isolation of Pt-DNA monoadducts.[36] The incorporation of a Pt-S bond in a well-defined complex prior to any administration may have considerable effects on this biology and the fact that Pt-S complexes are antitumor active may be relevant to the mechanisms of some of the above-mentioned effects. An interesting point to note here also is that albumin, the predominant agent responsible for tissue binding of Pt complexes, has been used (as protein BSA) as a template for the preparation of chiral sulfoxides.[37] It is therefore axiomatic that chiral sulfoxide complexes will react differently with this enzyme. The chemical aspects of the biodistribution, tissue binding, and metabolism of platinum antitumor complexes are not as well understood as those of DNA binding, but it is clear that these properties may vary quite widely between, e.g., a neutral dichloride complex and a cationic sulfoxide species.

In conclusion, independent of the detailed mechanism of action of these complexes, we report on a new class of active platinum antitumor complexes based on sulfoxide ligands, with a unique effect of the chirality of the sulfoxide ligand upon the antitumor activity.

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Registry No. I, 124442-33-7; II (isomer 1), 124460-83-9; II (isomer 2), 124578-26-3; III (isomer 1), 124460-85-1; III (isomer 2), 124578-28-5; IV, 124442-35-9; V, 124442-37-1; VI, 124442-39-3; VII, 124442-41-7; VIII, 124442-43-9; IX, 124442-45-1; X, 124442-47-3; XI, 124442-53-1; XII, 124442-49-5; XIII, 124442-51-9; XIV, 124510-92-5; XV, 124460-87-3; XVI, 124578-30-9; XVII, 124578-32-1; XVIII, 124578-34-3; (*R*,*R*)-dach, 20439-47-8; en, 107-15-3; (*S*,*S*)-dach, 21436-03-3; damch, 4441-55-8; pn, 109-76-2.

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Biphasic Kinetics of Aurothionein Formation from Gold Sodium Thiomalate: A Novel Metallochromic Technique To Probe Zn^{2+} and Cd^{2+} Displacement from Metallothionein[1]

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A method has been developed to exploit the metallochromic dyes pyridylazoresorcinol and Zincon as monitors of the rate of zinc and cadmium displacement from Zn-, Zn,Cd-, Zn,Cd,Cu- and Cd-thioneins. In this report, the antiarthritic drug gold sodium thiomalate (AuSTm) is the displacing agent. In 5 mM Tris-HCl/100 mM $NaClO_4$ buffer, pH 7.4, at 25 °C, the reactions are biphasic. The fast and slow components are first order and independent of the choice of dye and its concentration. The reaction kinetics for each of the thionein preparations (except one Zn,Cd,Cu-thionein preparation) were also independent of the gold concentration. Thus, the rate law for aurothionein formation from AuSTm is rate = k_f[MT] + k_s[MT]. The averaged rate constants of the fast and slow steps obtained by using PAR with six different protein preparations were $k_f = (2.7 \pm 1.2) \times 10^{-2}$ s^{-1} and $k_s = (6.9 \pm 0.9) \times 10^{-4}$ s^{-1} and by using Zincon, were $k_f = 2.4 \pm 0.6 \times 10^{-2}$ s^{-1} and $k_s = 9.6 \pm 1.7 \times 10^{-4}$ s^{-1}. The same rate laws for metal displacement describe the reactions generating Au,Cd-Th or Au,Zn,Cd-Th (for which gold is the limiting reagent) and those forming $(TmSAu)_{20}$-Th with complete loss of protein-bound zinc and cadmium (for which the protein is the limiting reagent). Differences in the kinetics due to the source of the metallothioneins and their metal contents were all within the experimental error. The biological implications of the kinetics for aurothionein formation during chrysotherapy are discussed.

Introduction

Metallothionein is a curious, cysteine-rich, metal-binding protein found in mammalian tissues. Twenty of its 61 amino acid residues are cysteines. As isolated, it may contain zinc, copper, or environmentally accumulated cadmium in ratios dependent on the tissue, the species, and the age and history of the organism from which it is isolated. Cadmium and zinc are bound in two clusters with exclusive thiolate coordination: M_3S_9 and M_4S_{11}, localized in the N and C terminal ends of the peptide chain, respectively.[2-4]

Gold(I) thiolates provide successful treatments for rheumatoid arthritis.[5] In animal models the gold binds to metallothioneins in vivo, generating aurothioneins.[6-10] Aurothionein formation can be modeled by in vitro reactions of gold complexes with Zn_7-Th, Zn,Cd-Th and Cd_7-Th.[10-12] Gold sodium thiomalate (AuSTm)[13] displaces Zn^{2+} completely and Cd^{2+} in an equilibrium competition, forming Au,Zn,Cd-Th, Au,Cd-Th, and $(TmSAu)_{20}$-Th at progressively higher Au to protein ratios.[10,11] In the first two forms, gold is coordinated to two MT cysteines with loss of the thiomalate carrier ligand. In the latter, one AuSTm moiety

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(13) Abbreviations: apo-CA, apo-carbonic anhydrase; AuSTm, gold sodium thiomalate; DTNB, dithionitrobenzoic acid; EDTA, ethylenediamminetetraacetic acid; hrs, horse; kid, kidney; liv, liver; MT, metallothionein; PAR, pyridylazoresorcinol; rbt, rabbit; rds, rate-determining step; Th, thionein; ZI, Zincon.

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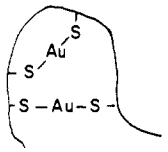
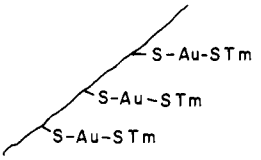
binds to each cysteine, unfolding the protein and increasing its hydrodynamic radius.[11] Recent studies have used cultured human cells to demonstrate that aurothioneins have unusually fast turnover rates compared to other metallothioneins[14] and that aurothionein formation can confer drug resistance and reduce gold-induced cytotoxicity.[15-17] These results have stimulated new interest in aurothionein formation.



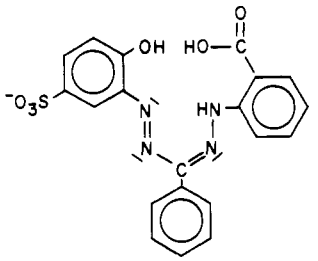
bidentate chelation



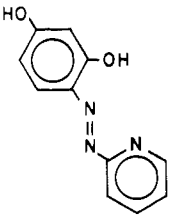
monodentate coordination

Metallothioneins lack the intense visible chromophores of heme or copper blue proteins. Although the MT metal–thiolate absorbances are sometimes useful kinetic probes,[18,19] they are often masked by other components of the reaction mixture. Chromophores in metal-extracting ligands and in electrophiles attacking the metal-bound thiolates have been exploited as kinetic probes.[20,21]

Recently, we used two metallochromic reagents, pyridylazoresorcinol (PAR) and Zincon (ZI), to verify Zn and Cd displacement from MT.[11] PAR binds zinc and cadmium, forming 2:1 complexes at neutral pH. Conditional stability constants[22] at pH 7.4, $K_{app} = 10^{12.6}$ and $10^{14.3}$ for Zn^{2+}, and $K_{app} = 10^{10.6}$ for Cd^{2+} were calculated from published pH-dependent equilibrium constants.[23-26] Zincon complexes with zinc ions ($K_{app} = 10^{4.9}$ at pH 7.4, forming a 1:1 complex.[27] A novel application of these metallochromic reagents for monitoring the kinetics of zinc and cadmium displacement from MT is reported here. The displacing agent is the inorganic pharmaceutical agent AuSTm. This is the first detailed study of the kinetics of aurothionein formation.



Zincon



pyridylazoresorcinol

Experimental Section

Materials. Reagents were obtained as follows: from Aldrich Chemical Co., gold(I)thiomalate disodium salt (AuSTm) and 4-(2-pyridylazo)resorcinol monosodium salt monohydrate (PAR); from Sigma Chemical Co., Tris (Trizma base), Zincon (2-carboxy-2-hydroxy-5-(sulfoformazyl)benzene sodium salt or ZI); from Fisher Scientific Co., purified sodium perchlorate monohydrate; from Bio-Rad Laboratories, Chelex-100 (analytical grade, 200–400 mesh, sodium form).

Table I. Metal to Protein Ratios of Metallothioneins

Mt-II[a]	source	metal content Zn	Cd	Cu	tot.
Zn,Cd-Th	horse kidney	2.47	4.23		6.7
Zn-Th_a	rat liver	7.14			7.14
Zn-Th_b	rabbit liver	6.43			6.43
Cd-Th	rat liver	0.44	6.12		6.56
Zn,Cd,Cu-Th_a	rabbit liver	2.60	1.43	2.52	6.55
Zn,Cd,Cu-Th_b	rabbit liver	0.69	4.86	0.66	6.21

[a] In each case, metallothionein iso-form II (Mt-II) was used for these studies.

Analyses. The metal contents of solutions were analyzed on an Instrumentation Laboratory 357 atomic absorption flame spectrophotometer. All samples were assayed against serial dilutions of reference standards. UV–visible spectra were recorded on a Cary 17 UV–visible 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 by using the published absorptivity coefficient of $\epsilon_{220} = 47\,300$ L/(mol cm).[28]

Isolation of Native Metallothioneins. Rat liver Zn-thionein (Zn-Th_a),[20] horse kidney Zn,Cd-thionein,[29] and rabbit liver Zn-thionein (Zn-Th_b)[2] were prepared as described elsewhere. The metal content of each preparation is given in Table I.

In Vitro Preparation of Cd- and Zn,Cd,Cu-Thioneins. Rat liver Cd-thionein (Cd-Th) was prepared from native rat liver Zn-Th_a. $Cd(NO_3)_2$ in 5 mM Tris/100 mM $NaClO_4$, pH 7.5, was added directly to a Zn-Th_a solution, yielding a 3/1 Cd/Zn mole ratio. The excess free metal was removed immediately by passing the solution over Chelex-100 (1.5 × 0.3 cm column, contact time ~ 1 min). Cd displaced 94% of the native Zn in a 1/1 ratio. Elution of the product over Sephadex G-50 gave the same chromatographic profile and K_d value as native rat liver Zn-Th. Rabbit liver Zn,Cd,Cu-thioneins (Zn,Cd,Cu-$Th_{a,b}$) were obtained by passing two different preparations of rabbit liver Zn-Th over a Sephadex G-25 column (2.5 × 60 cm) that had been previously exposed to cadmium and copper. The protein was eluted from the column by 10 mM NH_4HCO_3, pH 7.8.

Colorimetric Monitoring of the Metal Exchange. Because formation of the dye–metal complexes is pH- and concentration-dependent,[23,24,27,30] the Beer's law behavior was verified under the conditions used here. The following extinction coefficients in L/(mol cm) were determined: Cd-$(PAR)_2$, $\epsilon_{485} = 32\,000$; Zn$(PAR)_2$, $\epsilon_{485} = 43\,300$; Zn-ZI, $\epsilon_{620} = 23\,200$. No absorbance due to a Cd-ZI complex was detected in the 1–5 μM concentration range used here. Stock solutions of metallothionein, chromophore, and buffer (5 mM Tris-HCl/100 mM $NaClO_4$, pH 7.4) were mixed, and then AuSTm was added to give the desired concentrations of reagents: typically, 2 μM protein-bound metal; 20 μM chromophore (PAR or ZI); 2 μM to 1 mM AuSTm in a total volume of 1.00 mL. Kinetic analysis of the reactions at 25 °C commenced immediately after adding AuSTm and mixing the reagents. The significant changes in absorbance occurred within 20 min, but the reactions were followed for 80 min to determine A_∞. Each reaction was run at least twice, and the average k_{obs} values are reported.

Analysis of Kinetic Data. Data were digitized manually, and all calculations were performed on an IBM PC microcomputer using programs written by J.E.L. One program calculates ln $(A_\infty - A_t)$ and plots these values vs time. For a biphasic reaction, the corrected rate constant for the fast component of the biphasic plot is calculated by plotting ln $f(t)$ versus time, where $f(t)$ is defined by the equation $f(t) = (A_\infty - A_t)_{obs} - (A_\infty - A_t)_{II}$, and $(A_\infty - A_t)_{II}$ is determined by extrapolating the slow step. The rate constants (s^{-1}) of the fast and slow components and the intercepts are reported. A second program plots k_{obs} vs concentration with either 99% or 95% confidence bands about the calculated slope, reports the slope and intercept, and tests whether or not the slope is significantly different from zero.

PAR Extraction of Zn^{2+}. The kinetics of the direct, slow reaction between PAR and Zn_7-Th, in the absence of AuSTm, was studied by

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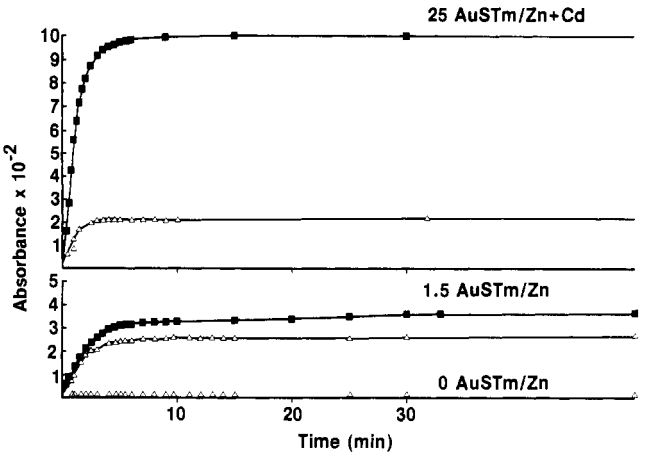

Figure 1. Time-dependent formation of the metal chromophore complexes during the AuSTm + Zn,Cd-Th reactons. Conditions: $[Zn]_{Th}$ = 2 μM; $[Cd]_{Th}$ = 3.4 μM; PAR or ZI = 20 μM, [AuSTm] = 3 or 136 μM; 5 mM Tris-HCl/100 mM $NaClO_4$; pH 8.6; 25 °C. Zincon reacts only with Zn^{2+}, PAR reacts with Zn^{2+} and Cd^{2+}. At the lower gold concentration, only zinc is displaced; at the high concentrations, both metals are displaced. Key: (■) PAR; (△) ZI.

using 20 μM Zn^{2+} as Zn_7-Th and PAR concentrations between 100 and 1500 μM in 50 mM Tris-HCl buffer with 100 mM $NaClO_4$, pH 7.5. The reactions were monitored for 60 min, and final absorbances (A_∞) were determined after 2-5 days. Because these reactions are extremely slow, they were kept under argon and in a thermostated water bath.

Conditional Stability Constants. A consistent set of conditional stability constants at pH 7.4 for the ligand-metal interactions central to this study were calculated from various equilibrium constants in the literature. Because different conventions of nomenclature and choice of ligand species (fully protonated, partially protonated, or deprotonated) were used by the original authors, a specific relationship was required for each metal-ligand pair. In citing the original constant used, we have in each case used the original authors' symbols. C_L and in some cases C_{ML} refer to the total concentrations of free ligand and complex in various states of protonation. For ZnZI, $K_{app} = [ZnL]/[Zn]C_L = 10^{4.9}$, derived from $K^{(1)} = 10^{-9.8}$.[27] For Zn(STm), $K_{app} = [ZnL_2]/[Zn]C_L^2 = 10^{8.7}$, calculated from $K_1K_2 = 10^{14.56}$.[31]

The calculations for the PAR complexes required care because the 3'-hydroxy group ($pK_a'' = 12.4$) is deprotonated upon complexation, while the more acidic 1'-hydroxy group ($pK_a' = 7.0$) is not, yielding complexes $M(HL)_2$, which are themselves weak acids at neutral pH; e.g., $pK_{a1} = 7.7$ and $pK_{a2} = 9.3$ for $Zn(HL)_2$.[23-26] Further, the original values of K_1[23] were in error by 1 order of magnitude (too large), leading to a 2 order of magnitude error in β_2 ($=k_1K_1^2/k_{a2}^2$).[24,26] Thus for $Cd(PAR)_2$, $K_{app} = C_{ML}^2/[M]C_L^2 = 10^{10.6}$ was obtained by using the corrected value, $\beta_2 = 10^{19.6}$, and for $Zn(PAR)_2$, $K_{app} = 10^{14.3}$ from the corrected value, $\beta_2 = 10^{23.3}$.[23,24] A somewhat smaller value for $Zn(PAR)_2$, $K_{app} = 10^{12.6}$, was calculated by using the independently measured data of Corsini et al.,[25,26] $k_1k_2 = 10^{23.5}$.

Results

In order to use the metallochromic dye Zincon as a probe for the rate of Zn displacement from MT, we first demonstrated that it does not extract zinc (or cadmium) from horse kidney Zn,-Cd-Th. Next we verified that ZI does not react with AuSTm or with cadmium under the conditions used here (pH 7.4 or 8.6 in 5 mM Tris-HCl buffer with 100 mM $NaClO_4$). Since it reacts with aqueous zinc ions within the conventional mixing time, it should provide a useful probe of zinc displacement from MT. Figure 1 shows that the development of the chromophore (Zn/ZI λ_{max} = 620 nm) is complete 5-10 min after adding AuSTm to a mixture of ZI and Zn,Cd-Th at pH 8.6 and 25 °C. (The metal contents and sources the thionein preparations used in this work are given in Table I.) Two ratios of gold to thionein were used. The lower ratio (Au/Zn = 1.5) is sufficient to displace the zinc but not cadmium from the MT and, with concomitant loss of the gold-bound thiomalate, form Au,Cd-Th.[11] The higher ratio (Au/(Zn + Cd) = 25) causes zinc and cadmium to be totally displaced, forming $(TmSAu)_{20}$-Th.[11] The extent of chromophore development is similar in each case since it is determined by the amount of zinc present.

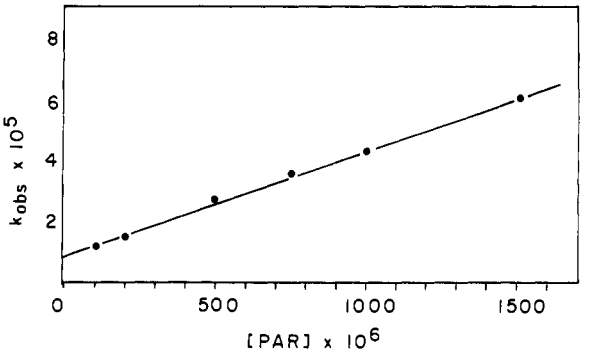

Figure 2. Pseudo-first-order rate constants (k_{obs}) vs [PAR] for the direct reaction of PAR with Zn_7-Th in the absence of AuSTm. Conditions: $[Zn]_{Th}$ = 20 μM; [PAR] = 100-1500 μM; 50 mM Tris-HCl/100 mM $NaClO_4$ buffer; pH 7.5.

PAR, a metallochromic agent sensitive to both cadmium and zinc, was also used (Figure 1). PAR does not react with AuSTm to give a colored metal/dye complex. Although it does not detectably extract Zn or Cd from Zn,Cd-Th under the conditions used for the AuSTm reactions ($[Zn]_{Th}$ = 2 μM; [PAR] = 20 μM, 10 min), we did observe a slow reaction of PAR with rat liver Zn_7-Th at higher concentrations and over longer times. A detailed kinetic study ($[Zn]_{Th}$ = 20 μM; [PAR] = 100-1500 μM) determined that, at concentrations up to 500 μM PAR, the reaction is monophasic and pseudo first order and that the observed rate constant depends on the PAR concentration (Figure 2). Thus, from the nonzero intercept, the direct (AuSTm-independent) reaction obeys the rate law

$$\text{rate} = k_2[PAR][Zn] + k_1[Zn]$$

The values of $k_2 = 3.8 \times 10^{-2}$ L/(mol s) and $k_1 = 8.5 \times 10^{-6}$ s^{-1} establish that less than 1% of the zinc ions would be extracted from Zn_7-Th under conditions of the AuSTm reactions. We did not detect any reaction between PAR and rabbit liver Cd-Th under the same conditions employed for the Zn-Th.

When PAR was used to monitor the reaction between AuSTm and Zn,Cd-Th, the absorbance changes occurred on the same time scale as the reactions monitored with ZI. At the lower gold concentration (Au/Zn = 1.5), the reaction profile was similar to that with ZI, but with excess gold (25 Au/(Zn + Cd)), the absorbance increase was much greater, since both cadmium and zinc are displaced to form chromophores. The extent of chromophore development, determined from A_∞ values (e.g., Figure 1), is less than that predicted from the known stoichiometries of the AuSTm reactions.[11] The discrepancy, when gold is the limiting reagent, results from competition between the displaced thiomalate and dye for Zn^{2+} and Cd^{2+}. When excess AuSTm is used, it also competes with the dyes for Zn_{2+} and Cd^{2+}. This may be due to the excess thiomalate (1.04-1.08 thiolates per gold) present in AuSTm.[32] As expected from the conditional formation constant[22] at pH 7.4 for $Zn(STm)_2$, $K_{app} = 10^{8.6}$, the discrepancy is greater for Zincon than PAR. The incomplete formation of dye-metal chromophore does not interfere with the first-order kinetic analysis described below, since the standard treatment requires only that the absorbance changes be proportional to the amount of metal displaced.

Since UV-visible spectrometry is convenient for monitoring these reactions, further development of this metallochromic method was undertaken. When the data for reactions of Zn,Cd-Th with AuSTm in the presence of ZI or PAR at pH 8.6 or 7.4 were analyzed as first-order reactions (plots of ln $(A_\infty - A_t)$ versus time), the reactions were with few exceptions biphasic, as illustrated in Figure 3. The corrected first-order rate constants were obtained

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Table II. Averaged Rate Constants (s^{-1}) for Metallothionein Preparations[a]

		pyridylazoresorcinol (PAR)			Zincon (ZI)		
thionein	source	10^2k_f	10^4k_s	$(n)^b$	10^2k_f	10^4k_s	$(n)^b$
Zn,Cd–Th	hrs kid	1.6 ± 0.6	5.8 ± 0.5	(4)	1.4 ± 0.3	6.4 ± 1.0	(3)
Zn–Th_a	rat liv	2.4 ± 0.5	7.3 ± 1.7	(14)	2.8 ± 0.1	10.7 ± 0.5	(10)
Zn–Th_b	rbt liv	2.4 ± 0.7	6.2 ± 0.2	(6)	2.5 ± 0.4	10 ± 2	(6)
Cd–Th	rbt liv	2.2 ± 1.1	7.3 ± 1.2	(10)			
Zn,Cd,Cu–Th_a	rbt liv	1.9 ± 0.8	8.0 ± 1.0	(10)			
Zn,Cd,Cu–Th_b	rbt liv	5.1 ± 1.3	5.5 ± 2.3	(10)			
av		2.7 ± 1.2	6.9 ± 0.9	(54)	2.4 ± 0.6	9.6 ± 1.7	(18)

[a] Includes data from Figures 4 and 5, where AuSTm or PAR concentrations were varied. [b] Number of independent measurements.

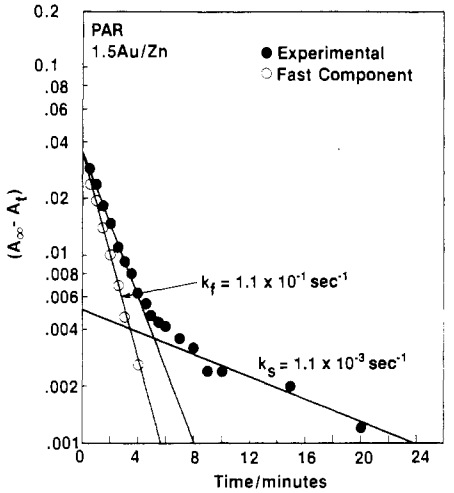

Figure 3. First-order kinetic treatment (ln $(A_\infty - A_t)$ vs time), showing biphasic kinetics for AuSTm displacement of metal ions from Zn,Cd–Th monitored by PAR. Conditions: $[Zn]_{Th}$ = 2 μM; $[Cd]_{Th}$ = 3.4 μM; [PAR] = 20 μM; [AuSTm] = 136 μM; 5 mM Tris–HCl/100 mM $NaClO_4$; pH 8.6; 25 °C.

by extrapolation and subtraction of the slow component according to established procedures. The resulting rate constants, k_f and k_s, were similar whether measured with PAR or ZI. Biphasic kinetics would be observed if k_f and k_s were each associated with a cooperative reaction of one of the clusters, M_3S_9 or M_4S_{11}. Alternatively the two rate constants might result from different rates of displacement for Cd and Zn ions, e.g., k_f for Zn^{2+} and k_s for Cd^{2+}. Data to test these hypotheses are described below.

To determine whether the chromophores ZI and PAR innocently probe the metal displacement or, au contraire, accelerate it, the reactions were carried out in the presence of increasing dye concentrations: 2–200 μM PAR or 2–80 μM ZI, holding the concentrations of AuSTm and rat liver Zn,Cd–Th constant. In these and all the subsequent studies reported here, the pH was maintained at 7.4. Analysis of the data yielded biphasic first-order kinetics for each run. Attempts to treat the data as having first- and second-order components, $k_{obs} = k_2[dye] + k_1$, yielded second-order components with unreasonably large error limits that were not significantly different from zero. Plots of the slow and corrected fast rate constants versus dye concentrations demonstrated that they are independent of both the concentration and the choice of the dye (Figure 4). The small discrepancy in the k_s values measured by PAR and ZI (Figure 4) is less than a factor of 2. Since there is no PAR or ZI concentration dependence, we do not attribute any significance to the differences.

Next, the dependence of the reaction rates on the gold concentration was studied. [AuSTm] was varied from 2 μM to 1 mM. Both dyes and three MT preparations, Zn–Th_a, Zn,Cd–Th, and Zn,Cd,Cu–Th_a (Table I), were examined at 25 °C and pH 7.4. The data yielded biphasic first-order plots in each case. The values for k_f plotted vs [AuSTm] fell on a single line with a slope indistinguishable from zero; the k_s points fall on a separate, parallel line (Figure 5). Thus, over 3 orders of magnitude, the reaction rates are independent of the AuSTm concentration. Even when the gold is not present in pseudo-first-order excess (i.e., at the left side of Figure 5, where [AuSTm] is 2–10 μM and is comparable

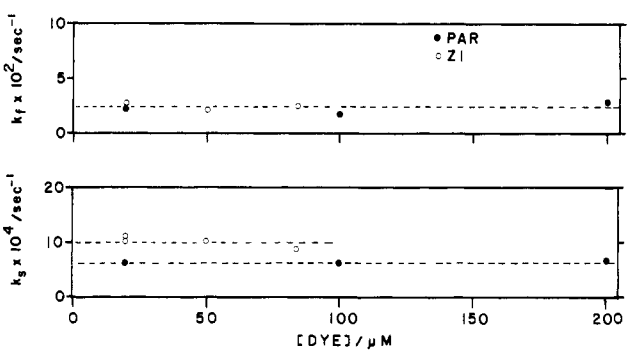

Figure 4. Dependence of the observed rate constants, k_f and k_s, of various thioneins on the concentration of PAR or ZI. Conditions: $[metal]_{Th}$ = 2 μM; [AuSTm] = 20 μM; [PAR] = 20–200 μM; [ZI] = 20–84 μM; 5 mM Tris–HCl/100 mM $NaClO_4$; pH 7.4; 25 °C.

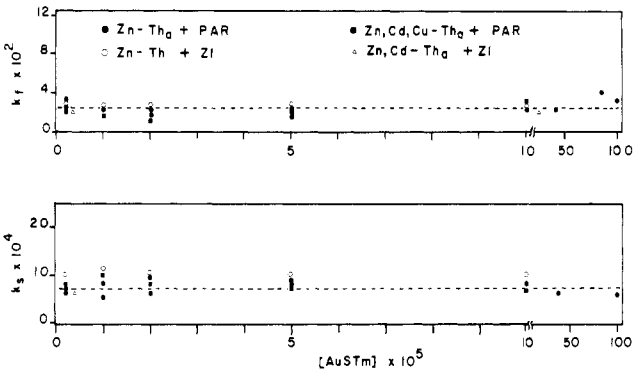

Figure 5. Dependence of the observed rate constants k_f and k_s on the concentration of AuSTm, using PAR or ZI. Conditions: $[metal]_{Th}$ = 2 μM; [dye] = 20 μM; [AuSTm] = 20 μM to 1.0 mM; 5 mM Tris–HCl/100 mM $NaClO_4$; pH 7.4; 25 °C. Data for three protein preparations are plotted: Zn–Th_a using PAR (●) and ZI (○), Zn,Cd,Cu–Th_a using PAR (□), and Zn,Cd–Th_a using ZI (△).

to $[M^{2+}]_{Th} \sim 2$ μM), well-behaved biphasic first-order plots were obtained. The data in Figure 5 obtained with either PAR or ZI fall on the same plots for k_f and k_s, further substantiating that the reaction rates are independent of the metallochromic ligand. The averaged values for each combination of dye and protein are listed in Table II.

A metal dependence of the rate constants would be expected if metal–thiolate bond breaking were the rate-determining step. Experiments were performed with three additional metallothioneins, Zn–Th_b, Cd–Th, and Zn,Cu,Cd–Th_b. For each, the rate constants measured at several gold concentrations were averaged. Table II compares the rate constants k_f and k_s for all the MT preparations studied. There are no significant changes in the rate constants as Zn^{2+} is replaced by Cd^{2+} or Cu^+. Thus, the rate-determining steps are not dependent on the nature of the thionein-bound metals.

Finally, the possibility that the two metal ions of Zn,Cd–Th might give rise to the biphasic kinetics of Figure 3 can be tested by the results for thioneins with homogeneous metal content. If Zn–Th_a, which had 7.14 Zn/protein and no detectable cadmium or copper (Table I), is used with either PAR or ZI, first-order biphasic kinetics were observed (Table II). Biphasic first-order

kinetics and similar rate constants were also obtained when Cd–Th, formed in vitro, reacted with PAR. Thus, the biphasic kinetics of the mixed-metal thioneins are due to an intrinsic property of the metallothionein metal binding sites and not to differences in the reactivity of cadmium and zinc.

Discussion

For a metallochromic ligand, such as PAR or ZI, to function as a probe of Zn^{2+} or Cd^{2+} displacement from MT, it should meet the following criteria:

(1) The metallochromic ligand must be thermodynamically incapable of removing the thionein-bound metals or must do so at a negligible rate under the conditions used.

(2) The reaction of the ligand and displaced metal ions must be rapid compared to the rate of the metal displacement.

(3) The ligand must not complex to or react with the attacking species.

(4) The ligand must not participate in forming the transition state of the rate-determining step.

The apparent stability constant at pH 7.4 for Zn–ZI, $K_{app} = 10^{4.9}$, establishes that it cannot extract zinc from metallothionein ($K_{app} = 10^{11.2}$ per zinc).[21] The conditional stability constant at pH 7.4 for $Zn(PAR)_2$ ($K_{app} = 10^{14.3}$ or $10^{12.6}$)[2], indicates that PAR has the thermodynamic capability to extract Zn^{2+} completely from metallothionein. Yet, the extraction of Zn^{2+} from Zn_7–Th proceeds very slowly, requiring days to remove the metal ions under the conditions used here. Thus, there must be a substantial kinetic barrier hindering direct PAR extraction of the Zn ions. The conditional stability constant for $Cd(PAR)_2$ ($K_{app} = 10^{10.6}$) indicates that large excesses of PAR are required to extract Cd^{2+} from metallothionein ($K_b = 10^{14.6}$ per cadmium).[21] We confirmed that, under the conditions used here, PAR did not detectably extract any Cd from Cd–Th. PAR and ZI react with free zinc or cadmium ions within the mixing time, but they do not detectably react with AuSTm. Thus, criteria 1–3 are clearly satisfied.

The fast step of the AuSTm–metallothionein reaction is found to be the same whether measured by PAR or ZI, and it is independent of the dye concentration. The marginal differences in k_s values obtained with ZI or PAR are not significant, and the values do not increase with dye concentration. Thus, the rates are clearly independent of PAR or ZI concentrations, verifying that they function as innocent probes of the reaction and do not participate in the rate-determining steps (rds), criterion 4. That is, k_f and $k_s \ll k_c$

$$\text{AuSTm} + \text{M}_7\text{–thionein} \xrightarrow[\text{rds}]{k_f,\, k_s} \text{aurothionein} + \text{M}^{2+}$$

$$\text{dye} + \text{M}^{2+} \xrightarrow[\text{fast}]{k_c} \text{M–dye}$$

Metallothionein, despite its small size, is an extremely complex protein, containing seven distinct metal binding sites grouped into two metal–thiolate clusters (M_4S_{11} and M_3S_9). Assuming that each site can be occupied by zinc or cadmium, there are a minimum of 14 rate constants for metal displacement (and even more, if electronic or structural effects of neighboring metal ions are transmitted through the thiolate bridges). The finding that only two first-order rate constants can fit the data greatly simplifies the task of associating the reaction kinetics with structural features of the protein and developing a mechanistic explanation.

The biphasic reactions of $Zn–Th_a$, $Zn–Th_b$, and Cd–Th with AuSTm rule out the hypothesis that the slow and fast steps are due to displacement of cadmium and zinc, respectively. Reasonable alternative explanations are (1) that each cluster is associated with one of the observed reactions (k_f or k_s) and the metal sites within each cluster react cooperatively or (2) that there are two sets of metal sites distributed between the clusters, each set having identical or, at least, indistinguishable rates for reaction with AuSTm.

This is the first report of a biphasic metal-exchange reaction for MT, although biphasic kinetics have been observed for reactions with other electrophiles and various metal-chelating agents.[18,20,21,33] Several studies point to a greater lability or reactivity of the three-metal cluster.[2,34] Thus, it is attractive to postulate that k_f is associated with the three-metal cluster and k_s with the four-metal cluster. While our data can not unambiguously eliminate the possibility that the fast-reacting sites might be distributed between both clusters, independent evidence of cooperativity in metal binding has recently been reported. Four equivalents of cadmium reacts with apothionein, binding cooperatively to form the four-metal cluster.[35]

The absence of gold dependence for k_f and k_s can be explained either by rate-determining steps localized in the metallothionein and preceding the binding of gold (eq 1) or by a rapid and tight

$$\text{M}_7\text{–Th} \xrightarrow[\text{rds}]{k_1} \text{M}_7\text{–Th*} \xrightarrow[\text{AuSTm}]{k_2} \text{products} \qquad (1)$$

preequilibrium that generates a Michaelis–Mentan-like complex that reacts in the rate-determining step (eq 2). For either case,

$$\text{M}_7\text{–Th} + \text{AuSTm} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{M}_7\text{–Th·(AuSTm)}_x \xrightarrow[\text{rds}]{k_4} \text{products} \qquad (2)$$

there would be separate rate-determining steps corresponding to k_f and k_s. Equation 2 predicts a saturation curve for k_{obs} as [AuSTm] is increased. If $k_3, k_{-3} \gg k_4$ and if, even at low AuSTm concentrations, k_3 [AuSTm]$/k_{-3} > 1$, then all the data points of Figure 4 could lie on the plateau region of such a saturation curve (i.e., k_f or $k_s = k_{obs} = k_4$). Equation 1, in which the rate-determining step occurs before reaction with the gold, provides a simpler explanation of the kinetics. While the simpler scheme seems more likely, neither one can be rejected with the data presently available. The lack of metal dependence for the different metallothioneins suggests that M_7–Th* is not formed by simple metal–thiolate bond cleavage.

Other reactions of metallothionein leading to metal displacement (or extraction) have been studied kinetically:[33] DTNB,[20] EDTA,[18] and apo-carbonic anhydrase.[18] The EDTA and apo-CA reactions, which are ligand-exchange reactions centered at the metal, show strong dependence on the metal: $k_{Zn} \gg k_{Cd}$. In contrast, DTNB reacts biphasically via electrophilic attack on the coordinated thiolates, and the rates are independent of the metals (Cd, Zn, or Cu) bound to MT.[20] Thus, the DTNB reaction in many respects resembles the AuSTm reaction. Both are biphasic reactions of electrophiles with the metal-coordinated thiolates. Both lead to metal displacement and occur at rates independent of the cadmium and zinc content of MT. They differ in that the DTNB reaction is slower and has first- and second-order components to the observed fast and slow steps.[20]

The kinetic rate constants determined here explain the ability of injected gold to bind almost immediately to preexisting MT in the liver and kidney of laboratory animals.[7,36] The levels of aurothioneins immediately after a 1 mg of Au/kg dose of AuSTm are 0.2–0.3 μg of Au/g of tissue. In the kidney (but not the liver), MT-bound gold and copper increase concomitantly over a week, reaching ca. 6 μg of Au/g of tissue. The rate constants for transfer of gold from the blood (central compartment) to the peripheral ($(2.3 \pm 0.1) \times 10^{-4}$ s^{-1}) and deep ($(9.0 \pm 0.9) \times 10^{-5}$ s^{-1}) compartments of rat tissue[36] are smaller than the constants for aurothionein formation, demonstrating that factors other than the kinetics of gold binding to MT control the rates of gold deposition into tissues.

Gold binds to metallothionein rapidly, and the rate is essentially independent of the metal content of the metallothionein. Thus, within any tissue, thermodynamic rather than kinetic factors must govern the distribution of metals between MT and other sites in equilibrium with it. Previous results of ours show that AuSTm

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displaces zinc more easily than cadmium.[11] From the known order of metal-binding affinities for metallothionein, Cu > Cd > Zn, one can infer that gold will displace Zn^{2+} in preference to Cu^{+}. Indeed, shortly after gold administration, the zinc content of metallothionein is decreased, while the copper and gold levels continue to increase.[7] The continued presence of zinc and copper in metallothionein suggests that $(TmSAu)_{20}$-Th does not form and that mixed-metal aurothioneins (e.g., Au,Cu,Zn-Th) are the principal in vivo forms.

Note Added in Proof. The kinetic study of the direct reaction of PAR (100-1500 μM) and Zn_7Th (20 μM Zn) was reexamined under the same reaction conditions with a better spectrophotometer, which enabled us to observe an initial, fast step that was not previously resolved at PAR concentrations below 500 μM. This fast step is independent of the PAR concentration from 100 to 1500 μM and the first-order rate constant, k_{1f}, is 2.1×10^{-3} s^{-1}. The fraction of the zinc ions reacting in the fast step was 0.03-0.07 for 100-500 μM PAR and never exceeded 0.17 at the highest concentration (1500 μM). Thus it corresponds, at most, to one of the seven zinc ions in MT. Thus, the direct PAR reaction consists of two steps, the fast step described here and the slow step described in the Results section:

$$\text{rate}_f = k_{1f}[Zn] \qquad \text{rate}_s = k_{1s}[Zn] + k_{2s}[PAR][Zn]$$

The contribution of the direct PAR reactions to the overall reaction in the presence of AuSTm was recalculated by using these rate constants for the direct reaction and the average rate constants for the AuSTm reaction from Table II, here designated $k_{f,Au}$ and $k_{s,Au}$:

$$\text{fraction} = \frac{\sum k_{PAR}}{\sum k_{PAR} + \sum k_{Au}} = \frac{(1/7)k_{1f} + k_{1s} + k_{2s}[PAR]}{(1/7)k_{1f} + k_{1s} + k_{2s}[PAR] + k_{f,Au} + k_{s,Au}} = 0.011$$

Although the relative magnitudes of k_{1f} and $k_{f,Au}$ suggest that a substantial amount of Zn^{+2} might be extracted directly by PAR, the small fraction of the zinc reacting in the k_{1f} step (taken as $^1/_7$ of the total in the calculation, renders it insignificant. Thus, the claim in the text that only about 1% of the zinc is directly extracted by PAR remains valid, despite the existence of the k_{1f} step that was not known to us at that time.

Acknowledgment. This work was supported by the National Institutes of Health (Grants GM 29583 and ES 04026) and Smith Kline & French Laboratories. The AuSTm was a generous gift of SK&F Laboratories.

Registry No. Cd, 7440-43-9; Zn, 7440-66-6; Au, 7440-57-5; AuSTm, 33796-26-8; pyridylazoresorcinol, 1141-59-9; Zincon, 135-52-4.

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Aluminum Citrate: Isolation and Structural Characterization of a Stable Trinuclear Complex

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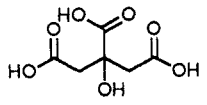
The complex anion $[Al_3(H_{-1}Cit)_3(OH)(H_2O)]^{4-}$ has been isolated from the reaction of $Al(NO_3)_3 \cdot 9H_2O$ with citric acid (H_3Cit) in aqueous solution between pH 7 and 9. The complex has been characterized by ^1H, ^{13}C, and ^{27}Al NMR spectroscopy and X-ray crystallography. The complex $[NH_4]_5[Al_3(H_{-1}Cit)_3(OH)(H_2O)][NO_3] \cdot 6H_2O$ ($C_{18}H_{47}Al_3N_6O_{32}$) crystallizes in the monoclinic space group $P2_1/a$ with the following crystal parameters: a = 17.289 (5) Å, b = 13.210 (6) Å, c = 17.705 (5) Å, β = 115.34 (2)°, Z = 4. The data was refined by using 5301 reflections to R = 8.46, R_w = 9.48. The complex anion consists of a trimeric Al_3O_4 core with each citrate ligand coordinated to two or more aluminum atoms. Variable-pH ^{27}Al NMR has been used to study the degradation of the trimer at low pH.

During the past decade, an ever increasing volume of evidence has been presented to associate aluminum with a variety of neurotoxic conditions.[1] Increased aluminum levels have been detected in patients suffering from Alzheimer's disease[2] as well as those undergoing dialysis treatment for chronic renal failure.[3] The toxicity of Al^{3+} raises questions concerning the possible route of its absorption into the body and its binding modes after ingestion. It has been suggested that citric acid (H_3Cit), which occurs at about 0.1 mM in blood plasma, is the most likely small molecule plasma binder of Al^{3+}.[4]



H_3Cit

Citrate binding of Al^{3+}, in aqueous solution, has been described in several recent studies,[5-8] where agreement is limited to acknowledging the formation of the complexes $[Al(HCit)]^+$ and Al(Cit) between pH 1 and 3. A definitive study by Öhman and co-workers[9] covering all the points of the speciation suggests that a stable trimeric aluminum citrate complex is the major species in an equimolar Al:Citric acid solution between pH 4 and 9. The trimer was formulated as either $[Al_3(Cit)_3(OH)_4(H_2O)]^{4-}$ or $[Al_3(H_{-1}Cit)_3(OH)(H_2O)_5]^{4-}$ and was proposed to be structurally similar to the magnesium citrate decahydrate complex.[10]

The tendency of aluminum alkoxide and carboxylate compounds to maximize the aluminum coordination number by associating to give aggregates containing tetrahedral and octahedral centers is well documented.[11] The aim of the present study is to investigate the possibility of polynuclear aluminum citrate complexes from aqueous solution.

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