtrates, reaction mixtures, and stock AuSTm solutions were suspended in Aquassure liquid scintillation coctail (New England Nuclear Corp.). Counting efficiency was 85% and 10^3–10^6 counts were recorded in 4- or 10-min intervals, depending on the sample activity. The background was typically 50 cpm.

Gold Assays. Gold was determined by atomic absorption spectroscopy on an IL 357 spectrometer using solutions, or appropriate dilutions, with concentrations in the range 0.2–4 μg/mL. Spex $KAu(CN)_2$ standards, appropriately diluted, were used to calibrate the instrument.

Ba Precipitation/Filtration Technique. A chemical separation of the AuSTm from unreacted albumin and albumin–gold complex was effected by precipitation of AuSTm with Ba^{2+}. At intervals up to 24 h after mixing the AuSTm and BSA, 1.0-mL samples of the reaction mixtures were pipetted into 2 mL of ice-chilled 200 mM $Ba(NO_3)_2$ in 50 mM Tris–HCl/150 mM KCl buffer, pH 8.6, to precipitate the free AuSTm. The precipitation mixture was incubated for 2 min in an ice bath, and then 2 aliquots (1.5 mL) were filtered through Millipore 0.45-μm HAWG filter disks on a filtration manifold to separate the precipitated BaAuSTm from the protein-bound AuSTm. The gold concentration in the filtrate of a control sample containing the same concentration of AuSTm was used to correct each filtrate gold concentration.

^{35}S and Au Uptake Studies. The Ba^{2+} filtration/precipitation technique was applied to measure the ratio of ^{35}S to Au in the albumin–gold complex. Bovine serum albumin (2.36 mM) in 50 mM Tris/150 mM KCl buffer, pH 8.6, was chilled to 3 ± 1 °C. [^{35}S]AuSTm solutions, with specific activities of ca. 0.25 mCi/mmol, were prepared by dilution of 1.8 mCi/mmol [^{35}S]AuSTm with nonradioactive AuSTm in the same buffer. Three-milliliter aliquots of the gold solutions were transferred to serum bottles, stoppered, and refrigerated at 3 ± 1 °C. Introduction of 3 mL of the chilled protein solution to the gold aliquots gave final concentrations of 118 μM BSA and 118 μM, 1.18 mM, or 2.95 mM AuSTm. The reaction mixture was maintained at 3 ± 1 °C. The unreacted AuSTm was precipitated with Ba^{2+} and the gold and ^{35}S in the filtrates were determined as described.

(L-Cysteinato)gold(I) (AuSCy). AuSCy was prepared as previously described.[15,33]

(Glutathionato)gold(I) (AuSGt). $KAuBr_4$ (0.50 g, 0.898 mmol) in 10 mL of 20:1 EtOH/H_2O was reduced by bubbling SO_2 through the so-

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Table I. ^{197}Au Mössbauer Spectra

compd	IS,[a] mm s^{-1}	QS, mm s^{-1}	Γ_1/Γ_2, mm s^{-1}	ref
BSA(AuSTm)$_{1.3}$	1.8 (1)	6.6 (1)	2.9/3.2 *c*	
	1.88 (2)	6.68 (2)	2.4/2.7 *d*	*e*
	1.70[b]	6.50[b]	2.4/2.7	*e*
[AuSGt]$_n$	1.71 (2)	6.36 (4)	1.9/2.4	*e*
	1.64 (3)	6.43 (3)	1.91/2.12	28
[AuSCy]$_n$	1.58 (1)	6.18 (2)	1.9/2.3	*e*
	1.67 (1)	6.33 (2)	2.1/2.4	12
	1.65 (3)	6.47 (3)	2.12/2.36	28
[$Na_2AuSTm]_n \cdot H_2O$	1.74 (2)	6.5 (1)	2.2/2.2	12
[$Na_2AuSTmH_2O]_n \cdot (n/3)C_3H_8O_3$	1.69 (3)	6.43 (3)	2.0/2.4	12

[a] Measured at 4.2 K and reported relative to ^{197}Au in the Pt source (gold foil = −1.22 mm/s). [b] Values fixed during deconvolution into two components (see Figure 1). [c] One-site fit. [d] Two-site fit. [e] This work.

lution until it was pale yellow in color. After the precipitated KBr was filtered out, reduced glutathione (0.28 g, 0.889 mmol) in 10 mL of DD H_2O was added. The resulting precipitate was collected by centrifugation, washed once in 10 mL of DD H_2O, recollected by centrifugation, redissolved in absolute ethanol, and precipitated by evaporation of solvent. The collected precipitate was oven-dried at 85 °C; yield 0.466 g (100%). Anal. Calcd for $C_{10}H_{16}N_3OSAu$: C, 23.82; H, 3.18; N, 8.33; Au, 39.16. Found: C, 23.89; H, 3.23; N, 8.07; Au, 39.06.

The solubility of AuSGt in 0.1 M PO_4^{2-}/KOH buffer at various pH values was determined by atomic absorption measurements on solutions anaerobically equilibrated with solid AuSGt at 32 °C: pH 2.6, 30 μM; 3.0, 13; 4.0, 61; 5.0, 132; 6.0, 171; 7.0, 135.

Preparation of BSA(AuSTm)$_{1.3}$ for Mössbauer Spectroscopy. BSA (2.92 g, 43 μmol; 0.65 SH/BSA) was incubated overnight with a 10:1 mole ratio of AuSTm in 10 mL of $(NH_4)_2CO_3$ buffer, pH 7.4, and then fractionated on a large Sephadex G-75 column (5 × 100 cm) eluted with the same buffer. The pooled albumin fractions were lyophilized, saving a sample for analysis. The preparation contained 1.3 Au/BSA (2.0 Au/mercaptalbumin). An aliquot of the lyophilized and frozen gold–albumin complex was redissolved, chromatographed, and analyzed: the UV spectrum, Au/BSA ratio, and chromatographic elution profiles (Au and A_{280}) were unchanged.

Preparation of BSA(AuSTm)$_{0.44}$ for EXAFS/XANES. To 0.70 g (10.3 μmol) of BSA (crystallized, lot 41F-9300; 0.58 SH/BSA) dissolved in 2 mL of $(NH_4)_2CO_3$ buffer was added AuSTm (4.46 mg, 10.9 μmol) in 2 mL of $(NH_4)_2CO_3$ buffer, pH 7.4, at 4 °C. After a 4-h incubation, 2 mL of the sample was fractionated over Sephadex G-75 to remove unbound gold. The albumin fractions were lyophilized. The sample contained 0.44 Au/BSA (0.76 Au/mercaptalbumin).

Preparation of BSA(AuSTm)$_{2.65}$ for EXAFS/XANES. To AuSTm (44.6 mg, 106 μmol) in 4.0 mL of $(NH_4)_2CO_3$ buffer, pH 7.4, was added 0.740 g (10.9 μmol) of BSA (fatty acid free, lot 10F-9371, 0.36 SH/BSA). The mixture was incubated for 4 h at 4 °C, fractionated, and lyophilized as above. This sample contained 2.65 Au/BSA (7.4 Au/mercaptalbumin).

Isolation of Au(III) Albumin Reaction Product. $HAuCl_4$ (11.6 mg, 30.8 μmol) in $(NH_4)_2CO_3$ buffer (pH 7.4) was added at 4 °C to 0.350 g (5.15 μmol) of BSA (crystallized, lot 10F-9371, 0.36 SH/BSA) in a 570 μM solution over a 2-h period in order to avoid precipitating a gold–protein complex. After being stirred for an additional hour, the solution was lyophilized, redissolved in $(NH_4)_2CO_3$ buffer, and chromatographed. The albumin fractions were pooled and lyophilized. The sample contained 5.0 Au/BSA.

Results

^{197}Au Mössbauer Spectra of AuSGt and AuSCy. Because no ^{197}Au Mössbauer spectra were available for gold complexes of biologically important thiol ligands when this work was initiated, (*S*-glutathionato)gold(I), AuSGt, and (*S*-L-cysteinato)gold(I), AuSCy, were prepared and measured. The isomer shifts (IS), quadrupole splittings (QS), and line widths (Γ_1/Γ_2) of the quadrupole doublets (Figure 1 and Table I) were consistent with values for polymeric gold(I) thiolates that were reported while this work was in progress.[12,34,35] Previous Mössbauer and EXAFS

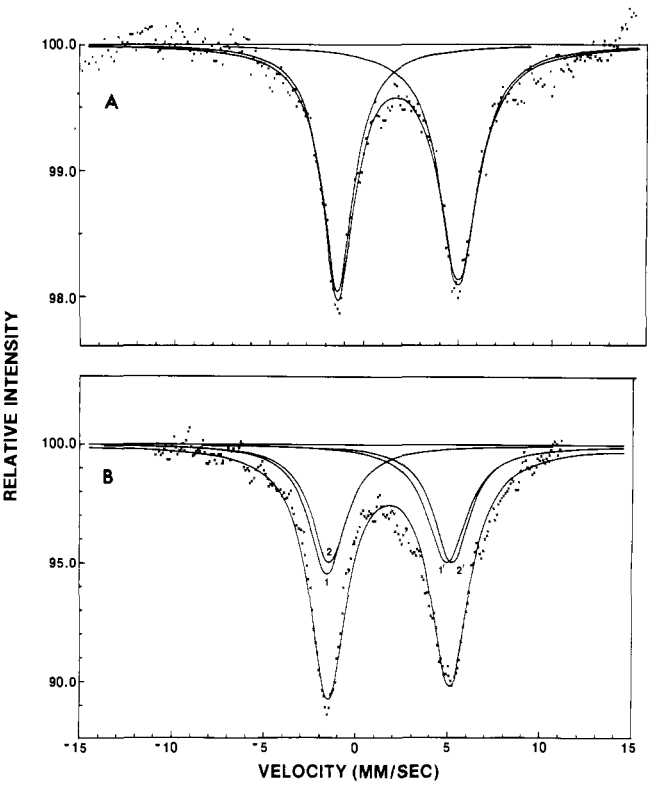


Figure 1. ^{197}Au Mössbauer spectra of AuSGt and BSA(AuSTm)$_{1.3}$ obtained at 4.2 K. IS = 0.00 for ^{197}Pt source. The AuSGt spectrum was fit with a single pair of Lorentzian lines; the serum albumin spectrum was fit with two pairs.

studies have assigned linear two-coordinate geometries to the gold(I) ions in these species.[11,12,34,36] The QS and IS values found here are in good agreement with the least-squares lines calculated for two-coordinate gold(I) species with SAuS and SAuP coordination by Hill et al. (QS = 1.11IS + 4.55).[12] The IS and QS values are also in the range of values reported for complexes with SAuS (1.0 < IS < 2.0 mm/S and 5.5 < QS < 7.0 mm/S). Complexes with Au(III)S_4 coordination have much lower IS and QS values. On the basis of the QS and IS values three-coordination can also be eliminated,[28] as can any structures in which nitrogen or oxygen donors of the ligands are coordinated to the gold. Thus the gold in AuSGt and AuSCy can be assigned as two-coordinate gold(I) with AuS_2 coordination geometry.

An intriguing aspect of the Mössbauer spectra of gold(I) thiolates is the pronounced broadening of the high-velocity component of the quadrupole doublet, Γ_2, which was observed here for AuSGt and AuSCy (Table I). Broadening of Γ_2 has also been reported for other gold(I) thiolates and is apparently a general property of this class of compounds.[12,34,35] The same phenomenon has been observed in the spectra of polymeric gold nucleosides.[37] The absolute values of Γ_1 and Γ_2 may vary due to experimental factors which affect the spectral resolution. However, their ratio, Γ_2/Γ_1, is determined by the distribution of IS and QS values: because of the linear relationship of QS to IS, Γ_2 will be greater than Γ_1 when there are two quadrupole-split resonances with slightly different isomer shifts. Thus, the broadening of the high-velocity components of gold(I) thiolate spectra must arise from the existence of two or more AuS_2 coordination environments.

Gold(I) thiolates are oligomeric species of variable size in solution and the solid state.[1-3,11,16] Efforts to crystallize them for X-ray structure determination have been unsuccessful, which may

(34) Brown, K.; Parish, R. V.; McAuliffe, C. A. *J. Am. Chem. Soc.* **1981**, *103*, 4943–4945.
(35) Calis, G. H. M.; Trooster, J. M.; Razi, M. T.; Sadler, P. J. *J. Inorg. Biochem.* **1982**, *17*, 139–145.
(36) Mazid, M. A.; Razi, M. T.; Sadler, P. J.; Greaves, G. N.; Gurman, S. J.; Koch, M. H. J.; Phillips, J. C. *J. Chem. Soc., Chem. Commun.* **1980**, 1261–1263.
(37) Calis, G. H. M.; Hadjiliadis, N., submitted for publication.

result from the absence of a single well-defined structural unit. The existence of more than one gold environment within the oligomeric structures has been postulated as explanation of the broadening of the high-velocity band of the quadrupole doublet.[12,18] An alternative explanation is partial oxidation of the thiolate ligands to the sulfenate (RSO^-) or sulfinate (RSO_2^-) derivatives. Gold thioglucose, AuSTg, prepared by the literature methods or obtained commercially as the solid or an oil suspension, contains an oxidized form of the ligand, tentatively identified as the sulfonic acid derivative.[13] The ^{197}Au Mössbauer spectra of three independent samples of AuSGt have subtly different isomer shifts, quadrupole splittings, and line widths.[12,34] Partial oxidation of the ligand would produce additional spectral components with slightly different QS and IS values which, because of their linear relationship, would increase the width of the high-velocity component, Γ_2, compared to the low-velocity component, Γ_1. Either explanation (different sites in the oligomer or incomplete oxidation of the thiolate ligand) could account for the small variations in IS and QS and the broadening of the high-velocity components of the quadrupole doublet. Further experiments are necessary to test these postulated explanations.

Mössbauer Spectrum of BSA(AuSTm)$_{1.30}$. A large-scale preparation of the gold–albumin complex was undertaken to provide enough albumin-bound gold to measure the spectrum. A 10-fold excess of the AuSTm was employed to ensure that the albumin would be saturated. Analysis of the starting material showed that it contained a mole ratio of 0.65 mercaptalbumin to total albumin. After fractionation by gel-exclusion chromatography to remove unbound gold, the albumin–gold complex had a mole ratio of 1.3 gold per albumin, which corresponds to approximately 2.0 gold/mercaptalbumin, Alb–S(AuSTm)$_2$. After the gold–albumin complex was desalted and lyophilized to produce a powdered sample for the Mössbauer analysis, a small aliquot was redissolved and chromatographed to ensure that it was not altered during handling. The chromatographic profile, gold to protein ratio, and UV spectrum of the sample were unaffected.

The Mössbauer spectrum of the albumin–gold complex consisted of a pair of broad resonances due to quadrupole splitting (Figure 1). The signal-to-noise ratio was much lower than for the model compounds AuSCy and AuSGt because the former has 1.3 gold atoms per 66 000 molecule weight units while the latter has 1.0 per 400–500. The initial analysis as a single quadrupole doublet yielded the parameters QS = 6.6, IS = 1.8, Γ_1 = 1.9, and Γ_2 = 3.2 mm/s, which were consistent with a linear two-coordinate AuS_2 environment for the gold. This analysis assumed that there was only one gold-binding site, while the stoichiometry of the complex showed two golds per mercaptalbumin. Subsequent computer analysis deconvoluted the very broad quadrupole doublet into two pairs of overlapping doublets (Figure 1 and Table I). One set of Mössbauer parameters was fixed at the values for AuSTm determined by Hill.[12] The intensities of the resulting two doublets were equal within experimental error. The QS and IS data of the second computer-resolved doublet were also consistent with the Viegers[26] and Hill[12] equations for linear two-coordinate gold(I) spectra and within the range of IS values between 1.4 and 2.23 mm/s for AuS_2 coordination environments reported by Hill.[12]

The Mössbauer parameters are clearly inconsistent with any proposed bonding models which involve nitrogen or oxygen ligands or three- or four-coordinate gold(I). Coordination by nitrogen or oxygen ligands would produce smaller values of QS and IS since the electron density at gold would be much less. Three-coordinate gold(I), AuS_3, would require similar values of QS but strongly reduced IS values, and on the plot of QS vs. IS, points would lie well above the line for two-coordinate complexes. Four-coordinate gold(I) would have a tetrahedral structure, yielding a spectrum with very small quadrupole splitting, if it were resolved at all. Thus the Mössbauer spectrum eliminates from consideration models **2**, **3**, and **6**. Models **1** and **4**–**9** are consistent with the Mössbauer data, although **4** and **5** are readily dismissed on the basis of the monomeric nature of the complex (vide infra). Since methionine is a very weak ligand for gold(I) and does not interact with [AuSTm]$_n$ to disrupt the polymeric structure,[2] any ligation of gold

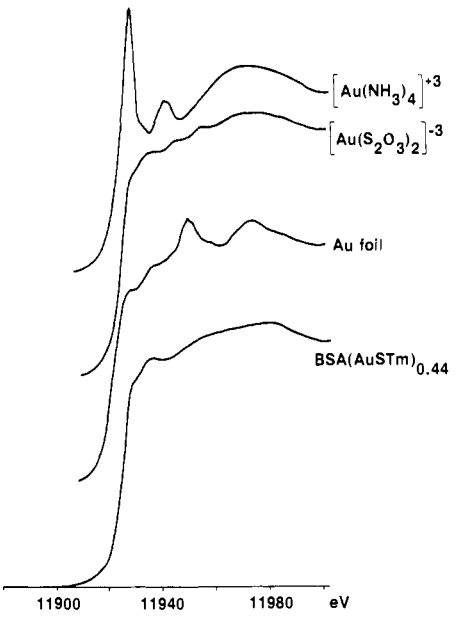


Figure 2. XANES transmission spectra of the model compounds, with gold(III), gold(I), and gold(0), compared to the XANES fluorescence spectrum of BSA(AuSTm)$_{0.44}$. They are plotted on an arbitrary absorption scale vs. the X-ray energy over the range 11.9–12 keV.

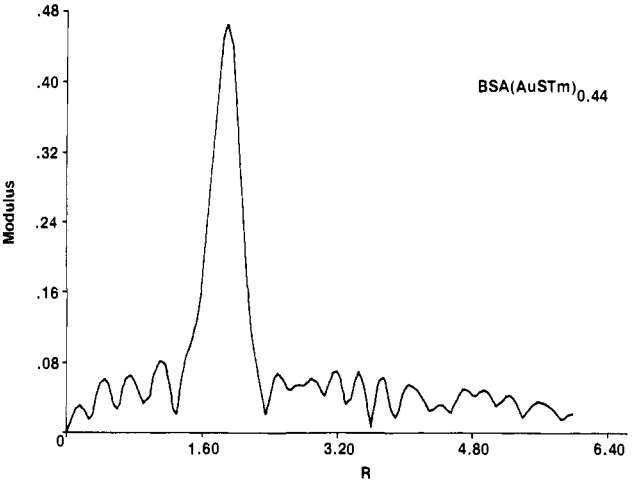


Figure 3. Fourier-transformed spectrum (in phase-shifted angstrom space) of BSA(AuSTm)$_{0.44}$. The single peak indicates that only a single scatterer (ligand atom) is needed to describe the first coordination sphere of the gold(I) ion.

by the protein involves cys-34. Thus the Mössbauer spectrum provides the first spectroscopic evidence consistent with cys-34 as the probable binding site for gold(I).

EXAFS/XANES Spectroscopy. Two complexes, BSA-(AuSTm)$_{0.44}$ and BSA(AuSTm)$_{2.65}$, were prepared for EXAFS/XANES measurements. The first albumin sample was incubated with an equimolar concentration of AuSTm and then separated from unreacted gold by gel-exclusion chromatography. It contained 0.44 Au/mol, significantly less than the SH content, 0.58/mol, which should maximize gold binding at the tight-binding site and minimize occupation of the weak-binding site. The XANES spectrum of BSA(AuSTm)$_{0.44}$ (Figure 2) lacked the sharp bound-state transition at 11.923 keV characteristic of gold(III) and the broader EXAFS features at 11.945 and 11.967 keV diagnostic of gold(0). Their absence established that the gold remained gold(I) in the complex.

The Fourier transform (in phase-shifted angstrom space) of the EXAFS data contained only a single major peak (Figure 3), indicating that only a single shell of scattering atoms would be needed for a curve fit. After retransforming the data in Figure 3, the Fourier-filtered EXAFS spectrum (Figure 4) was fit by

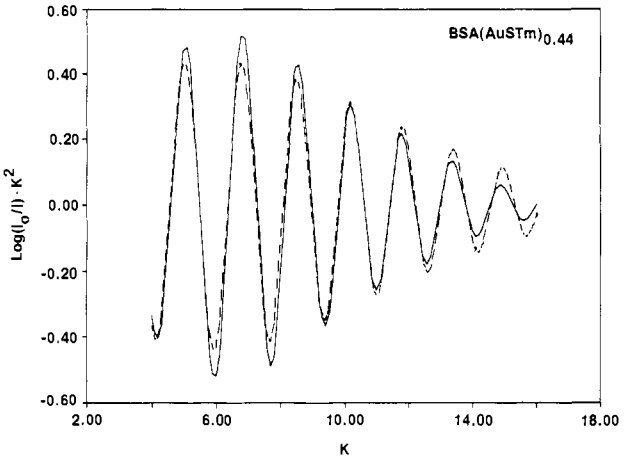


Figure 4. Fourier-filtered EXAFS spectrum of $BSA(AuSTm)_{0.44}$. A segment of transformed spectrum from 1.30–2.37 Å (Figure 3) was retransformed and fit by a parameterized EXAFS equation, assuming AuS bonds. The coordination number and bond distances were computed by the equations given in the Experimental Section.

Table II. Calculation of Single Shell Distances and Coordination Numbers[a]

	albumin preparations	
	BSA-$(AuSTm)_{0.44}$	BSA-$(AuSTm)_{2.65}$
distance (model A),[b] pm	228	230
distance (model B),[c] pm	228	230
coord. no. (model A)	2.1	2.0
coord. no. (model B)	2.3	2.2

[a] Analysis range ($Å^{-1}$) = 4–16. [b] Model A: $Na_3[Au(S_2O_3)_2]$ d_{Au-S} = 227.6 pm. [c] Model B: $[(etu)_2Au]Cl·H_2O$ d_{Au-S} = 227.8, 227.9 pm.

using phase-shift and amplitude parameters obtained for the AuS scattering from $[Au(S_2O_3)_2^{3-}]$ and also $[Au(etu)_2^+]$. Excellent agreement was obtained between the calculated and observed data, yielding a coordination number of 2.1–2.3 depending on the model chosen and a bond distance of 228 pm (see Table II). These results are consistent only with models retaining an AuS_2 coordination environment for the strong-binding site, and eliminate any model such as **2** or **3**, which entails coordination by oxygen, nitrogen, or other donor atoms.

The second complex was prepared by incubating an albumin sample having an SH content of 0.36/mol with a 10-fold excess of AuSTm. After gel-exclusion chromatography it contained 2.65 Au/mol. In this complex, $BSA(AuSTm)_{2.65}$, most of the gold cannot be bound directly to cys-34. The absorption spectrum lacked the bands characteristic of gold(III) and gold(0), indicating that gold is taken up into the weak-binding sites as gold(I). The transform was similar to that of $BSA(AuSTm)_{0.44}$ and the Fourier-filtered spectrum was satisfactorily fit assuming that only sulfur was present in the first coordination sphere. The calculated coordination number was 2.0–2.2 depending on the choice of model, and the bond distance was 230 pm. Thus even with extensive occupation of the weaker binding site(s) the AuS_2 coordination geometry was maintained and binding at histidine or other nitrogen- or oxygen-donor ligands was not evidenced in the EXAFS spectrum. The structure around the gold ions in BSA-$(AuSTm)_{2.65}$ is in excellent agreement with the Mössbauer data for $BSA(AuSTm)_{1.3}$ which resolved two slightly different Au^IS_2 coordination environments. In both complexes the weak- and strong-binding sites must be populated.

The data are consistent with structure models which entail only sulfur ligands, but cannot distinguish between bridging or terminal thiolates. The $AuS_{terminal}$ bond length of 228 pm in $Na_3[Au(S_2O_3)_2]$ is indistinguishable from the $AuS_{bridging}$ distance of 230 pm determined for AuSGt.[11] Therefore, bond distances cannot be used to assign the mode of sulfur coordination in the albumin complex.

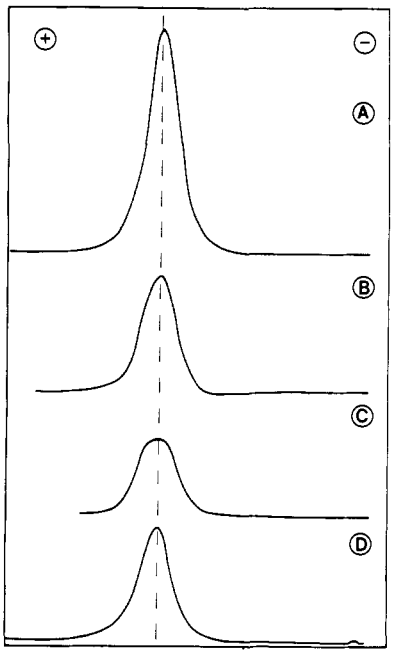


Figure 5. Electrophoresis. BSA (600 μM) was incubated with AuSTm or AuSTg and subjected to acetate strip electrophoresis in Tris/barbital buffer, pH 8.6, 1 mA/strip for 1 h: (A) BSA control; (B) 1.2:1 AuSTm/BSA; (C) 12:1 AuSTm/BSA; (D) 12:1 AuSTg/BSA. Half-height peak width of the densitometer tracings (average of three tracings each of two independent separations): (A) 12.0 mm; (B) 14.5 mm; (C) 15.3 mm; (D) 11.0 mm. The broadening of the AuSTm-incubated albumin samples indicates that a second species of greater net charge is present.

Electrophoresis. To further examine the possible bonding models, acetate strip electrophoresis studies were conducted. The electrophoretic mobility of a protein is proportional to the net electrical charge and inversely proportional to the frictional coefficient which depends on the shape and degree of aggregation of a protein. Albumin, which has a net charge of 18–, migrates rapidly toward the positive pole of the electric field. In particular it was necessary to definitively test model **2**, which is inconsistent with the chemistry of gold(I), but is still occasionally cited in the literature.[38] The electrophoresis also provided a means to test models **4** and **5** which are dimeric and polymeric, respectively. Model **2** predicts that there should be no change in the net charge of the protein and therefore no alteration of its mobility. Model **4** increases the negative charge by $^1/_2$– per albumin and model **5** does not alter the charge, but for both models **4** and **5** the aggregation of the protein should decrease its mobility. The remaining models all increase the net negative charge by an amount depending on the number of gold and thiomalate moieties bound. At the pH of the electrophoresis experiments, the charge of the thiomalate is approximately 3– when the thiol is deprotonated. For the remaining models, the increased charge upon complexation would be **1** (3–), **3** (1–), **6** ($-1 - 3m + n$), **7** ($-2n - 3$), **8** ($-2n + 2$), and **9** ($2n$–), where n and m are defined by the stoichiometry of the models.

The commercial defatted BSA preparations used for these studies were microheterogeneous mixtures of mercaptalbumin (up to 70%), various disulfides (ca. 30%), dimer (ca. 2%), and various minor components.[9] The symmetrical BSA electrophoresis pattern (Figure 5) demonstrated that the disulfide forms and mercaptalbumin had similar mobilities, as previously reported.[9] When the BSA was incubated with 1.2 AuSTm per albumin, the electrophoresis band was significantly broadened, with a distinct front-running (more mobile) component, indicating that the gold–albumin complex was more negative than the albumin. Incubation with 12 AuSTm per mol of albumin produced a more pronounced front-running component. The experiments were

(38) Lorber, A.; Simon, T. M. *Gold Bull.* **1979**, *12*, 149–158.

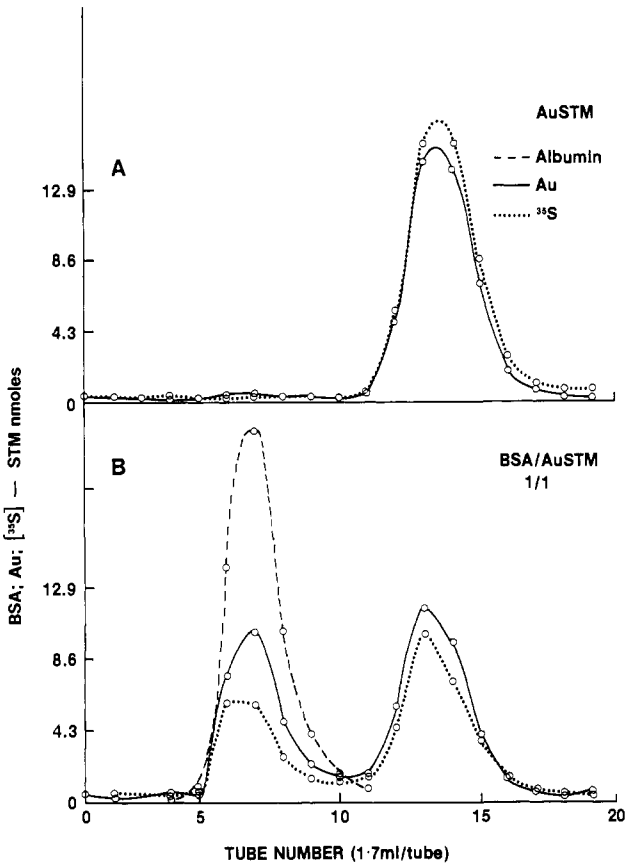


Figure 6. Gel-exclusion chromatography. (A) 100 μM [^{35}S]AuSTm was chromatographed on Sephadex G-75, yielding a single peak. Recoveries: Au, 74%; ^{35}S, 79%. (B) BSA (100 μM) incubated with [^{35}S]-AuSTm (100 μM, 0.166 μCi/mmol) for 13 h and factionated, demonstrating the incorporation of the thiomalate into the gold–albumin complex. Recoveries: BSA, 97%; Au, 80%; ^{35}S, 57%.

repeated with nondefatted albumin, producing identical results. The incomplete conversion of the albumin into the more mobile component, even at very large AuSTm/albumin ratios, was consistent with the expected low affinity of methionine, disulfides, and other amino acid side chains for gold(I) and provided indirect evidence to support cys-34 as the principal binding site.

The increased mobility of the complex is consistent with a model having a more negative charge than the albumin, by which models **2** and **5** are unambiguously eliminated. Also the slightly increased negative charges predicted by models **3** and **4** would not alter their mobility sufficiently to permit them to be resolved from uncomplexed albumin. The increased mobility is consistent with model **1** and, where n and m are small values, models **6–9**. Values of n greater than 3 or 4 would substantially increase the negative charge, resulting in greater resolution of the complex from unreacted albumin than was observed here.

Gel-Exclusion Chromatography. To investigate directly whether the thiomalate ligand is displaced from gold or incorporated into the gold–albumin complex (in the absence of other ligands and chemical species which are present in serum), ^{35}S-labeled AuSTm was allowed to react with BSA. At various times after initiation of the reaction, the mixture was fractionated by gel-exclusion chromatography to separate the gold–protein complex from the unreacted gold and any ligand which might be displaced. Both gold and [^{35}S]thiomalate were progressively taken up by the protein (Figure 6). Thus, the presence of the ligand in the gold–protein complex was clearly demonstrated. The gold–protein complex was not resolved from the unreacted BSA, confirming previous findings that the complex is monomeric with respect to the albumin.[5-7] Complexation to one or several units of AuSTm would not increase the effective hydrodynamic volume of the protein, and resolution of the complex from unreacted protein would not be expected, unless aggregation of the protein occurred. Thus the dimer and oligomer, models **4** and **5**, are not credible

Table III. Gold and [^{35}S]TmSH Binding to BSA: nmol/nmol of Protein[a]

	AuSTm/BSA					
	25:1[b]		10:1[b]		1:1[b]	
time, h	[^{35}S]-Tm-SH	Au	[^{35}S] TmSH	Au	[^{35}S] TmSH	Au
0.5	0.12	0.10	0.051	0.051	0.015	0.020
1.0	0.21	0.224	0.10	0.13	0.043	0.057
3.0	0.38	0.38	0.20	0.23	0.10	0.15
5.0	0.47	0.45	0.26	0.29	0.15	0.20
8.0	0.53	0.52	0.32	0.37	0.19	0.25
20.0	0.59	0.55	0.36	0.46	0.28	0.42

	AuSTm/GSA			
	1:1[c]		1:1[b]	
time, h	[^{35}S]TmSH	Au	[^{35}S]TmSH	Au
2	0.30	0.25	0.11	0.09
7	0.50	0.51	0.25	0.26
9	0.49	0.50	0.25	0.33
13	0.60	0.61	0.32	0.38

[a] BSA solutions 118 μM were incubated at 3 ± 1 °C with various mole ratios of AuSTm and sampled at stated intervals by the Ba^{2+} precipitation–filtration technique. [b] BSA (lot 12F-9365) 0.45 SH/BSA. [c] BSA (lot 41F-9300) 0.60 SH/BSA.

models for the albumin–gold complex. The presence of thiomalate in the complex is inconsistent with models **2**, **3**, **4**, and **5**, but consistent with models **1** and **6–9**.

The recoveries of gold and ^{35}S in the chromatographic fractions were low (Au, 70–90%; ^{35}S, 50–80%; vs. 97–100% for albumin) and, therefore, precluded the accurate stoichiometric measurements which would be required to distinguish model **6** from models **1** or **7–9**. A cursory investigation established that large amounts of ^{35}S label were taken up by the Sephadex, in an apparently irreversible manner. The extent of binding increased with the time of exposure. Neither extensive flushing with buffer nor treatment with unlabeled AuSTm, thiomalate, dithiothreitol, or BSA reversed the binding. DEAE-Sephadex, polystyrene ion exchange, and Bio-gel exclusion resins, which showed similar or greater avidity for the gold and label, could not be substituted. Therefore, the effort to undertake quantitative chromatographic studies was abandoned.

Precipitation–Filtration Studies. To circumvent the problems with chromatographic techniques, an alternate method to separate free and bound gold was developed. It depends on the ability of the dipositive cation, Ba^{2+}, to react with the carboxylate groups of the thiomalate forming an insoluble precipitate, whereas if the gold thiomalate is already bound to albumin, no precipitate results.

$$n\mathrm{Ba}^{2+} + [\mathrm{AuSTm}^{2-}]_n \rightarrow n\mathrm{BaAuSTm}\downarrow \quad (1a)$$

$$\mathrm{Ba}^{2+} + \mathrm{AlbSAuSTm} \rightarrow \text{no reaction} \quad (1b)$$

After precipitation of the AuSTm by Ba^{2+}, filter-disk ultrafiltration of the precipitate yielded a solution of albumin and albumin–gold complex. In the presence of 167 mM Ba^{2+}, the residual concentrations of gold and ligand in the filtrates of protein-free AuSTm solutions were negligible. The protein was not precipitated by this concentration of Ba^{2+}. The time for incubation with Ba^{2+} and filtration (2–4 min) was fast compared to the time for gold uptake by albumin (6–8 h) and was kept as short as possible to prevent the slow loss of bound gold which occurred during prolonged incubation.

By use of the Ba^{2+} precipitation–filtration method, gold and ligands were found to be taken up in a ratio approximating 1:1 when 118 μM BSA and 2.95 mM AuSTm were employed (Table III). Uptake continued over a period of 10–12 h before a plateau was reached. The ratios of gold bound approached the level of mercaptalbumin and never exceeded a 1:1 Au:BSA ratio, even in the presence of the 25-fold excess of AuSTm. This result indicated that the weak-binding site was not populated after the Ba^{2+} reaction.

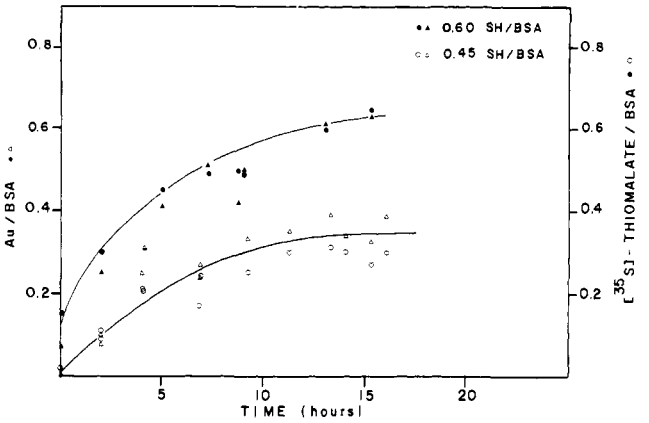


Figure 7. Gold uptake for two albumin preparations (118 μM) having different SH titers. Samples were incubated with [^{35}S]AuSTm (118 μM) and aliquots were withdrawn periodically over 16 h and separated by the Ba^{2+} precipitation–filtration procedure.

With the same BSA and lower AuSTm concentrations, the initial parallel uptake of ligand and gold over 5–8 h was followed by a plateau in the level of bound ^{35}S and a kinetically distinct uptake of gold without the ligand.

The uptake studies were conducted aerobically, and it is possible that the second step, uptake of gold without ligand, is due to a spurious oxidation of the gold. XANES spectra demonstrated that gold(III) reacts with and binds to albumin undergoing partial reduction to gold(I). With constant [BSA], the second step was most rapid at the 1:1 ratio of AuSTm to BSA, slower at the 10:1 ratio, and very slow or negligible at the 25:1 ratio, suggesting that excess AuSTm might inhibit the putative oxidation. Detailed kinetic studies of the two processes are in progress and will be reported elsewhere.

During the early stages, the reaction can be represented by

$$\text{AuSTm} + \text{BSA} \rightarrow \text{BSA–AuSTm} \qquad (2)$$

This result directly confirmed Gerber's pioneering study on the stoichiometry of the complex and established that that ligand was intimately bound to the gold(I) moiety during the initial uptake period.

To examine the influence of cys-34 on the binding of gold, the reaction was carried out under identical conditions (118 μM BSA and 118 μM AuSTm) using two different commercial BSA preparations (Figure 7 and Table III). One had a SH/BSA ratio of 0.60 ± 0.02 and the second 0.45 ± 0.02. Gold and ^{35}S binding were monitored for 16 h. The former reached a constant value of ca. 0.61 Au/BSA and the latter a value of 0.38 Au/BSA, demonstrating that gold binding is proportional to the level of reduced SH in the protein.

AuSTm–Dansyl Dye Competition Studies. Gold thiomalate exists in solution as oligomers, probably 6- to 14-mers, which might bind to albumin at one of its anion-binding sites. In particular, the second and weaker binding constant might be due to a loose association of this nature (model **9**). Two anion-binding sites on albumin, conventionally labeled I and II, can be distinguished and studied by their respective specificities for the fluorescent dye probes, dansylamide (5-(dimethylamino)-1-naphthalenesulfonamide, abbreviated DA) and dansylsarcosine (5-(dimethylamino)-1-naphthalenesulfonyl-*N*-methylglycine, abbreviated DS).[24,25] Competition of drugs and chemical agents for either site and allosterically generated conformational changes in either site can be monitored fluorimetrically since DA and DS fluoresce in response to excitation at 335 nm only when they are protein bound. The concentrations of BSA, and DA or DS, each ca. 20 μM, were chosen to give approximately 50% bound dye and 50% complexed albumin, conditions which maximized the sensitivity of the fluorescent measurement to displacement of the dyes from albumin.

When the albumin–dye complexes were allowed to react with AuSTm at 0.55, 1.10, or 1.65 mol ratios of AuSTm per albumin,

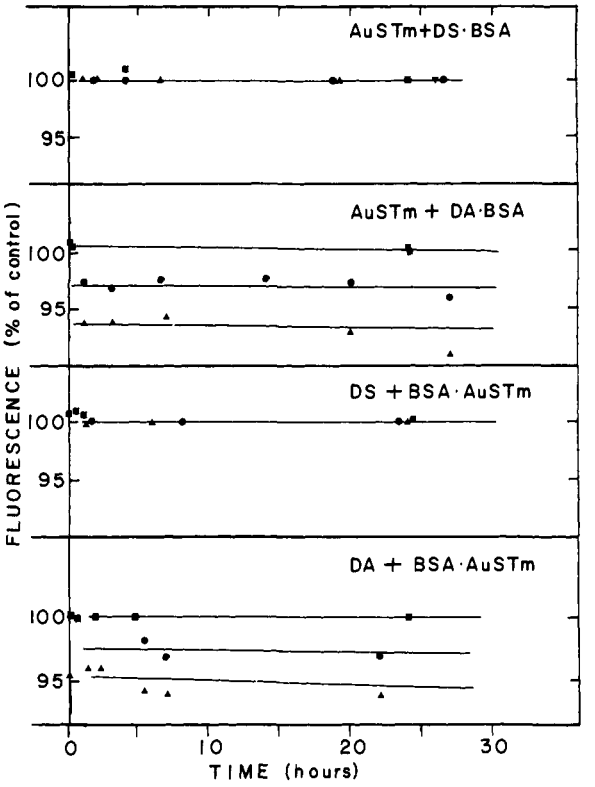


Figure 8. Fluorescent dye/AuSTm competition studies. The fluorescence of the BSA (19 μM)/AuSTm (10 μM–9.5 mM)/DA (20 μM) or DS (19.5 μM) system presented as % of control (without AuSTm) vs. time. Over 24 h, no change is observed in DS fluorescence with 0.55–500-fold AuSTm. Small decreases (2 and 5%) in the fluorescence of DA were observed for 100- and 500-fold AuSTm, in both the reaction of DA with preequilibrated AuSTm/BSA and the reaction of AuSTm with preequilibrated BSA/DA. The changes occurred instantaneously and direct quenching of DA fluorescence by AuSTm cannot be excluded as a possibility. ((■) 0.55, 1.10, and 1.65 AuSTm/BSA; (●) 100 AuSTm/BSA; and (▲) 500 AuSTm/BSA.)

the DA·BSA and DS·BSA complexes maintained the same degree of dye binding as controls to which only buffer was added (Figure 8). There was no change in the percent of bound dye over a 24-h period. Thus, AuSTm does not displace the DA from site I or DS from site II nor does it induce allosteric changes which enhance or decrease the extent of dye binding. Since these results could be caused by the inability of the gold to displace one or the other of the dyes once they are bound, the reactions of the dyes with preequilibrated albumin–gold complex were measured. The extent of dye binding to the albumin–gold complex was the same as to albumin alone immediately upon mixing and after 24 h. Thus, low levels of gold bound to the protein neither prevent nor kinetically retard the binding of DA at site I and DS at site II and do not induce any conformational changes to which the dyes are sensitive.

Only with very large excesses of AuSTm (100–500-fold) was a small decrease in fluorescence of the bound DA observed (Figure 8). The fluorescence was 97% of control at 100:1 and 92% at 500:1 AuSTm/BSA. The effects were observed immediately and did not change significantly over 24 h. There was no change in the emission profile of albumin-bound DA or DS in the presence of the 500-fold excess of AuSTm.

When the weakest published binding constant for gold, $K_2 = 2 \times 10^2$, and the DA binding constant, $K_b(\text{DA}) = 5.6$ μM, were used, it was calculated that for direct competition a 25% decrease of fluorescence would be observed at the 100:1 ratio and 55% at the 500:1 ratio. Thus, gold did not bind directly at anion sites I or II. The UV–visible spectra of DA and DS were perturbed very slightly and to the same extent by incubation with 100- and 500-fold excesses of AuSTm, thus ruling out as the cause of reduced fluorescence with DA any direct unique reaction of it with AuSTm (which would have shifted the equilibrium for BSA·DA

formation toward free DA). The reduction in fluorescent intensity for DA may result from weakly bound AuSTm at a site near drug binding site I, from an allosterically induced change in the conformation of drug binding site I, or from quenching of the DA fluorescence by AuSTm. Since it occurred only with very large amounts of AuSTm and more rapidly than the binding of AuSTm to albumin, further experiments to ascertain its origin were not undertaken.

Discussion

Spectroscopy. The experiments reported here demonstrate the utility of EXAFS/XANES and ^{197}Au-Mössbauer spectroscopies for studying macromolecular complexes of gold. The data obtained by the two techniques are complementary, and for the albumin–gold thiomalate complexes are in complete agreement regarding the coordination sphere of gold.

The XANES spectrum determines the oxidation state of gold by the presence or absence of characteristic peaks in the near-edge region of the spectrum, 11 900–12 000 eV. EXAFS determines the nearest-neighbor environment of gold, including the atomic number of the neighboring atoms and the number of each type of atom in the coordination sphere. It does not, however, provide information about bond angles and sometimes cannot distinguish adjacent elements of the periodic table, e.g., P and S, or O and N. The ^{197}Au-Mössbauer isomer shifts and quadrupole splitting parameters are sensitive to the chemical nature of the ligands (e.g., phosphine vs. thiolate), although the range of values overlap for some classes of ligands. Coordination numbers and geometries are determined by empirical comparisons of the IS and QS parameters to those for crystallographically characterized compounds. Because the values of QS and IS differ for linear two-coordinate Au(I), trigonal-planar three-coordinate Au(I), tetrahedral four-coordinate gold(I), and square-planar gold(III), the geometric arrangement of the ligands can be inferred. When EXAFS and Mössbauer are utilized in tandem, the uncertainties inherent in either technique are minimized. For example, the Mössbauer spectra for the gold–albumin complexes rule out chloride or phosphine ligation and the EXAFS conclusively eliminates the presence of 2nd period ligand atoms, O or N. Thus the conclusion that gold at the weak- and strong-binding sites is gold(I) coordinated by two sulfur ligands is very firmly established by the EXAFS and Mössbauer data.

Gold-Binding Sites. The results obtained from the spectroscopic and protein chemistry experiments can be used to test structures **1–9** as models for the thermodynamically strong and weak gold-binding sites on albumin. The models must account for the following new observations: (1) Gold is taken up into the complex in the +1 oxidation state. (2) More than 1 mol of gold can be taken up per mol of mercaptalbumin. (3) All of the bound gold, whether more or less than 1 Au/Alb–SH, is present in AuS_2 coordination environments. (4) Thiomalate is incorporated into the complex in ratios approximating one thiomalate per gold(I). (5) A substantial proportion but not all of the albumin molecules react with gold to form a more negatively charged complex. (6) Not all albumin molecules react with AuSTm, even when it is present in excess. (7) Below a ratio of 1 bound gold per albumin, AuSTm does not compete with or alter the binding constant of DA at anion site I or DS at anion site II; at very large ratios (100-fold) of AuSTm to albumin, limited displacement or quenching of DA, but not DS, was observed. (8) The amount of gold which binds tightly is determined by the SH titer of the protein.

It is immediately possible to eliminate conclusively five of the models. Model **2**, in which gold is presumably hydrated, and model **3**, involving coordination by a non-sulfur ligand, are inconsistent with the Mössbauer and EXAFS data requiring an AuS_2 coordination environment and also cannot explain the incorporation of thiomalate into the complex. The dimer and oligomer, models **4** and **5**, although consistent with the EXAFS and Mössbauer data, are conclusively ruled out by the incorporation of thiomalate into the complex and by the electrophoresis and chromatographic data establishing that the complex contains only a single albumin. They are also implausible on steric grounds, particularly in regard to the ESR spin-label study, concluding that cys-34 is located in a cleft approximately 950 pm (9.5 Å) deep.[39] Model **6** is consistent with the uptake of thiomalate into the complex and the increased charge of the gold complex. However, clusters analogous to copper structures, e.g., $Cu_4(SR)_7^{3-}$, which would involve three-coordinate gold, are not in agreement with the spectroscopic data. The remaining models, **1**, **7–9**, are consistent with the EXAFS, Mössbauer, electrophoresis, and ^{35}S tracer studies.

The possible sulfur donor groups of the protein are cysteine, cystine, and methionine. BSA contains 6 methionines and a total of 35 cysteine residues, which are present in 17 disulfide linkages essential to the tertiary structure and the single free sulfhydryl, cys-34. The latter is sometimes bound in disulfide linkages which are partially responsible for the microheterogeneity of the albumin. Unlike thiols, neither disulfides nor thioether residues react directly with $[AuSTm]_n$ to form simpler complexes.[16] Thus, the finding that gold binds to albumin in AuS_2 coordination environments with retention of the thiomalate ligands must result from (i) binding of intact AuSTm oligomers or (ii) reactions at cys-34. Reaction at cys-34 has generally been assumed, and two previous experiments provide supporting, but not conclusive, evidence for it. Modification of cys-34 with *N*-ethylmaleimide and other reagents reduced the amount of gold taken up by the albumin.[5] After AuSTm reacted with albumin, the sulfhydryl interchange-dependent denaturation of albumin at 95 °C was blocked.[18] Although both experiments can be explained by reaction at cys-34, neither experiment provides conclusive evidence, since the same results could be obtained if the gold-binding site were in close proximity to cys-34.

The Strong-Binding Site. Considering first the tight-binding site, model **8**, in which albumin replaces a thiomalate from an $[AuSTm]_n$ cluster, and model **9**, in which intact $[AuSTm]_n$ clusters bind noncovalently, predict that a small fraction of the albumin molecules should each be associated with large aggregates of AuSTm. The number of albumin molecules complexed by gold given by eq 3, where n is the average number of gold(I) ions in

$$\text{fraction of complexed albumin} = (1/n)[\text{Au}]_{\text{bound}}/[\text{BSA}] \quad (3)$$

the aggregates, would be a small fraction of the total. For a preparation in which $[Au]_{bound}/[BSA] \simeq 0.7$ and on the assumption that n is 8 (the average size of the oligomers of H_2-AuSTm in aqueous solution), approximately 10% of the albumins should be complexed. The net charge of $[AlbSAu_8(STm)_7]$ would be 34–. Thus, model **8** predicts that a very small fraction of the albumin should undergo a dramatic increase in electrophoretic mobility (approximately doubling) upon complexation with AuSTm. Figure 5 demonstrates that approximately half the albumin molecules have a marginally (15–20%) increased mobility after reacting with AuSTm, a result which is inconsistent with the prediction. The same argument also precludes binding of long chains of AuSTm (model **7** when n is large) for the tightly bound gold. Therefore models **7–9** with large values of n are inconsistent with the electrophoresis result. The fluorescent probe (DA and DS) studies demonstrate that AuSTm does not bind at the anion sites I or II, further strengthening the case against **9** for the strong-binding site.

For the tight-binding site, structure model **1** is consistent with all of the data gathered to this point: the Mössbauer and EXAFS data requiring gold(I) coordinated by two sulfur atoms, the presence of thiomalate in 1:1 ratio with the gold; the electrophoresis data showing that a large fraction of the albumin molecules bind gold with a consequent increase in the charge and electrophoretic mobility, while some molecules do not react at all; and most importantly, the increased amount of gold taken up by albumin preparations with larger SH/BSA ratios.

The EXAFS and Mössbauer results are the first spectroscopic evidence regarding the ligation of gold in the complex and are fully consistent with cys-34 as the site of gold binding on albumin.

(39) Hull, H. H.; Chang, R.; Kaplan, L. J. *Biochem. Biophys. Acta* **1975**, *400*, 132–136.

Thus we propose that the reaction occurs at cys-34, with the formation of a mixed bis(thiolato)gold(I) species:

$$\text{AlbSH} + (1/n)[\text{AuSTm}]_n \rightarrow \text{AlbS–Au–STm} + \text{H}^+ \quad (4)$$

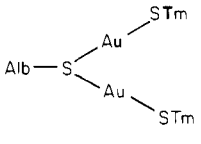
This conclusion agrees with the models previously put forth by this[1,40] and other laboratories.[18]

The Weak-Binding Sites. The preparation and isolation of albumin–gold complexes with more than 1 mol of gold per protein molecule definitively confirm the predictions of the thermodynamic studies that two classes of binding sites exist. For the Mössbauer and EXAFS studies, complexes of formula $Alb(AuSTm)_{1.3}$ and $Alb(AuSTm)_{2.65}$, respectively, were obtained. From the characterization it is clear that the additional gold atoms bind in AuS_2 coordination environments, that the albumin remains monmeric, and that there is no gold binding at anion sites I or II. From these observations and the logic applied to the strong-binding site, models **2–6** can be eliminated. It is not possible to maintain the AuS_2 coordination environment unless thiomalate is taken up with each gold and, therefore, only models **7–9**, which maintain the 1:1 stoichiometry of gold and thiomalate and AuS_2 coordination geometry, will be considered further.

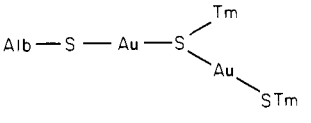
The evidence does not allow a totally unambiguous choice between models **7–9** for the weak-binding site. In the precipitation filtration studies using Ba^{2+} to remove unbound AuSTm from solution, the weakly bound gold was apparently precipitated along with free oligomer, since the $[Au]_{bound}/[BSA]$ ratio approached the fraction of mercaptalbumin (Table III), whereas the chromatographically separated samples yielded ratios greater than 1. This result indicates that the loosely bound gold is labile and may explain why a small increase in the amount of complexed albumin, but not a significant increase in its mobility, was observed during electrophoresis of samples incubated with a 12-fold excess of AuSTm. Thus neither the Ba^+ precipitation–filtration nor the electrophoresis results provide insight into the charge, stoichiometry, or ligation of gold bound at the weak-binding sites.

The lack of any effect of gold on DS binding demonstrated that AuSTm oligomers do not bind at anion site II. The marginal decrease in fluorescence of DA at site I is considerably smaller than that predicted for even the weakest gold-binding constant reported ($K_2 = 5 \times 10^2$), ruling out direct competition at site I. $[AuSTm]_n$ aggregates would experience electrostatic repulsions due to the net −18 charge of albumin which would counter weak noncovalent interactions. Although the data do not disprove the possibility of noncovalent binding (i.e., $(AuSTm)_n$·AlbSAuSTm), the fluorescence experiments which directly probe the most probable sites—i.e., anion sites I and II—and electrostatic considerations argue strongly against it.

Model **7** will accommodate the binding of an additional AuSTm monomer unit with linear AuS_2 coordination geometry, if the sulfur of cys-34 or the thiomalate in model **1** acts as a bridging sulfur atom (**7a** or **7b**, respectively).



7a

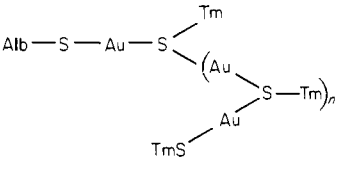


7b

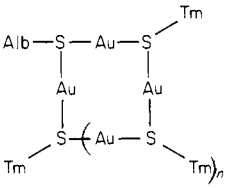
Additional AuSTm units can be accommodated in the same manner by binding to the terminal AuSTm thiolate sulfur atoms of **7a** or **7b**. Bridging sulfur atoms have been postulated to explain the oligomeric structure of $[AuSTm]_n$[11,12] and the formation of thiolate-bridged mixed-metal gold–mercury clusters of albumin and cysteine.[41]

In the Mössbauer data for $BSA(AuSTm)_{1.30}$, the two gold coordination environments obtained by computer deconvolution of the broad signal had equal populations, in agreement with model **7** and the stoichiometry of the complex: the mercaptalbumin fraction, 0.65, and gold/protein ratio, 1.30, corresponded to the formula $Alb–S(AuSTm)_2$ for the complex. The isomer shift difference of the two gold environments, 0.18 mm/s, could arise from **7a** only if the protein environment about cys-34 were sufficiently asymmetric. The unique gold environments in **7b**, where one gold has albumin as the terminal thiol and the other has a thiomalate, are more reasonable as the source of the 0.18 mm/s isomer shift difference. The data do not eliminate either possibility.

The EXAFS sample $BSA(AuSTm)_{2.65}$ corresponds to 7.3 gold thiomalates per mercaptalbumin, $AlbS(AuSTm)_{7.3}$. Although the number of gold units is large, the thermodynamic study of Mason predicted 1 tight-binding site and 6.6 weak sites.[7] Kamel et al. have reported the binding of 3.5 gold atoms to albumin after incubation with AuSTm.[19] From consideration of these results and the three binding studies, it is apparent that the number of weakly bound gold atoms will vary with the experimental procedures employed to prepare and isolate the complex. Structures consistent with the variable extent of gold binding and gold coordination chemistry can be obtained by successive addition of AuSTm units to the terminal thiolate sulfur of the previously bound thiomalates or cys-34, to generate a linear array, **7c**, or a cyclic structure, **8a**. Model **8a** differs from model **7c** by the



7c



8a

loss of one thiomalate with concomitant closure to a cyclic structure in which each thiomalate bridges adjacent gold atoms. Although the open structure of **7c** is more consistent with the lability of the weakly bound gold, model **8a** cannot be ruled out by the data presently available.

Relationship to Chrysotherapy. The concentration of albumin in serum is 590 μM, while gold, during chrysotherapy, rarely exceeds 50 μM. Thus gold should be bound primarily at the strong-binding site, cys-34. The structure proposed for the strong-binding site, AlbSAuSTm, predicts retention of the thiomalate ligand, contrary to the results obtained in vivo by Jellum and Munthe, who observed displacement or dissociation of thiomalate from the albumin and other protein-bound forms of gold.[21] The dissociation of the thiomalate, eq 5, contradicts the result obtained in this study, formation of AlbSAuSTm, eq 4. The displacement of thiomalate in vivo by ligand exchange at the protein-coordinated gold could occur according to eq 6, where

$$\text{AuSTm} + \text{AlbSH} \rightleftharpoons \text{AlbSAu} + \text{TmSH} \quad (5)$$

$$\text{AlbSAuSTm} + \text{RSH} \rightleftharpoons \text{AlbSAuSR} + \text{TmSH} \quad (6)$$

RSH represent an endogenous thiol, such as glutathione or cysteine.

Reaction 6 is expected from the inorganic chemistry of gold(I) thiolates.[1,2,3] Although the endogenous levels of reduced cysteine and glutathione in the serum are very small, they are in equilibrium with large body pools, which should drive the equilibrium of reaction 6 to the right. The metabolic degradation of thiomalate, as reported by Turkall,[42] must also have the effect of driving the reaction to the right. Danpure has reported that the nonprotein-bound forms of gold in serum 24 h after the administration of AuSTm are complexed to ligands other than thiomalate,[43] possibly cysteine and glutathione.

Abbreviations. AuSCy, AuSGt, AuSTg, and AuSTm represent the 1:1 gold(I) thiolates of cysteine, glutathione, thioglucose, and thiomalate, respectively. The corresponding thiols are abbreviated

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(41) Danpure, C. J.; Fyfe, C. A. *Biochem. Soc. Trans.* **1976**, *4*, 631.
(42) Turkall, R. M. Ph.D. Thesis, The Ohio State University, 1979.
(43) Lawson, K. J.; Danpure, C. J.; Fyfe, D. A. *Biochem. Pharmacol.* **1977**, *26*, 2417–2426.

CySH, etc. BSA represents the microheterogeneous preparation of bovine serum albumin; Alb–SH, the mercaptalbumin component (0–65%) in which cys-34 is present in reduced form. Other abbreviations include the following: DS = dansylsarcosine, DA = dansylamide, IS = isomer shift, QS = quadrupole splitting, Γ = Mössbauer line width, and RSH = generic thiol.

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Registry No. AuSTm, 4846-27-9; AuSCy, 74921-06-5; AuSGt, 89827-22-5; Au, 7440-57-5.

Supplementary Material Available: Fourier-filtered EXAFS spectrum of $BSA(AuSTm)_{2.65}$ and transformed spectrum of $BSA(AuSTm)_{0.44}$ (2 pages). Ordering information is given on any current masthead page.

Influence of Temperature on the Conformation of Ions Undergoing Electron Impact Induced Fragmentation

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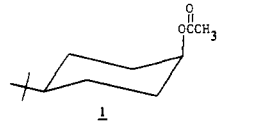
Abstract: The conformational mixture from which hydrogen abstraction occurs during the electron impact induced elimination of diethyl malonate from diethyl cyclohexylmalonate (**8**) was determined at several ion source temperatures by comparison of the mass spectra of several deuterated analogues of **8** to the mass spectra of the deuterated analogues of diethyl *trans*-4′-*tert*-butylcyclohexylmalonate (**4**) and diethyl *cis*-4′-*tert*-butylcyclohexylmalonate (**3**). It was found that the conformational distribution is closely correlated with that predicted from solution chemistry data and ion source temperature.

Mass spectroscopists have developed a variety of interesting and powerful instrumental techniques to permit study of the structure and mechanisms of fragmentation of ions after electron impact.[1] However, conceptually simpler approaches rooted in the techniques used by chemists for the study of neutrals and their reactions in more conventional environments have been less thoroughly exploited. For example, an approach frequently used in the study of conformationally mobile systems in solution is to compare their properties and behavior to conformationally immobile systems frozen into the specific conformations attainable by the mobile system. Since most cyclohexyl derivatives can exist in two chair conformations of well-defined geometry, and since accurate conformationally immobile models for these structures are available, this approach has been particularly useful when applied to the solution chemistry behavior of cyclohexyl and related systems.[2] Nevertheless, this methodology has found comparatively little application to mass spectrometric studies.

Recently, a variety of studies have suggested that this general approach might be applicable to studies of the mass spectral behavior of cyclohexyl derivatives.[3] It has been established, for example, that the McLafferty rearrangement of *cis*- and *trans*-4-*tert*-butylcyclohexyl acetate (**1** and **2**) and diethyl *cis*- and *trans*-4′-*tert*-butylcyclohexylmalonate (**3** and **4**) involve hydrogen abstraction predominantly from the most stable chair conformations and proceed with high but opposite stereochemistries.[3a,e]



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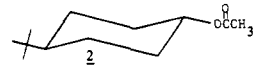

2

3

4

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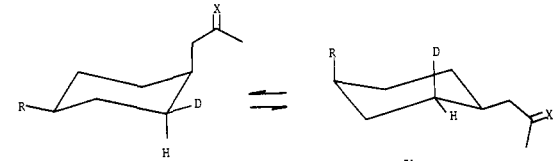
The high stereospecificities demonstrate that the 4-*tert*-butyl group effectively locks the conformation of the cyclohexyl ring prior to hydrogen abstraction and that the ring structure retains its integrity, necessary requirements for use of this technique. The observation of very predominant cis hydrogen abstraction from axial derivatives (e.g., **1** and **3**) and trans hydrogen abstraction from equatorial derivatives (e.g., **2** and **4**) provides a simple diagnostic for the stereochemistry of a reacting, conformationally mobile system. Thus, the relative amounts of cis and trans elimination from conformationally mixed systems such as **5** will



5a 5b

be revealed by the relative amounts of deuterium vs. protium elimination observed. This methodology has been used to study the mass spectral behavior of a series of 4-substituted cyclohexylmalonates and acetates,[3e] leading to the important empirical observation that the influence of structural variation on the population of fragmenting ions could be accurately predicted,

(1) See, for example: (a) Kingston, D. G. I.; Bursey, J. T.; Bursey, M. M. *Chem. Rev.* **1974**, *74*, 215–242. (b) Budzikiewicz, H.; Djerassi, C.; Williams, D. H. "Mass Spectrometry of Organic Compounds"; Holden-Day: San Francisco, CA, 1967. (c) Beynon, J. H.; Saunders, R. A.; Williams, A. E. "The Mass Spectra of Organic Molecules"; Elsevier: Amsterdam, 1968.

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