Formation and Characterization of Aurothioneins: Au,Zn,Cd-Thionein, Au,Cd-Thionein, and (Thiomalato-Au)$_x$-Thionein†

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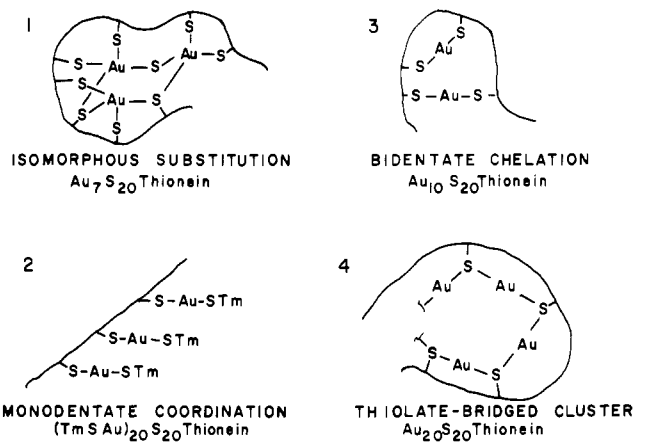
ABSTRACT: Three gold-containing thioneins (Au,Zn,Cd-Th, Au,Cd-Th, and (TmSAu)$_x$Th, where Th = thionein and TmS = thiomalate) have been prepared by the reactions of horse kidney Zn,Cd-thionein with gold thiomalate (AuSTm). When thionein was present in excess, the thiomalate ligand was displaced and the protein chelated the gold in a bidentate fashion. Primarily zinc but also some cadmium was displaced to form Au,Zn,Cd-Th or Au,Cd-Th. Excess AuSTm reacted to form (TmSAu)$_x$-thionein with monodentate coordination of the protein to each bound gold, retention of the thiomalate, loss of zinc and cadmium, and an increase in the Stokes radius of the product. EXAFS/XANES studies of Au,Zn,Cd-Th and (TmSAu)$_x$Th established that the oxidation states and coordination environments of gold were Au(I)S$_2$ and that the gold–sulfur bond distances were 229 and 230 pm, respectively. Radioimmunoassay established that the aurothioneins retained their antigenicity to native metallothionein antibodies. Metal exchange reactions with gold were complete within 5–10 min when Zincon or 4-(2-pyridylazo)resorcinol was used to monitor Cd^{2+} and Zn^{2+} displacement.

Gold(I) thiolates are important chemotherapeutic agents in the treatment of rheumatoid arthritis (David & Harth, 1981), but only recently has their biochemistry been studied extensively (Sadler, 1976; Shaw, 1979; Brown & Smith, 1981). They react with proteins and low molecular weight ligands in the serum (Danpure et al., 1979), kidneys (Shaw, 1979; Sharma & McQueen, 1982), and liver (Lawson et al., 1977), and a chemical model for the reactions of gold(I) drugs with protein sulfhydryl groups (e.g., Cys-34 of serum albumin) has been proposed (Schaeffer et al., 1980; Shaw, 1981).

Metallothionein, a low molecular weight, thiol-rich protein of the kidney and liver, has been implicated in the metabolism of cadmium, zinc, copper, and other metals (Kägi & Nordberg, 1979). After the administration of gold(I) or gold(III), liver and kidney contain mixed-metal thioneins with bound gold and copper, zinc, or cadmium depending on the experimental condition (Mason, 1983; Sharma & McQueen, 1982; Lawson et al., 1977; Schmitz et al., 1980; Thompson et al., 1978; Shaw et al., 1979). Thus, it may be a site of interaction of gold with the essential trace elements, particularly zinc. Several studies have examined in vivo the interactions of gold(I) with zinc, copper, and cadmium including the changes in levels of thionein-bound metals (Mason, 1983; Sharma & McQueen, 1982; Lawson et al., 1977; Schmitz et al., 1980; Thompson et al., 1978; Shaw et al., 1979). Metallothionein contains 20 cysteines and no aromatic amino acids among its 61 residues and binds seven zinc or cadmium ions (Kägi & Nordberg, 1979). The ^{113}Cd NMR spectrum of the rabbit liver protein was interpreted in terms of two proposed metal clusters, M_4S_{11} and M_3S_9, where S represents the protein cysteinato ligands (Otvos & Armitage, 1980). Gold(I) thiomalate (AuSTm)[1] reacts with native horse kidney Zn,Cd-thionein to displace zinc, and when large excesses of AuSTm were employed, cadmium could be displaced in an equilibrium reaction (Schmitz et al., 1980; Shaw, 1980).

Three possible bonding models have been previously discussed for thionein-bound gold(I) (Shaw, 1980; Schmitz et

Chart I



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[1] Abbreviations: AAS, atomic absorption spectroscopy; AtgSH, 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (or tetraacetylthioglucose); AuSTm, sodium ((*S*)-thiomalato)aurate(I); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EXAFS, extended X-ray absorption fine structure; hk, horse kidney; hmw, high molecular weight; lmw, low molecular weight; PAR, 4-(2-pyridylazo)resorcinol; rl, rat liver; Th, thionein; Tm and TmSH, thiomalate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; XANES, X-ray absorption near-edge spectroscopy; ZI, Zincon or 2-carboxy-2-hydroxy-5′-sulfoformazylbenzene sodium salt. Braces represent the moles of metal or ligand.

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al., 1980) (Chart I). Model 1 is isomorphous displacement of M^{2+}, which, according to the Otvos–Armitage model, would lead to $Au^{I}S_4$ coordination environments. [In our earlier publication, following the hypothesis of Kägi et al. (1974), we described this possibility as AuS_3.] Model 2 is monodentate coordination of gold to a single cysteine with retention of the thiomalate ligand. Model 3 is chelation of gold(I) by two protein sulfhydryl ligands leading to displacement of the thiomalate. Recent structural models for gold(I) thiolates, based on EXAFS and Mössbauer data, suggest a fourth model, a thiolate-bridged gold cluster containing bridging cysteinato ligands and gold(I) in a 1:1 ratio (Hill et al., 1983; Elder et al., 1983).

To learn more about the stoichiometry of the reaction of Au(I) with metallothionein and the structure(s) of the gold(I) coordination environment(s) in the protein, we have isolated and studied the aurothioneins prepared by the reaction of excess AuSTm with metallothionein to produce an all-gold thionein and the reaction of AuSTm with excess thionein to produce mixed-metal (Au,Zn,Cd) thioneins. The characterization included (1) determination of the oxidation state and coordination environment of gold by XANES (X-ray absorption near-edge spectroscopy) and EXAFS (extended X-ray absorption fine structure) spectroscopy, (2) radioimmunoassay examination of the cross-reactivity between native thioneins and aurothioneins, and (3) accurate determination of the stoichiometry of metal exchange with milligram quantities of protein and [^{35}S]AuSTm to trace the ligand.

Experimental Procedures

Materials. Reagents were obtained as follows: from Aldrich Chemical Co., AuSTm and 4-(2-pyridylazo)resorcinol monosodium salt monohydrate (PAR); from Sigma Chemical Co., Tris (Trizma base), DTNB, Sephadexes G-50 and G-75, Bio-Gel P-60, Zincon (2-carboxy-2-hydroxy-5′-sulfoformazylbenzene sodium salt or ZI), and 2-mercaptoethanol. [^{35}S]AuSTm (1.88×10^{-3} mCi/mmol) custom-synthesized by New England Nuclear Corp. was added to unlabeled AuSTm to produce solutions with specific activities of 4.84×10^{-4} mCi/mmol and 2.15×10^{-4} mCi/mmol.

Analyses. Metal contents of solutions were analyzed on Perkin-Elmer 360 and Instrumentation Laboratory 357 atomic absorption flame spectrophotometers. All samples were assayed against serial dilutions of reference standards. Scintillation counting was performed on a Chicago Nuclear Unilux III under standard conditions for $^{14}C/^{35}S$ β radiation (Shaw et al., 1979).

UV–visible spectra were recorded on a Cary 17 UV–vis spectrophotometer. To quantitate the native metallothionein, the ultraviolet absorption spectrum of thionein in 0.1 M HCl was recorded at 220 nm, and the concentration was calculated from the published absorptivity coefficient of $\epsilon_{220} = 47\,300$ L mol^{-1} cm^{-1} (Bühler & Kägi, 1974). For aurothioneins, however, this method systematically overestimates the thionein concentration, since the gold–mercaptide absorbance in the 220-nm region is not eliminated by acidification.

Preparation of Horse Kidney Zn,Cd-Thionein. Immediately after frozen horse kidneys (hk) were thawed, the cortex was diced, homogenized in 2.0 volumes of 250 mM sucrose/20 mM Tris-HCl buffer, pH 8.6, containing 10 mM 2-mercaptoethanol. As previously described, the 23500*g* supernatant was used to homogenize successively a second, third, and fourth kidney (Li et al., 1981). From the resulting supernatant, a 45000*g* supernatant was prepared, subjected to heat treatment, recentrifuged at 45000*g*, and fractionated on an 8-L bed of Bio-Gel P-60 eluted with 20 mM Tris-HCl, pH 8.6. The thionein-containing fractions were lyophilized, redissolved in double-distilled H_2O, and desalted over Sephadex G-25 (2.5 × 60 cm column) eluted with double-distilled H_2O.

After ion-exchange chromatography over DEAE-Sephadex A-25 (5–500 mM Tris-HCl gradient, pH 8.6), the thionein-containing fractions were pooled, analyzed for metal content, and divided into small aliquots that were stored frozen (−30 °C) for further use. Typically, 12.0 mL of solution containing 1–3 mg/mL horse kidney thionein (based on zinc and cadmium content) was obtained and divided into 1- or 2-mL aliquots.

Preparation of Au,Cd-Thionein. In a typical preparation, AuSTm (0.57 μmol) was added to a 2.0-mL solution of hk Zn,Cd-thionein (0.34 μmol of Zn; 1.33 μmol of Cd) in 5 mM Tris-HCl buffer, pH 8.6. After incubation for 1 h, one drop of 0.9% NaCl was added as a marker, and the mixture was placed on a Sephadex G-50 chromatographic column (1.5 × 40 cm) and then eluted with the same buffer at a flow rate of 20 mL/h. Fractions of 3.3 mL were collected and analzyed for Au, Cd, and Zn by AAS. The pooled thionein-containing fractions [$K_d = 0.43$, where $K_d = (V_s - V_0)/(V_t - V_0)$ and V_s, V_0, and V_t refer to the elution volumes of the sample and totally included and totally excluded fractions, respectively] were lyophilized, dissolved in a minimum amount of double-distilled H_2O, and desalted on a Sephadex G-25 column (2.5 × 60 cm) eluted with double-distilled H_2O. This preparation contained gold and cadmium but only a trace of zinc. The thionein fractions were pooled, lyophilized, and stored at −30 °C in an air-tight vial.

The basic procedure was repeated with Au/Zn ratios from 1.0 to 3.0 with 2.0-mL samples of native hk Zn,Cd-Th containing 0.55–18 μmol of Zn + Cd. In two reactions, [^{35}S]-AuSTm (4.84×10^{-4} mCi/mmol) was used.

Colorimetric Analysis of Displaced TmSH and Zn. Aliquots (1.0 mL) of each fraction were added to 1.0 mL of 0.1 M DTNB in 50 mM phosphate buffer, pH 7.2, incubated for 1 h to allow complete reaction with the metal-bound cysteines of thionein (Li et al., 1981), and then scanned between 410 and 430 nm on a Cary 17 UV–vis spectrometer. The absorbance (λ_{max} 412 nm) was used with the published molar extinction coefficient ($\epsilon_{412} = 13\,600$ L mol^{-1} cm^{-1}) to calculate the thiol concentrations of the fractions. The remaining 2.3 mL of each fraction was placed in a nitric acid washed glass test tube, to which Zincon (0.1 mL of 1.0 mM) was added. After 10-fold dilution, the Zn–Zincon complex was determined from the absorbance at 620 nm. (It was independently determined that no detectable absorption resulted from the possible reaction of Cd^{2+} or AuSTm with Zincon at concentrations exceeding by one order of magnitude those employed here.) The extinction coefficient at pH 8.75 was determined to be 17 500 L mol^{-1} cm^{-1} for Zn–Zincon and Beer's law behavior was observed up to $[Zn^{2+}] = 10$ mM.

Preparation of $(TmSAu)_x$-Thionein. Following the procedures for Au,Cd-Th, but using excess AuSTm (25–28 AuSTm per Zn + Cd), a single thionein peak ($K_d = 0.23$) containing principally gold and traces of zinc or cadmium was obtained. Several preparations using 0.35–4.5 μmol of Zn + Cd in the original thionein dissolved in 1–2 mL of buffer were carried out. In two reactions, [^{35}S]AuSTm (2.15×10^{-4} mCi/mmol) was used.

In Situ Chromophoric Monitoring of Metal Displacement. Zincon (2-carboxy-2-hydroxy-5′-sulfoformazylbenzene sodium salt) and PAR [4-(2-pyridylazo)resorcinol sodium salt] were used to monitor the rate of zinc and cadmium displacement from thionein. Zincon reacts only with Zn^{2+} under the con-

ditions used, while PAR reacts with Zn^{2+} (λ_{max} 485; ϵ = 38 750) and Cd^{2+} (λ_{max} 485; ϵ = 21 666). Neither ligand reacted with AuSTm under these conditions. In the absence of AuSTm they did not extract zinc or cadmium from native hk thionein.

In a typical experiment, a thionein sample containing 2.0 nmol of Zn and 3.4 nmol of Cd dissolved in 0.8 mL of 5 mM Tris-HCl, pH 8.6, was mixed with 0.1 mL of 100 μM PAR or Zincon in the same buffer. Immediately after addition of 0.1 mL of buffer containing 3 or 136 nmol of AuSTm, the mixture was placed in a Cary 17 spectrometer and monitored at 620 (Zincon) or 485 nm (PAR). The reactions were performed in duplicate. Final concentrations were as follows: [ZI] or [PAR], 10 μM; $[Zn]_{Th}$, 2.0 μM; $[Cd]_{Th}$, 3.4 μM; [AuSTm], 3 or 136 μM.

XANES/EXAFS Spectra. The X-ray absorption spectra were measured at the Stanford Synchrotron Radiation Laboratory, on Beam Line VII-3. Fluorescence and absorbance measurements were simultaneously collected. The lyophilized protein samples were packed into sample cells with a 5-mm path length. The spectra of the model compounds, $Na_3[Au(S_2O_3)_2]\cdot 2H_2O$, $[(etu)_2Au]Cl\cdot H_2O$ (etu = ethylenethiourea), $[Au(NH_3)_4](NO_3)_3$, and $KAuCl_4$, were recorded on powdered solid samples diluted with Li_2CO_3 and with a path length of 1 mm. A gold foil spectrum was measured periodically for calibration purposes.

The EXAFS was extracted from the absorption spectrum, following the technique of Hodgson (Cramer & Hodgson, 1979; Cramer, 1977; Eccles, 1977) using programs modified for compatibility with an Amdahl 470/V7A computer and TSO IBM software in use at the University of Cincinnati. Details of the data collection and analysis were published previously (Elder et al., 1983; Shaw et al., 1984).

Radioimmunoassay. Samples of (TmSAu)x-Th, Au,Zn,-Cd-Th, and the hk Zn,Cd-Th from which they were prepared were dissolved in buffer, quantitated by their absorbance at 220 nm and metal content, then lyophilized, and shipped frozen in dry ice to Syracuse University. Two different native hk metallothionein preparations were used to generate independent sets of aurothioneins, which were shipped and assayed separately. They were redissolved in double-distilled H_2O and appropriate serial dilutions were made and assayed in a double-antibody radioimmunoassay (RIA) following a protocol as previously described (Vander Mallie & Garvey, 1978, 1979; Garvey et al., 1982). Both the ^{125}I-labeled antigen (Bolton & Hunter, 1973) and a reference antigen (S) were prepared from the same rat liver (rl) Cd,Zn-thionein. This isoform is known to be cross-reactive with equine thionein. Inverse variance weighted logit–log standard curves were developed from the data following established protocols (Rodbard et al., 1968, 1976; Rodbard & Lewis, 1970; Rodbard, 1971, 1974). Changes in the sequence or in conformation affecting particularly the principal determinants of metallothionein (Winge & Garvey, 1983), and thus the affinity of the altered determinant for the primary antibody, may be evaluated in terms of either changes in slopes of the standard curves with respect to that of the reference isoform or changes in concentration of the competing antigen required to reduce the fraction bound of labeled antigen to 0.5.

Results

Stoichiometry of Reaction. Previous studies of the stoichiometry of reactions between gold thiomalate and metallothioneins were hampered by poor recoveries of metals (Schmitz et al., 1980). We have now used much larger samples of the protein, up to milligram quantities of zinc and cadmium, to determine definitively the metal exchange ratios and to isolate the products for EXAFS/XANES, radioimmunoassay, and chemical examination.

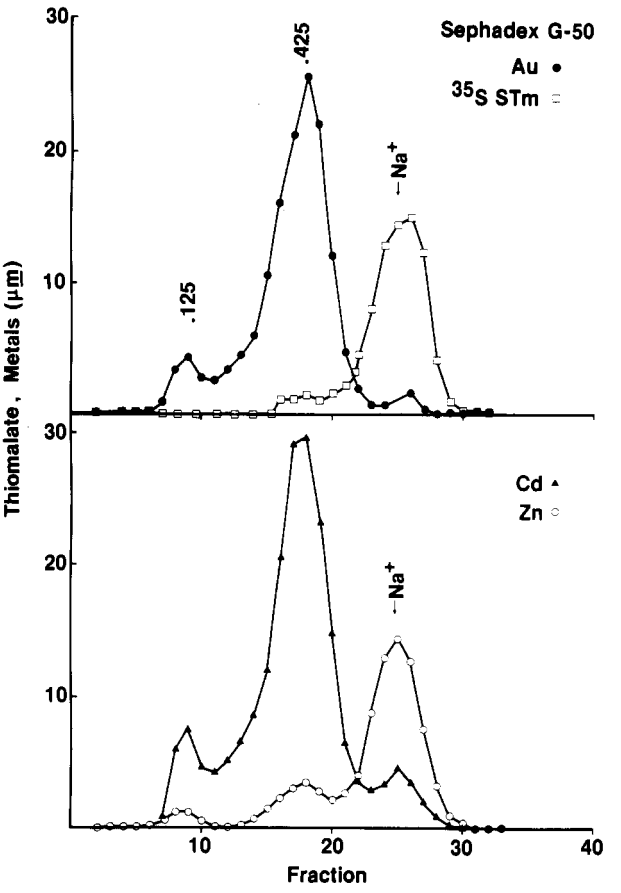

FIGURE 1: Gel exclusion chromatogram for preparation IB: [^{35}S]-AuSTm (0.30 μmol) with Zn,Cd-Th (0.20 μmol of Zn). After incubation at 4 °C for 1 h, the mixture was fractionated over Sephadex G-50 in 5 mM Tris-HCl, pH 8.6. Fractions of 3.3 mL were collected. Gold and ^{35}S are plotted above Zn and Cd for clarity, but all were determined in a single separation. The values of K_d are given for the thionein and hmw peak.

The ratios of gold to thionein-bound zinc and cadmium in these studies were the previously identified limiting cases (Schmitz et al., 1980). Very low ratios (1–3 Au/Zn) were used to displace primarily zinc; large ratios [25 Au/(Zn + Cd)] were used to displace both cadmium and zinc, forming a product containing predominantly gold as the thionein-bound metal (Table I). In Table II, the resulting thioneins are compared. The details of the two reactions are discussed separately, below.

Reaction of Excess Metallothionein with AuSTm. Five reactions of AuSTm and hk Zn,Cd-thionein were conducted with the Au/Zn mole ratio in the range of 1–3, Figure 1 and Table I. As expected, the zinc was preferentially displaced. For example, in preparation IA, a thionein having a 1:4 Zn:Cd ratio was incubated with 1.7 AuSTm/bound zinc for 1 h and separated over Sephadex G-50 in Tris buffer at pH 8.6. All the zinc recovered (92%) was in the low molecular weight (lmw) fractions, while the cadmium was primarily protein bound (91%) with only a small amount displaced into the lmw fractions (8%). The product thionein contained 0.57 μmol of gold, 1.22 μmol of cadmium, and no zinc. The mole ratio of metals lost (0.31 μmol of Zn and 0.10 μmol of cadmium) to gold bound, $\{Zn + Cd\}_d/\{Au\}_{Th}$, was 0.71.

For each gold binding model presented in the introduction, the expected $\{Zn + Cd\}_d/\{Au\}_{Th}$ ratio can be predicted: (1) isomorphous substitution, 1.0; (2) monodentate coordination

Table I: Gel Exclusion Chromatography of Reactions of Zn,Cd-Thionein with AuSTm

prepn (metal ratios)[a]	species[b]	reactants (μmol) thionein	reactants (μmol) AuSTm	products (μmol) thionein	products (μmol) lmw	% recovery
		Excess Metallothionein (Au/Zn)				
IA (1.7)	Zn	0.34		0.001	0.31[c]	92
	Cd	1.33		1.22	0.10	99
	Au		0.57	0.57	0.015	104
	Tm		0.57	2.79[d]	0.52[d]	92
	SH_{Th}	4.76		4.64		97
IB (1.5)	Zn	0.20		0.05[e]	0.14	96
	Cd	0.35		0.34[e]	0.043	111
	Au		0.30	0.27[e]	0.018	95
	[^{35}S]Tm		0.30	0.010	0.17	58
	SH_{Th}	1.56		1.69		108
IC (3.0)	Zn	0.15		0.002	0.15	104
	Cd	0.67		0.56	0.15	105
	Au		0.43	0.38	0.038	97
	[^{35}S]Tm		0.43	0.065	0.34	93
	SH_{Th}	2.34		2.41		103
ID (1.0)	Zn	7.5		2.7	5.0	103
	Cd	10.9		10.6	0.32	100
	Au		7.5	7.6		101
	SH_{Th}	53		53		100
IE (1.2)	Zn	2.6		1.0	1.6	100
	Cd	6.6		6.2	0.41	100
	Au		3.2	3.0	0.019	100
	SH_{Th}	26		26		101
		Excess AuSTm [Au/(Zn + Cd)]				
IIA (25)	Zn	1.4		0.11	1.6	118
	Cd	3.1		0.23	3.0	104
	Au		113	14	121	119
	SH_{Th}	13		15		116
IIB (28)	Zn	0.20		<0.001	0.22	111
	Cd	0.34		0.063	0.27	98
	Au		15	1.4	13.7	101
	[^{35}S]Tm		15	1.5	14.5	107
	SH_{Th}	1.55		1.6		103
IIC (28)	Zn	0.20		0.024	0.17	99
	Cd	0.34		0.044	0.30	102
	Au		15	1.5	13.5	100
	[^{35}S]Tm		15	1.4	12.3	92
	SH_{Th}	1.55		1.7		107
IID (25)	Zn	2.9		0.019	2.7	94
	Cd	6.2		0.31	5.8	99
	Au		260	24	240	104
	SH_{Th}	26		25		98
IIE (36)	Zn	20		1.9	20	109
	Cd	53		5.1	49	101
	Au		2670	190	2370	96
	SH_{Th}	210		210		100

[a] Mole ratios of gold to Zn or Zn + Cd in reaction mixtures. [b] $\{SH\}_{Th}$ calculated by eq 2. [c] By use of Zincon and correcting for $Zn(STm)_2$ formation, a value of {Zn} = 0.39 μmol (115% recovery) was obtained. [d] Determined by using DTNB. [d] Includes an aggregated (hmw) band containing the following (μmol): Zn, 0.007; Cd, 0.024; Au, 0.021, [^{35}S]TmSH, 0.001. See text.

0.350; (3) bidentate coordination, 0.700; and (4) thiolate-bridged cluster, 0.350. The average value of $\{Zn + Cd\}_d/\{Au\}_{Th}$ for reactions IA–E is 0.69 ± 0.03, in excellent agreement with the bidentate coordination model.

Further insight can be obtained from the stoichiometric ratio of sulfhydryls available for binding to gold, calculated by assuming that the displaced metals were associated with thionein sulfhydryls in a 7:20 ratio according to the Otvos–Armitage model (Otvos & Armitage, 1980):

$$\{SH\}_{avl} = (20/7)\{Zn + Cd\}_d \quad (1)$$

An $\{SH\}_{avl}/\{Au\}_{Th}$ value of 2.03 was obtained for reaction IA, and the average of reactions IA–E is 1.97 ± 0.05 in striking agreement with the bidentate coordination model. However, this ratio measures only the stoichiometric relationship of sulfur and gold and cannot be interpreted as the coordination number, since bridging of thiolate sulfurs between two or three gold ions could increase the coordination number of each gold ion. The latter situation occurs for zinc and cadmium in metallothionein, where the stoichiometric ratio 20/7 = 2.86 is less than the coordination number of 4.

The possibility of losing or gaining protein sulfhydryl groups by oxidation–reduction reactions accompanying the metal displacements was examined by using eq 2 to calculate the total

$$\{SH\}_{Th} = (20/7)\{Zn + Cd\}_{Th} + n\{Au\}_{Th} \quad (2)$$

number of sulfhydryls in the reactant and product thioneins; n, the stoichiometric relationship of protein thiols coordinated

Table II: Analysis and Comparison of Aurothionein Preparations

prepn	reaction ratio	mole fractions Zn	Cd	Au	$\{SH\}_{avl}/\{Au\}_{Th}$[a]	$\{Tm\}_{Th}/\{Au\}_{Th}$	$\{Cd + Zn\}_d/\{Au\}_{Th}$
				Au/Zn			
IA	1.7	0.000	0.68	0.32	2.0		0.71
IB	1.5	0.077	0.52	0.41	1.9	0.037	0.68
IC	3.0	0.002	0.59	0.40	1.9	0.17	0.70
ID	1.0	0.13	0.51	0.36	2.1		0.70
IE	1.2	0.0097	0.61	0.30	1.9		0.65
av ± SD					2.0 ± 0.1	0.10 ± 0.09	0.69 ± 0.03
				Au/(Zn + Cd)			
IIA	25	0.008	0.016	0.98	0.93		0.32
IIB	28	0.001	0.042	0.96	0.99	1.06	0.35
IIC	28	0.016	0.029	0.96	0.93	0.98	0.33
IID	25	0.001	0.013	0.99	1.01		0.35
IIE	36	0.010	0.025	0.97	1.01		0.35
av ● SD					0.97 ● 0.04	1.02 ± 0.06	0.34 ± 0.01

[a] $\{SH\}_{avl}$ is calculated by eq 1.

to gold, is 2 in this case. The agreement between the sulfhydryl levels of the products and reactants in preparations IA–E of Table I demonstrated that there was neither extensive oxidation of the previously metal-bound thiolates nor extensive reduction of any disulfides, possibly present in the native metallothionein due to metal loss and thiol oxidation during its isolation.

Aurothionein preparations IA–C, which used 1.5–3.0 Au/Zn in the incubation mixtures, contained principally gold and cadmium. The average mole ratio of zinc (to total metal) in these preparations was 0.03 ± 0.04, while the values for cadmium and gold were 0.58 ± 0.08 and 0.36 ± 0.05. In preparation IC, which used the largest Au/Zn ratio, 22% of the cadmium was displaced. The $\{Zn + Cd\}_d/\{Au\}_{Th}$ and $\{SH\}_{avl}/\{Au\}_{Th}$ ratios were nonetheless consistent with bidentate chelation.

For the preparations using less than 1.5 Au/Zn, relatively little of the initially bound cadmium (<8%) was displaced, some zinc remained in the thionein, and the $\{Zn + Cd\}_d/\{Au\}_{Th}$ ratio was approximately 0.70. For example, with a 1.0 Au/Zn ratio, approximately one-third of the zinc remained bound to thionein. Thus, the aurothioneins produced in reactions ID and IE can best be described as Au,Zn,Cd-Th. The K_d values [where $K_d = (V_s - V_0)/(V_t - V_0)$ and V_s, V_0, and V_t refer to the elution volumes of the sample and totally excluded and totally included markers, respectively] were 0.41–0.43, which is the same as that of the native Zn,Cd-Th starting material.

In several preparations a minor peak at $K_d \simeq 0.10$–0.13 contained approximately 0.1 as much zinc, cadmium, and gold as the major peak and in approximately the same ratios. It was presumed to be aggregated metallothionein: for the stoichiometry calculations, the metals associated with it were considered to be thionein bound.

The $\{SH\}_{avl}/\{Au\}_{Th}$ ratio suggested that the thionein was effecting a ligand exchange reaction in which thiomalate was displaced from the gold. To confirm that thiomalate was being displaced, the chromatographic fractions of reaction IA were analyzed for sulfhydryl content by the DTNB reaction. Free thiols react rapidly with DTNB (Ellman, 1959), while the zinc- and cadmium-bound sulfhydryl groups of metallothioneins react much more slowly (Li et al., 1981). The value for thionein sulfhydryl determined by DTNB, 2.79 μmol, was lower than that calculated by the metal content, 4.76 μmol. This result is consistent with the previously reported inhibition of the DTNB reaction by gold (Danpure, 1976). In the low molecular weight fractions, where very little gold is present, the amount of thiomalate determined by the DTNB method, 0.52 μmol, was more than 92% of the value expected if the thiomalate were completely displaced from the protein-bound gold, 0.57 μmol.

Further confirmation that thiomalate was being displaced from the gold was obtained by using ^{35}S-labeled AuSTm to trace the distribution of the ligand in preparations IB and IC of Table I. The radiotracer was more accurate than the DTNB, since there was neither interference by gold nor complications due to the large thiol content of the protein itself. The recovery of the [^{35}S]thiomalate ([^{35}S]Tm) after chromatography of the reaction mixtures over Sephadex G-50 was sometimes erratic, consistent with parallel studies on the reaction of [^{35}S]AuSTm and serum albumin (Shaw et al., 1984). Most of the [^{35}S]Tm was present in the low molecular weight (lmw) fractions, and the ratio of thiomalate to gold in the aurothioneins $\{[^{35}S]Tm\}_{Th}/\{Au\}_{Th}$ was 0.10, indicating displacement of the thiomalate from the gold under the conditions of this reaction and confirming the results of the analysis using DTNB. Thus, the reaction of thionein with 1.5 Au/Zn can be assigned the following stoichiometry:

$$(10x/7)\text{AuSTm} + \text{Zn}_x\text{Cd}_y\text{Th} \rightarrow \text{Au}_{10x/7}\text{Cd}_y\text{Th} + x\text{Zn}^{2+} + (10x/7)\text{TmSH}$$

When the chromatographic fractions of reaction IA were analyzed with the zinc-specific chromophore Zincon (ZI), the zinc in the lmw fractions, 0.11 μmol, determined from the absorption at 620 nm was less than the amount present in the metallothionein, 0.34 μmol. The thiomalate present in these fractions, however, should bind tightly to zinc, $\log K_1 = 8.24$ and $\log K_2 = 6.32$ (Lenz & Martell, 1965), displacing Zincon which has a low equilibrium binding constant for zinc, $K_b \simeq 10^6$ (Rush & Yoe, 1954). When it was assumed that the thiomalate and zinc formed a complex $Zn(STm)_2$, the total displaced zinc, in equilibrium between Zincon and thiomalate-bound forms (0.39 μmol), was in reasonable agreement with the initial value, 0.34 μmol. The displacement by thiomalate of zincon from the Zn–ZI complex was independently verified by direct reaction.

Reaction of Metallothionein with Excess AuSTm. When large ratios of AuSTm to thionein-bound metals were employed, nearly complete displacement of the zinc and cadmium was observed, Table I. In Figure 2 (preparation IIC), 96% of the cadmium and zinc were shifted to the low molecular weight fractions and eluted with the large excess of unbound gold. The resulting aurothioneins eluted consistently at $K_d = 0.23$, indicating a significantly increased Stokes radius in comparison with native metallothionein and Au,Zn,Cd-Th.

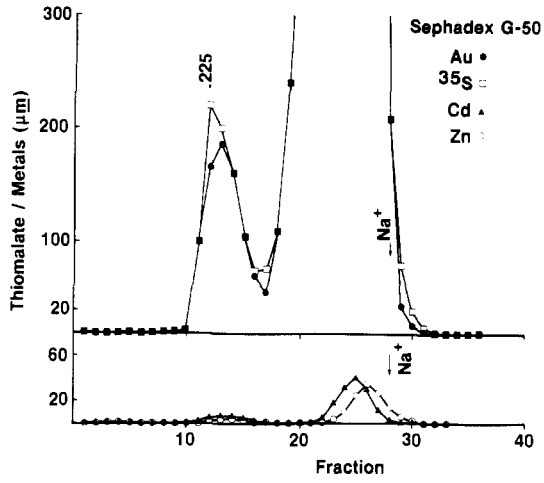

FIGURE 2: Gel exclusion chromatogram for preparation IIC: [^{35}S]AuSTm (15.0 μmol) with Zn,Cd-Th (0.20 μmol or Zn; 0.34 μmol of Cd). Other conditions are as in Figure 1.

For preparations IIA–E, the average mole fractions of the metals in the thionein were as follows: Zn, 0.008 ± 0.006; Cd, 0.02 ± 0.01; Au, 0.97 ± 0.01. Thus, the aurothioneins produced in these reactions contained almost exclusively gold.

The $\{SH\}_{avl}/\{Au\}_{Th}$ ratios were 0.97 ± 0.04, indicating that one gold was bound for each cysteine residue previously coordinated to zinc or cadmium. If one gold were bound per sulfhydryl, the ratio of $\{M^{2+}\}_d/\{Au\}_{Th}$ should be 0.350. For preparations IIA–D, the experimental ratios ranged between 0.32 and 0.35, with an average of 0.34 ± 0.01, in very good agreement with the predicted value. Therefore, the stoichiometry of the reaction with excess AuSTm was different from that for the reactions with low gold to metal ratios.

A second difference was the incorporation of the thiomalate ligand into the aurothionein. The ratios of ^{35}S to Au in the protein were 1.06 and 0.98 for preparations IIB and IIC, indicating the retention of one thiomalate per bound gold.

The number of sulfhydryl groups in the thionein, calculated from eq 2 with $n = 1$, appeared to increase slightly. The amounts of ^{35}S and Au bound to the thionein were in better agreement with $\{SH\}_{Th}$ of the starting material (e.g., preparation IIC: 1.47 μmol of gold bound vs. $\{SH\}_{Th} = 1.55$ μmol for the reactant thionein and $\{SH\}_{Th} = 1.66$ for the product.) The increase in the value of $\{SH\}_{Th}$ might be caused by AuSTm-induced reduction of the disulfide bonds adventitiously formed in the reactant thionein, although such reductions are not observed in the reaction of cystine with AuSTm (Danpure & Lawson, 1977). Alternatively, the implicit assumption of eq 2 that the residual Cd and Zn occupy unperturbed cluster sites, M_3S_9 or M_4S_{11}, may be invalid. They may bind weakly to the sulfhydryls of the thiomalate protein, which are already coordinated to gold. Assuming that to be the case and excluding Zn and Cd in using eq 3, values of the $\{SH\}_{Th}$ products were comparable to those of the reactants and to the amounts of gold and ^{35}S taken up by the thioneins.

From the data presented here, the product of the reaction of excess AuSTm with Zn,Cd-Th can be formulated as $(TmSAu)_xTh$. For a fully metal-saturated starting material, the value of x would be 20, and the stoichiometry of the reaction would be

$$20AuSTm + (Zn,Cd)_7\text{-}Th \rightarrow (TmSAu)_{20}Th + 7(Zn^{2+},Cd^{2+})$$

Since metal loss during the initial isolation of the native hk Zn,Cd-thionein is accompanied by sulfhydryl oxidation, the product contained less than 20 (thiomalato)aurate entities per mole and will be represented hereafter as $(TmSAu)_xTh$. The consistent elution of this product at $K_d = 0.23$, in contrast to the starting material at $K_d = 0.40$, indicates a substantial change in its hydrodynamic properties.

In Situ Chromophoric Monitoring of Metal Displacement. Metal displacement reactions of metallothioneins are difficult to follow directly because the aqueous Cd^{2+} and Zn^{2+} ions and the metal thiolate clusters of the protein lack UV–visible absorption bands of unique energy and sufficient intensity to follow the reactions. Chromatographic methods are slow and cumbersome. Therefore, we have developed a technique to monitor Cd^{2+} and Zn^{2+} displacement, using metal-binding chromophores that react with a metal ion only after it is displaced from the protein.

Zincon, a zinc-specific chromophore, does not react with cadmium or AuSTm to form a complex analogous to the zinc–Zincon chromophore under the conditions used here. More importantly, it does not react independently with metallothionein to extract the zinc and form the chromophore, but its reactions with aqueous Zn^{2+} are complete within the mixing time for conventional UV–visible measurements. Thus, it served as a monitor of the appearance of aqueous Zn^{2+} in the metallothionein/gold reactions. Applying this technique to a metallothionein sample with [Cd] = 3.4 μM and [Zn] = 2.0 μM, the displacement of zinc by 3 or 136 μM gold was complete within 5–10 min. The gold concentrations correspond to the same ratios, 1.5 Au/Zn and 25 Au/(Zn + Cd), used in the chromatographic preparations.

PAR reacts with Zn^{2+} and Cd^{2+} to form colored complexes but does not, by itself, react with thionein-bound metals. When the reactions of AuSTm with metallothionein were monitored with PAR, they proceeded rapidly, and the displacements of Cd and Zn were complete within 20 min. Thus, reactions of AuSTm with metallothionein are completed more quickly than the previously determined upper limit of 2 h (Schmitz et al., 1980).

Aurothioneins for EXAFS/XANES Studies. Large-scale preparations of aurothioneins, designed to produce ca. 10 mg of bound gold, were carried out. The reaction with excess thionein, ID, used an AuSTm/Zn ratio of 1.00 to retain the cadmium and displace preferentially some, but not all, of the zinc, leaving some bound for possible zinc EXAFS studies. The mole ratio of metals in the Au,Zn,-Cd-Th was Au:Zn:Cd = 0.36:0.13:0.51.

The $(TmSAu)_xTh$ preparation, IIA, used a smaller sample of Zn,Cd-Th, since the product binds significantly more gold per mole of protein. The mole fractions of metal were Zn:Cd:Au = 0.008:0.016:0.98. From the results with [^{35}S]AuSTm in preparations IIB and IIC, it can be inferred that thiomalate was present in 1:1 ratio with the gold. The $(TmSAu)_xTh$ had an off-white color, resembling that of the freshly prepared gold(I) thiolates of cysteine, thioglucose, and thiomalate.

EXAFS/XANES Spectroscopy. X-ray absorption near-edge spectroscopy (XANES) using the L_{III} absorption edge permits the oxidation state of gold in its compounds to be determined. Gold(III), with an $[Xe]6s^04f^{14}5d^8$ configuration, has a bound state transition at 11.923 keV, resulting from promotion of a 2p electron into a vacant 5d orbital. This transition clearly distinguishes gold(III) from gold(I) or gold(0). The latter can be identified by two absorption features at 11.945 and 11.967 keV. The absence of these features from a spectrum identifies the species being examined as gold(I) (Elder et al., 1983). The XANES spectra of Au,Zn,Cd-Th and $(TmSAu)_xTh$ prepared as above clearly demonstrated that gold(I) had been incorporated into both aurothioneins, Figure

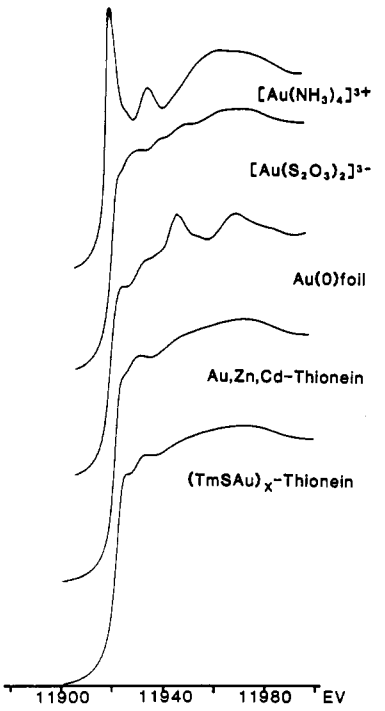

FIGURE 3: XANES spectra of (a) $[Au^{III}(NH_3)_4](NO_3)_3$, (b) $Na_3[Au^{I}(S_2O_3)_2]$, (c) Au(0) foil, (d) Au,Zn,Cd-Th, and (e) $(TmSAu)_xTh$. The absence of the Au(III) spike at 11.932 keV and the Au(0) features at 11.945 and 11.967 keV establish that the aurothioneins contain gold(I).

Table III: EXAFS Single-Shell Distances and Coordination Numbers[a]

	$Na_3[Au(S_2O_3)_2]$		$[Au(etu)_2]Cl\cdot H_2O$	
compd	d_{AuS} (pm)	coord no.	d_{AuS} (pm)	coord no.
Au,Zn,CdTh	229	2.2	229	2.4
$(TmSAu)_xTh$	230	1.7	230	1.9
AuSTm	230	1.8	230	2.0
AuSCy	231	1.9	231	2.1
AuSGt	230	1.7	230	2.0
$[Au(dtt)_2^-]^{b,c}$	230	4.1		
$[Au(dte)_2^-]^{b,c}$	229	3.9		

[a] Error estimates: distances ±2 pm and coordination number ±20%. [b] Eidsness & Elder (1985). [c] The abbreviation dte is used for 1,2-bis-(trifluoromethyl)ethene 1,2-dithiolate; dtt is used for 2,3-dithiotoluene.

3. The spike characteristic of gold(III) at 11.923 keV and the broader EXAFS features at 11.945 and 11.967 keV were absent. Thus, the gold(I) of AuSTm reacts to form the aurothionein species primarily via ligand exchange reactions and without any change of oxidation state.

By use of extended X-ray absorption fine structure (EXAFS) spectroscopy, the nature of the gold coordination environment, including the kind of nearest-neighbor atoms and the approximate number of each kind, as well as the gold–ligand atom bond distances, can be determined by curve fitting of model compound parameters to the spectral data beyond the absorption edge (Elder et al., 1983). Sulfur ligands can be clearly distinguished from nitrogen or oxygen donors by this technique. The transforms (in phase-shifted angstrom space) of the EXAFS spectra of Au,Zn,Cd-Th and $(TmSAu)_xTh$, Figure 4, each have only a single major peak suggesting that only a single shell of scattering atoms would be needed for a curve fit. Appropriate segments were retransformed to yield the Fourier-filtered EXAFS spectra shown in Figure 5. By use of parameters transferred from the model compound $Na_3[Au(S_2O_3)_2]$, excellent agreement between the observed and calculated spectra was obtained, Figure 5. The

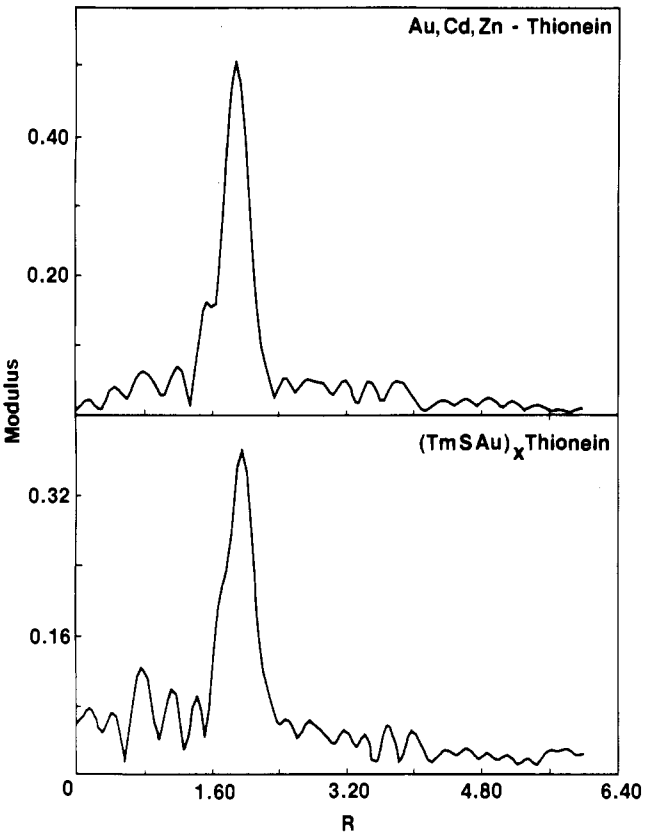

FIGURE 4: Transform (in phase-shifted angstrom space) of the EXAFS spectrum of (a) Au,Zn,Cd-Th and (b) $(TmSAu)_xTh$. Only a single absorber peak is present in either transform consistent with a single type of ligand atom.

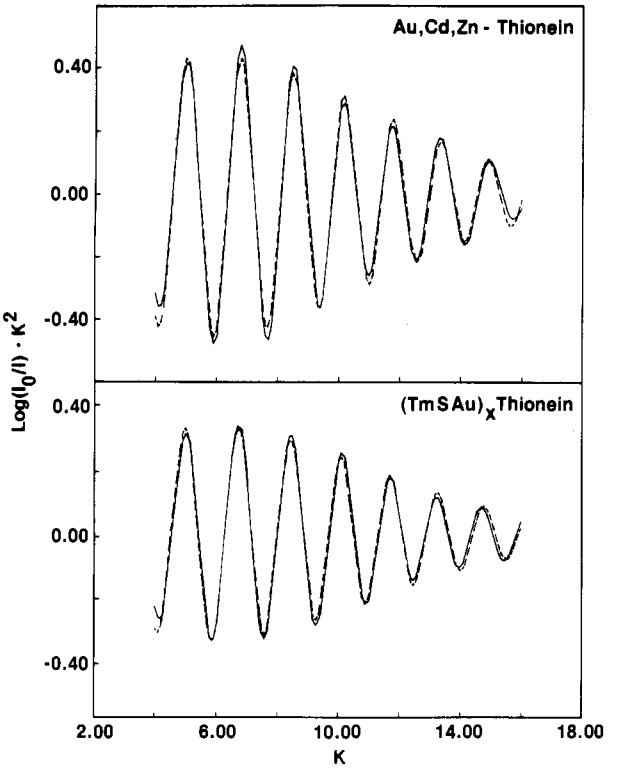

FIGURE 5: Fourier-filtered EXAFS spectrum of (a) Au,Zn,Cd-Th and (b) $(TmSAu)_xTh$: experimental (—) and calculated (--). The calculated spectrum, assuming only sulfur coordination, used empirical parameters transferred from the model compound $Na_3[Au(S_2O_3)_2]$.

calculated bond distances and coordination numbers are given in Table III. Calculations were performed by using both the

Table IV: Logit–Log Analysis of Radioimmunoassay Results[a]

		trial 1[b]			trial 2[b]		
Ag[c]	thionein	prepn	slope (SE)	r^d	prepn	slope (SE)	r^d
1	hk Zn,Cd-Th		−0.72 (0.02)	−0.990		−0.83 (0.03)	−0.993
2	Au,Cd,Zn-Th	ID	−0.66 (0.02)	−0.986	IE	−0.75 (0.01)	−0.999
3	$(TmSAu)_x$Th	IIA	−0.79 (0.02)	−0.990	IID	−0.88 (0.04)	−0.984

[a] Slopes and their standard errors (SE) as determined by regression analysis of the logit–log standard curves ($Z = a + bQ$, where Z = logit Y = log $[Y/(1 - Y)]$, Y is fraction of bound labeled antigen, Q is log X, and X is sample antigen concentration in pM). Each regression is based on quadruplicate determinations at eight dilutions. [b] Preparations ID and IIA are compared to the hk Zn,Cd-Th from which they are prepared, as are IE and IID. Significance levels of the slope differences are as follows: (trial 1) $b_1 - b_2$, not significant ($p < 0.10$); $b_1 - b_3$, $p < 0.05$; $b_2 - b_3$, $p < 0.01$; (trial 2) $b_1 - b_2$, $p < 0.05$; $b_1 - b_3$, not significant ($p < 0.40$); $b_2 - b_3$, $p < 0.02$. Note that the shift in slope value for the response of a particular competitor antigen (comparing values in trial 1 and trial 2) reflects both the use of competitor antigens from independent preparations and a slightly different protocol for the labeled antigen (source and concentration used) in the two trials. [c] Line in Figure 6. [d] Correlation coefficient of the regression analysis.

model parameters generated from $Na_3[Au(S_2O_3)_2]$ and from $[Au(etu)_2]Cl{\cdot}H_2O$. The gold atom is two-coordinate in each model; however, as seen previously, the coordination number appears marginally higher with $[Au(etu)_2]^+$ parameters. We believe that $[Au(etu)_2]^+$ is probably a better model and that the slightly higher values of coordination number are to be preferred. Note also that the errors in EXAFS determination of coordination number are large and that we are unable to distinguish the gold coordination number in Au,Zn,Cd-Th from that in $(TmSAu)_x$Th.

The coordination number of gold in $(TmSAu)_x$Th, Au,-Zn,Cd-Th, AuSGt and AuSCys is approximately 2. When EXAFS data are used to calculate coordination number for known four-coordinate gold–sulfur complexes, the EXAFS results are 4.1 and 3.9 (see Table III). The bond distances determined by EXAFS for these four-coordinate structures agree with the crystallographic distances within 1 pm. Thus, we have considerable confidence that the gold coordination number in both Au,An,Cd-Th and $(TmSAu)_x$Th is 2 and not 4. This is unusual since both zinc (Garner et al., 1982) and cadmium (Otvos & Armitage, 1980) are known to occupy tetrahedral sites in metallothioneins.

Since bridging and terminal gold–sulfur bond distances are similar, e.g., 228 pm for AuS_t in $Na_3[Au(S_2O_3)_2]$ and 230 pm for Au-S_{br} in the (glutathionato)gold(I) polymer, AuSGt, the bond distances from the EXAFS cannot distinguish between bridging or terminal thiolates in the aurothioneins.

Radioimmunoassay. To study the influence of Au substitution on the antigenicity of the product metallothioneins, two independent sets of samples of Au,Cd,Zn-Th, $(TmSAu)_x$Th, and the native Zn,Cd-Th from which they were prepared were assayed by RIA (see Experimental Procedures). The results of the assays are presented in Table IV and Figure 6. The various aurothioneins all cross-react (Figure 6) and are clearly identified as metallothioneins. In view of the uncertainty in the sample thionein concentrations (ca. 10% by the A_{220} absorption at pH 1) and the slight differences found in the concentrations of the various samples at the 50% bound level (log $[Y/(1 - Y)] = 0$ in Figure 6), the slope differences, because they are independent of the initial estimation of thionein concentration, are the superior method for comparing antigenicities in this instance.

The data (Table IV) indicate that both preparations (trials 1 and 2) of the mixed-metal Au,Cd,Zn-Th exhibited enhanced competition with the labeled antigen (a decrease in the absolute value of the slope of the standard curve) compared to that exhibited by the native Zn,Cd-Th; the $(TmSAu)_x$Th exhibited a slightly decreased antigenicity when similarly compared. Figure 6 illustrates this graphically. The slopes differed significantly in both trials ($p < 0.01$ and $p < 0.02$ in trials 1 and 2, respectively) and were significantly different for $(TmSAu)_x$Th and the native thionein in trial 1 ($p < 0.05$) and for Au,Cd,Zn-Th and the native thionein in trial 2 ($p < 0.05$). The enhancement of antibody–antigen affinity upon metal substitution, as observed for Au,Cd,Zn-Th, indicates that a slight conformational change has been induced in the principal determinant reverse bend(s) in the region of residues 19–27 (Winge & Garvey, 1983; Garvey, 1983). Such a phenomenon indicates a heteroclitic antibody (one exhibiting greater affinity for an antigen differing slightly in conformation or sequence from the antigen that induced the antibody in vivo). The decreased affinity, in both trials, of the more completely Au-substituted antigen, $(TmSAu)_x$Th, presumably reflects the negative influence of the additional Au atoms due to an unfavorable modification of the conformation of the principal determinant.

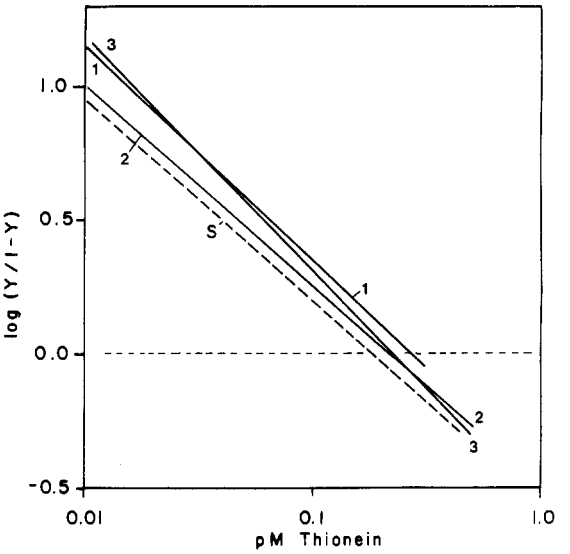

FIGURE 6: Radioimmunoassay of aurothioneins. Inverse variance weighted logit–log regressions, developed from the experimental data of trial 2, are plotted for antigens 1 (native hk Zn,Cd-Th), 2 (Au,-Cd,Zn-Th), and 3 [$(TmSAu)_x$Th]. See Table V for details. In addition, the standard curve for a rat liver Cd,Zn-Th (labeled S) that was assayed simultaneously with the antigens is shown. For antigen S, the slope of the standard curve is −0.75; the correlation coefficient (r) is −0.992; the slope differs significantly only from that of antigen 3 ($p < 0.05$).

Discussion

The properties of the aurothioneins produced in this study are compared in Table V to those predicted by the four models for gold coordination. Au,Cd-Th and Au,Zn,Cd-Th have properties that are consistent only with bidentate chelation of gold by the protein. The EXAFS result, two-coordination of gold, AuS_2, eliminates the possibility of isomorphous substitution of gold(I) for Zn^{2+} or Cd^{2+} in the intact three-metal

Table V: Comparison of Predicted and Observed Properties for Aurothioneins

bonding model or aurothionein	gold coord no.	$\{Zn + Cd\}_d/\{Au\}_{Th}$	$\{SH\}_{avl}/\{Au\}_{Th}$	$\{Tm\}_{Th}/\{Au\}_{Th}$
isomorphous substitution (1)	4	1.00	2.86	0.00
monodentate coordination (2)	2	0.250	1.00	1.00
bidentate chelation (3)	2	0.700	2.00	0.00
thiolate-bridged cluster (4)	2	0.350	1.00	0.00
Au,Zn,Cd-Th	2.2 ± 0.2	0.69 ± 0.03	1.97 ± 0.05	0.10 ± 0.09
$(TmSAu)_xTh$	1.7 ± 0.2	0.34 ± 0.01	0.97 ± 0.04	1.02 ± 0.06

and four-metal clusters. This result is expected since three- and four-coordinate gold(I) species have been reported mainly for phosphine complexes, AuL_3^+ and AuL_4^+, while all crystallographically characterized gold(I) thiolates are two-coordinate linear complexes (Shaw, 1979). Monodentate coordination is inconsistent with the displacement of thiomalate from the gold and with the stoichiometric relationships $\{Zn + Cd\}_d/\{Au\}_{Th}$ and $\{SH\}_{avl}/\{Au\}_{Th}$. The experimental values of the two ratios are quantitatively correct for bidentate chelation. Thus, the reaction using the stoichiometry of 1.5 gold atoms/initially bound zinc can be represented as

$$(10x/7)\text{AuSTm} + Zn_xCd_yTh \rightarrow Au_{10x/7}Cd_yTh + xZn^{2+} + (10x/7)\text{TmSH}$$

At the lowest ratios of gold to zinc employed, some zinc remains bound to the protein, and the product is Au,Zn,Cd-Th. At slightly higher ratios, cadmium is also displaced, with the same ratio of $\{Zn + Cd\}_d/\{Au\}_{Th}$. These results confirm the earlier study concluding that displacement of zinc is preferential to displacement of cadmium (Schmitz, 1980) but, due to better metal recoveries, demonstrate that the selectivity is not absolute and some cadmium is always displaced. The similarity of the K_d values of Au,Cd-Th and Au,Zn,Cd-Th to those of native Zn,Cd-Th (K_d = 0.40) suggests that only minimal alteration of the protein conformation has taken place upon gold binding and that the Stokes radius is similar to that of native metallothionein, 15.4 Å (Phillips, 1983).

For $(TmSAu)_xTh$ the properties observed are in excellent agreement with those predicted for monodentate coordination (model 4 in the introduction): retention of the ligand, thiomalate, in 1:1 ratio to the gold; $\{Zn + Cd\}_d/\{Au\}_{Th}$ = 0.34, and coordination of gold(I) to two sulfur atoms. Analogous monodentate coordination of AuSTm to a single sulfhydryl group has recently been documented for the tight gold binding site of serum albumin, Cys-34 (Shaw et al., 1984).

The elution of $(TmSAu)_xTh$ at K_d = 0.23 is consistent with the loss of the native conformation and a significant increase in the Stokes radius. On a given exclusion gel, linear polymers elute more rapidly than globular species of the same molecular weight. Vasak et al. (1980) concluded that when metals are removed from native thioneins to form apoprotein, the latter has a more random structure and a larger Stokes radius, 20.8 Å (Phillips, 1983). Likewise, monodentate coordination of gold (model 2) eliminates the metal clusters that provide the structural integrity of native thioneins, thereby explaining the dramatic change of elution profile observed here.

EXAFS/XANES analysis and the stoichiometric results indicated bidentate chelation of gold in Au,Cd,Zn-Th and Au,Cd-Th and monodentate coordination in $(TmSAu)_xTh$. However, these techniques do not eliminate the possibility that a small fraction of gold may be bound in a different manner. In fact, the [^{35}S]STm studies (IB and IC) suggest that mixed binding modes may occur. In reaction IB, using an AuSTm/Zn ratio of 1.53, there was only 0.04 [^{35}S]thiomalate per bound gold in the thionein peak, but for reaction IC, using an AuSTm/Zn ratio of 2.9, almost twice that of reaction IB, there was 0.17 [^{35}S]thiomalate per gold in the thionein product, indicating that while the majority of gold was bound in the bidentate chelation mode (i.e., with loss of thiomalate) some probably bound via monodentate coordination, retaining the ligand.

^{113}Cd NMR has demonstrated that all seven cadmium ions of Cd_7Th occupy similar, although spectroscopically distinguishable, CdS_4 binding sites (Otvos & Armitage, 1980). EXAFS studies of an all-zinc protein established that the metal ions occupied ZnS_4 sites, but the much lower resolution of EXAFS does not distinguish more subtle differences in environment (Garner et al., 1982). The aurothioneins are the first cases in which there is direct evidence to establish that a single metal ion binds to metallothionein in two distinct coordination modes. Gold is also the first metal for which structural evidence supports nontetrahedral geometry. This surprising difference between gold and cadmium or zinc coordination reflects the strong tendency of gold(I) to be two-coordinate (Shaw, 1979).

The observation that more than one coordination environment exists for gold raises the possibility that other metals such as Pt(II) for which four-coordinate tetrahedral structures are energetically unfavorable, or Cu(I) which can adapt two-, three-, or four-coordination, may bind in more than one manner depending on the conditions of formation. Indeed, Winge has reported that depending on the ratio of Cu^+ used in reaction with apothionein, products of stoichiometry $Cu_{6-7}Th$ and $Cu_{10-12}Th$ result (Nielson & Winge, 1984).

The reaction of AuSTm with thionein is fast, leading to complete reaction within 10–15 min. This result indicates that gold in cellular environments can react with preexisting metallothionein, as suggested by in vivo studies (Thompson et al., 1978; Sharma & McQueen, 1982; Lawson et al., 1977). Furthermore, the rapidity of the reaction implies that the incomplete binding of gold to thionein in kidney and liver cytosol, where it accounts for 20–40% of the gold present (Thompson et al., 1978; Sharma & McQueen, 1982; Lawson et al., 1977), is due to thermodynamic competition by other cellular ligands and not due to a kinetic limitation of the reaction between AuSTm metallothionein.

Registry No. AuSTm, 12244-57-4; Au, 7440-57-5; Zn, 7440-66-6; Cd, 7440-43-9; $Na_3[Au(S_2O_3)_2]$, 10233-88-2; $[(etu)_2Au]Cl$, 95407-71-9; AuSCys, 61593-59-7; $[Au^{III}(NH_3)_4](NO_3)_3$, 60117-73-9; sulfur, 7704-34-9.

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