where stabilization of *u-fac* and *s-fac* over *mer* correspond to 1.3 and 3.2 kJ mol^{-1}, respectively, compared to R_x values, derived from Figure 3 of ref 40, of 1.3 and 1.9.

These correlations suggest that if a given anion does not produce separation of the different isomers when used to elute a mixture down a column (i.e. $R_x \simeq 1$) then ion association with each isomer is similar and large environmentally induced changes in equilibrium distributions should not occur when these anions are present. Searle[38] has shown that water, chloride, nitrate, perchlorate, nor presumably hexafluorophosphate produce a separation of the three isomers of $[Co(dien)_2]^{3+}$ on SP-Sephadex chromatography columns. Therefore, isomer distributions determined with these species present should produce free energy differences approximating isolated-state values and in this context it is worth noting that distributions obtained with chloride, nitrate, and perchlorate present are all similar.[22]

(iv) Model for Electrochemical Reduction of Cobalt Complexes. Electrochemical data for kinetically inert systems such as $[Co(sep)]^{3+,2+}$ and $[Co(dien)_3]^{3+,2+}$ are amenable to a theoretical description. For many other complexes the cobalt(II) species is very labile and complicates the interpretation of data. Nevertheless, force field calculations on other systems demonstrate that minima in free energy do occur.[3,39-41] The electron-transfer scheme, under these conditions, must be interpreted in terms of reaction scheme 2. Support for this concept comes from data contained on other complexes in ref 42-44. Organic compounds studied by Evans et al. and other workers[45] also support this view.

The majority of studies describing the reduction process

$$Co(III) + e^- \overset{E^\circ, k_s, \alpha}{\rightleftharpoons} Co(II) \qquad (9)$$

treat the problem as a single step and no knowledge on conformer contribution is considered. This may be appropriate, fortuitously, as is the case of $[Co(sep)]^{3+,2+}$, where the same conformer is the most stable in both oxidation states, but if rearrangements of conformers with different E° values are involved in the electron transfer, then this cannot be correct. Studies are continuing in these laboratories to further understand the influence of conformation changes on redox processes. Clearly, homogeneous electron-transfer mechanisms are also affected by the above considerations, and importance of these concepts to this field will be considered at a later date.

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Registry No. $[Co(sep)]^{3+}$, 72496-77-6; $[Co(sep)]^{2+}$, 63218-22-4; *mer*-$[Co(dien)_2]^{3+}$, 38318-06-8; *mer*-$[Co(dien)_2]^{2+}$, 67145-46-4; *u-fac*-$[Co(dien)_2]^{3+}$, 38318-05-7; *u-fac*-$[Co(dien)_2]^{2+}$, 67145-47-5; *s-fac*-$[Co(dien)_2]^{3+}$, 38318-04-6; *s-fac*-$[Co(dien)_2]^{2+}$, 67145-48-6; $[Co(sep)](ClO_4)_3$, 88228-09-5.

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Cadmium Binding by Biological Ligands. 3. Five- and Seven-Cadmium Binding in Metallothionein: A Detailed Thermodynamic Study

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The anaerobic binding of cadmium ions by rat liver apometallothionein 2 was studied by pH-static (pH 4.5, 6, 7, 8.25, 9.5), pCd-metric techniques, employing a microcomputer-controlled two-buret, two-electrode titrator. The solutions (3-4 mL, 25 °C, 0.15 M KNO_3) were 0.13-0.16 mM in apoprotein. Several of the sulfhydryl groups in metallothionein were found to be unexpectedly acidic, with pK_a's as low as 7. At pH 6 and 7 several cadmium-protein species were identified: the corresponding binding constants were successfully refined by a weighted nonlinear least-squares procedure. A dianionic seven-cadmium complex forms at pH 6, with an apparent binding constant of 10^{66}. Surprisingly, only five-cadmium complexes form if the apoprotein solution is reacted with cadmium ions at pH 7. At pH 6, 18 protons are liberated by the addition of metal ion, which is consistent with the displacement of all of the sulfhydryl protons by the metal ions. At pH 7, however, only 11 protons are liberated by the metal ions, clearly indicating that not all of the sulfhydryl groups participate in coordination, under the experimental conditions. A discussion of the possibility of a conformational change in the apoprotein taking place when the pH is raised from 6 to 7 is presented.

Introduction

Biologically significant sulfhydryl-containing ligands, such as cysteine, can strongly bind cadmium ions to form a variety of complexes whose compositions in aqueous solutions depend in a sensitive manner on such factors as pH, total cadmium ion concentration, ionic strength, and the presence of other metal ions and non-sulfhydryl ligands. Until recently, studies of cadmium reactions with sulfhydryl ligands had led to oversimplified models concerning the nature of complexes present in solution: the tendency of thiolate groups to bridge metal ions to form polynuclear complexes had been frequently neglected. Factors governing the stoichiometry and the extent of formation of polynuclear complexes in aqueous solution are consequently poorly understood. In parts 1 and 2 of this series, we applied novel potentiometric techniques to study the equilibrium mechanisms of cadmium binding with simple monosulfhydryl ligands: penicillamine[2] and cysteamine.[3] Both model systems were found to exhibit a tendency to form tri- and tetranuclear ternary complexes under mildly acidic conditions (pH 4-6). The present study concerns cadmium binding by the protein metallothionein (MT).

Metallothionein is a small (M_r 6700-6800) intracellular, sulfur-rich protein that strongly binds a variety of metal ions, including Zn, Cd, Cu, and Hg. The protein was first isolated from horse kidney by Margoshes and Vallee in 1957;[4] since then its

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Table I. Composition of Titrated Solutions

expt no.	titration method[a]	pH	[protein], mM[b]	no. of SH[c]	$\bar{n}_H$[f]	[KNO_3], M
1	I	4.5	0.161	18.8	25.5	0.155
2	I	6.0	0.130	18.8	23.7	0.157
2′	I	6.0	0.143	18.6 (18.2)[d]	23.7	0.136
3	I	7.0	0.147	18.0 (15.7)[d,e]	22.2	0.174
3′	I	7.0	0.142	18.6 (18.0)[d]	22.2	0.136
4	I	8.25	0.128	18.8	16.6	0.157
5	I	9.5	0.140	18.0	6.8	0.155
6	II	3.0–6.0	0.135	18.8	29.3–23.7	0.166
7	II	3.0–8.2	0.135	18.8	29.7–17.2	0.166
8	II	3.0–10.9	0.135	18.8	29.9–0.0 (16.2)[g]	0.162
9	III	7.0–9.9	0.129	18.0	(8.8)[f]	0.153
10	III	2.1–11.8	0.107	17.2		0.142

[a] Conditions for I: pCd metric, pH static; two titrants, 0.0179 M $Cd(NO_3)_2$ (acidified with 0.0181 M HNO_3) and 0.0461 M KOH. Conditions for II: pH metric; KOH titrant. Conditions for III: pH metric, pCd metric; KOH titrant; experiment 9 contained 8.8 equiv of $Cd(NO_3)_2$ (added at pH 7); experiment 10 contained 7.8 equiv of metal salt (added at pH 2.1). [b] 1.5–2.1% relative error (triplicate determinations). [c] ±0.5 (standard deviation). [d] Determined *after* titration. [e] Exposed to air for 3 days, before analysis. [f] Average number of dissociable protons (pH 3–11 domain) bound to apothionein. [g] Number of protons titrated at pH 7.0–9.9.

widespread occurrence has been established in mammals, as well as in other vertebrates, invertebrates, and microorganisms. The major biological function of this protein is still a matter of controversy. The known physical and biological properties of MT have been comprehensively reviewed,[5-8] and the antigenic determinants of vertebrate MT's have recently been identified by radioimmunoassay.[9]

The most revealing structural probe to date in the study of MT has been ^{113}Cd NMR.[10-15] The elegant studies of Armitage and co-workers[11-15] have shown the presence of two thiolate-bridged cadmium clusters in the cadmium-exchanged protein. Winge and Miklossy[16] were able to enzymatically fragment MT into a 31-residue segment, one of the clusters. Garvey[17] has applied the detailed Chou–Fasman protocols[18,19] in elucidating the secondary structure of vertebrate MT's, and Boulanger et al.[15] applied these protocols[20] to produce a three-dimensional stereo diagram for the suggested backbone structure. Recently, initial X-ray structural studies have been performed by Melis et al.[21]

In the above NMR studies and in several other investigations, the reported number of cadmium ions bound to MT was less than the expected seven ions per mole of protein. The question of the number of bound metal ions and the conditions of binding could well be addressed by potentiometric studies.[22,23] Since further detailed examinations of cadmium binding and release by MT would be valuable contributions to a better understanding of the role the protein plays in metal metabolism, we decided to perform such experiments, using pH and Cd ion-selective electrodes (ISE), the results of which we report here.

Experimental Section

Metallothionein. Liver (Cd,Zn)-metallothionein, containing 4.12 Cd ions, 1.93 Zn ions, and 0.04 Cu ion per molecule, was isolated from adult female Wistar rats injected (subcutaneously) at 2-day intervals for 14 days with $CdCl_2$ (2 mg of Cd/kg of body weight/dose). The two isoforms of the protein, MT-1 and -2, were separated and purified as described previously.[24] Purity of the protein was verified by (a) determination of the amino acid composition (JEOL JLC 6AH analyzer), (b) metal analysis by atomic absorption spectroscopy (AAS) (Pye Unicam 192 and Perkin Elmer 603), (c) polyacrylat gel electrophoresis,[24] and (d) determination of the number of sulfhydryl (SH) groups present, using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 6 M guanidine hydrochloride.[25] The apoprotein stock solution was prepared and stored under N_2 in an inert-atmosphere glovebox (Vacuum Atmospheres). The metal ions were removed by ultrafiltration in an Amicon apparatus using UM-2 membrane and 0.1 M HCl. The absence of metal ions was confirmed by AAS (<40 ng of Cd/mg of protein). The sulfhydryl content in the protein was determined before and after titration (Table I). Two independent methods, micro Kjeldahl–Nessler[26] and Lowry,[27] were used to determine the apoprotein concentration. The nitrogen content in apometallothionein was assumed to be 16.4%[6] for the Kjeldahl method. A calibration coefficient of 2.5 was applied in the Lowry method.[28]

Reagents. The preapration and standardization of HNO_3, KOH, ethylenediamine, and $Cd(NO_3)_2$ stock solutions have been described elsewhere.[2,3] All solutions were prepared and stored under N_2 in the glovebox.

Titration Methods. Twelve titrations of rat apothionein 2 were performed by a two-buret, two-electrode computerized titrator[2,3,29,30] using three different methods. Type I titrations (experiments 1–5, Table I) were conducted at constant pH, with use of a Cd ISE, a combination glass electrode, and two titrants. Apoprotein solutions (3–4 mL, 0.13–0.16 mM), initially at pH 3, were brought to the desired pH automatically. Thereafter, variable-volume aliquots of $Cd(NO_3)_2$ titrant were added, each time producing decreases in pH. Following each addition of the metal titrant, the KOH-containing buret added variable amounts of base titrant to bring the pH gradually back to the desired constant value. (The 0.25-mL burets were capable of 50-nL minimum volume titrant additions.) After each addition of metal titrant and the

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(30) Avdeef, A.; Zabronsky, J.; Stuting, H. H. *Anal. Chem.* **1983**, *55*, 298–304. In the present study, $\sigma^2 = (0.05)^2 + (0.001 dpCd/dv)^2$, where v = volume of added metal titrant.

reestablishment of the desired pH, the metal and pH electrodes were sampled until stable readings were reached.

At pH 6 and 7, the titrations labeled 2′ and 3′ in Table I were performed under conditions nearly identical with those of experiments 2 and 3, to address reproducibility.

Type II titrations (experiments 6–8, Table I) were conducted to determine the apoprotein pK_a's. Only one buret (KOH) and one electrode (glass) were used by the autotitrator.

Type III titrations (expeiriments 9 and 10) involved reconstituted holometallothionein (containing cadmium ions exclusively). A one-buret (KOH) and two-electrode (pCd and pH) configuration was employed by the titrator.

During all titrations (which were conducted in a capped cell outside the glovebox), premoistened N_2 was passed gently over the solutions. The temperature was maintained at 25 °C. Each titrated solution contained enough KNO_3 to maintain the ionic strength approximaaely constant at 0.15 M. Each type I titration required about 18 h for completion. About 75% of this time was used in restoring the pH to the desired levels (without overshooting) following metal titrant additions. Methods II and III required about 2–5 h per titration.[31]

Electrode Calibrations. The Orion Ross glass electrode (81-03) was calibrated against a standard ethylenediamine solution, as described elsewhere.[29] (The reference half-cell in the latter combination electrode does not contain Ag^+ ions.) The Orion 94-48 cadmium ion-selective electrode was calibrated against $Cd(NO_3)_2$ solutions whose concentrations were determined by AAS. The Cd ISE response time[30,32] in the protein solutions seldom exceeded 3 min, which is indicative of both adequate ISE exchange current densities[33] and relatively labile metal–protein-exchange reactions. The "protein poisoning" effect observed by D'Orazio and Rechnitz[22] for the silver ISE exposed to solutions containing sulfur-rich proteins was not observed in the case of the cadmium electrode. There was no visible evidence of a protein coating on the highly polished sensing surface of the Cd ISE.

Methods of Calculation. The apometallothionein pK_a's were estimated from type II data by Bjerrum plots,[34] $\bar{n}_H$ vs. pH, where $\bar{n}_H$ refers to the average number of dissociable protons bound to the apoprotein at a given pH (pH 3–11 in the present study). It is the property of Bjerrum plots that the pH values at "$^1/_2$-integral $\bar{n}_H$" points approximately equal the pK_a's. The acidity constants were subsequently refined by nonlinear least-squares methods adapted from the computer program library STBLTY.[35]

Since our study draws specifically from potentiometric data, all determined equilibrium constants are termed "macroconstants", as distinguished from "microconstants".[36] To illustrate the distinction, consider the titration of cysteamine. In the corresponding Bjerrum plot, $\bar{n}_H$ takes on values $^3/_2$ at pH 8.3 and $^1/_2$ at pH 10.8 (the "$^1/_2\bar{n}_H$" points), and so the pK_a's are about 8.3 and 10.8.[3] Because the difference between the two macroconstants is not large, each constant cannot be precisely associated with a specific "microstate" process. Rather, one macroconstant describes the two simultaneously occurring "microstate" reactions

$$^+H_3NCH_2CH_2SH = {}^+H_3NCH_2CH_2S^- + H^+$$
$$^+H_3NCH_2CH_2SH = H_2NCH_2CH_2SH + H^+$$

while the other macroconstant describes the two microstate reactions

$$H_2NCH_2CH_2SH = H_2NCH_2CH_2S^- + H^+$$
$$^+H_3NCH_2CH_2S^- = H_2NCH_2CH_2S^- + H^+$$

The distinction between the doublet microstates is not based on toichiometric considerations, and thus the macroconstant cannot be precisely assigned to single microstate ionizations. (Macroconstants describe microstate reactions only when the difference between successive macroconstants is large.) In proteins, the situation is further complicated. Macroscopic constants can be closely spaced, and each can represent a linear combination of many microstates, in principle. Although pK_a's of proteins are precise measures of acidity and describe accurately the stoichiometries of ionization, the assignment of macroconstants to microstate processes becomes imprecise. Nevertheless, such macroscopic measurements can reveal important properties about the macromolecule, as we will attempt to show.

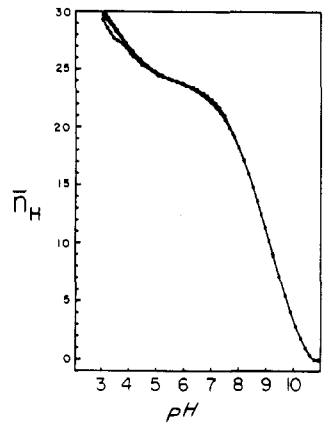

Figure 1. Bjerrum formation curves for apometallothionein. The number of dissociable protons bound to the apoprotein is represented by $\bar{n}_H$. Up to pH 8.25, the figure contains three (superimposed) curves, corresponding to titrations 6–8 (Table I).

From the shapes of the titration and proton release curves (vide infra), and from the refined apoprotein pK_a's, it was possible to identify the stoichiometries of several likely ternary (metal–protein–hydrogen) complexes, along with estimates of the corresponding equilibrium constants, for the constant-pH titrations.

The deductive process is illustrated with the following example (as we parenthetically draw on some of the results described in detail in the next section). At pH 7 the proton release curve (Figure 3) shows 3 and 2 H^+ liberated for each added Cd^{2+}. In addition, the pH-static, pCd-metric curve (Figure 2) clearly shows MT binding 5 Cd^{2+}. Thus one may test the presence of two species: one that forms by liberating 15 protons and another that forms by liberating 10 protons. The apoprotein pH titration suggests the presence of the MT species of the stoichiometry LH_{22} (L = macroligand, namely, MT, and the subscript refers to the dissociable proton count). The pK_a analysis and the readily interpretable information in the two above-mentioned curves suggest the following two equilibrium reactions:

$$5Cd^{2+} + LH_{22} = Cd_5LH_7 + 15H^+$$
$$5Cd^{2+} + LH_{22} = Cd_5LH_{12} + 10H^+$$

Thus, the initial model contains the species (5,1,7) and (5,1,12), where the indices refer to the stoichiometric coefficients in the order (metal,-macroligand,proton).

During the least-squares fitting analysis, the above stoichiometries were tested first; in addition, a systematic testing was made with alternate species of similar stoichiometries. Rough estimates of the equilibrium constants were determined in much the same way as in small-ligand metal binding studies.[2,3,35]

Given a set of constants and stoichiometric coefficients (the "equilibrium model"), along with the total reagent concentrations (corrected for dilution), it was possible to calculate pCd and pH values, by employing the same methods used in studying small-ligand systems. Refinement of the equilibrium model had the objective of making as close a match as possible between the calculated dependent variables pCd and pH and those which were measured. The devised least-squares refinement procedure specifically minimized the weighted nonlinear function

$$S = \sum_{N_0}[(pCd^{obsd} - pCd^{calcd})^2 + (pH^{obsd} - pH^{calcd})^2]/\sigma^2$$

where N_0 is the number of metal titrant additions in a single titration and σ^2 values are the estimated variances.[30]

The "goodness of fit", GOF, an index of how well a model predicts the observed data, was defined as

$$GOF = [S/(2N_0 - N)]^{1/2}$$

where N is the number of refined constants. GOF < 3 generally implies a good fit.[2]

(31) Due to technical difficulties, the experiment 1 solution, while at pH 3, was exposed to air for about 10 s. Although the titration was resumed under a nitrogen atmosphere, a white precipitate developed within 5 min, accompanying a slight decrease in pH. The pH effect, along with a visual estimate of the slight quantity of solid present, suggested that about 5–15% of the sulfhydryl groups were air oxidized. In no other titrant was any precipitate observed.

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Table II. Refined pK_a's of Rat Apometallothionein 2[a]

equil expression	log $K \pm$ SD	pH_{max}[b]
$L^{20-} + 3H^+ = LH_3^{17-}$	30.9 ± 0.2	10.1
$LH_3^{17-} + 3H^+ = LH_6^{14-}$	29.2 ± 0.2	9.7
$LH_6^{14-} + 2H^+ = LH_8^{12-}$	19.0 ± 0.2	9.2
$LH_8^{12-} + 3H^+ = LH_{11}^{9-}$	27.5 ± 0.1	8.9
$LH_{11}^{9-} + 3H^+ = LH_{14}^{6-}$	26.6 ± 0.1	8.6
$LH_{14}^{6-} + 5H^+ = LH_{19}^{-}$	41.2 ± 0.1	7.8
$LH_{19}^{-} + 3H^+ = LH_{22}^{2+}$	22.4 ± 0.1	6.9
$LH_{22}^{2+} + 2H^+ = LH_{24}^{4+}$	12.9 ± 0.1	5.7
$LH_{24}^{4+} + 2H^+ = LH_{26}^{6+}$	9.6 ± 0.1	4.4
$LH_{26}^{6+} + 2H^+ = LH_{28}^{8+}$	7.7 ± 0.1	3.7
$LH_{28}^{8+} + 2H^+ = LH_{30}^{10+}$	6.8 ± 0.2	3.0

[a] GOF = 1.5; N_0 = 50; average $|pH^{obsd} - pH^{calcd}|$ = 0.035; data from experiment 8. The charges are deduced from the reported amino acid composition[24] and have a probable estimated error of ±2 units. [b] pH where concentration of the formed species is at a maximum.

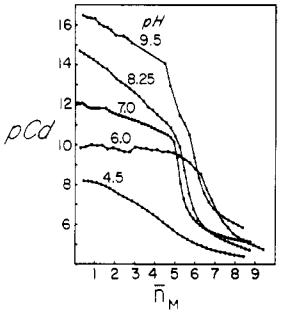

Figure 2. pH-static, pCd-metric titration curves for metallothionein. $\bar{n}_M$ refers to the average number of bound cadmium ions per protein molecule.

Results

Thionein pK_a's. The Bjerrum protonation curves, $\bar{n}_H$ vs. pH, for apoprotein titrations 6–8 (Table I) are shown in Figure 1. For pH 3–11, there appear to be 30 titratable protons in thionein. All three (superimposed) curves show a distinct inflection point at pH 5.6, corresponding to the titration of six protons. The 30 graphically estimated pK_a's were subsequently subjected to least-squares refinement. In the iterative procedure, those constants whose estimated standard deviations exceed 10 log units were dropped from consideration. (Such cases arise when the data are not consistent with the presence of particular tested species: the partial derivatives dpH/d log K tend to zero, leading to a mathematically ill-conditioned least-squares symmetric matrix.) It was possible thus to refine 11 groups of pK_a's; Table II summarizes the results. It is worth noting that there are possibly some sulfhydryl residues with $pK_a > 11$. The titration analysis could not be reliably extended beyond pH 11. Thus when cadmium ions liberate protons, some may be from residues with pK_a's >11. (By analogy, in small-ligand metal binding studies, with amino acids, for example, the peptide protons displaced by metal ions are not usually assigned intrinsic pK_a's. Peptide pK_a's in ligands not coordinated to metal ions are very high and cannot be reliably determined by simple pH titration. Metal complexes formed by displacement of peptide protons usually are denoted with negative proton coefficients, as for example, $CuLH_{-1}$.)

Constant-pH Titration Curves. Figure 2 shows the five constant-pH metal titration curves for metallothionein (type I titrations). The calibrated pCd electrode readings are plotted as a function of $\bar{n}_M$, the apparent number of bound cadmium ions per thionein:

$$\bar{n}_M = ([Cd]_{total} - [Cd^{2+}]_{free})/[thionein]_{total}$$

The above variable is linearly related to the volume of metal ion titrant added, since at no time is there a significant concentration of free metal ions, relative to the concentration of complexes. For $\bar{n}_M < 5$, the titration curves clearly show increasing cadmium–thionein apparent binding strength with increasing pH, indicating the pH dependence of the metal binding process. Sharp drops in pCd with increasing $\bar{n}_M$ are exhibited prominently at several pH levels, corresponding to conditions of saturation of strong metal-binding sites. (Similar inflection breaks in titration curves are used in analytical procedures to determine concentrations of species of known stoichiometry; conversely, when concentrations are known, stoichiometries may be determined.) These "end points" correspond to five, six, or seven bound cadmium ions. *Evidently, thionein continues to bind cadmium ions beyond these points, albeit with considerably decreased affinity.* (Similar weak binding has been observed in copper superoxide dismutase titrations and is thought to be associated with adventitious metal binding to the surface of the protein.[37])

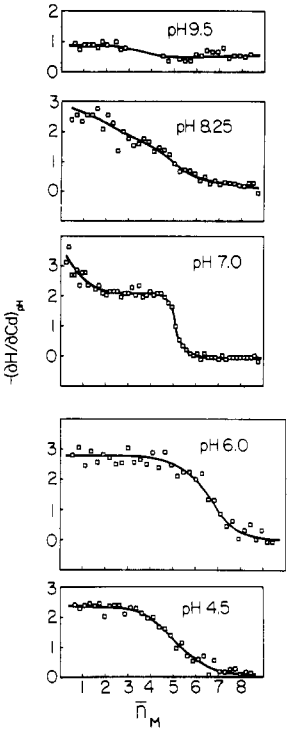

Figure 3. Number of protons released by metallothionein per added cadmium ion, $-(\partial H/\partial Cd)_{pH}$, as a function of the average number of bound metal ions.

Deprotonation Curves. The plots of the average number of protons displaced at constant pH by each added cadmium ion, as a function of $\bar{n}_M$, are shown in Figure 3. For pH 4.5–8.25, two to three protons are liberated by each cadmium ion, up to the fifth or so added metal ion. Additional metal ions do not affect significant proton release. The cessation of proton release in the deprotonation curves correlates precisely with the metal saturation end points (vide supra) observed independently in the pCd titration curves. Thus, the end of strong metal binding coincides with the end of proton release.

A different response is observed at pH 9.5. Proton release, although considerably diminished overall, does not follow critical features in the corresponding pCd–$\bar{n}_M$ curve. Apparently, adventitious cadmium binding is accompanied by proton release at high pH.

The area under the deprotonation curves correspond to the total number of protons liberated by the metal ions at a given pH. Integration of the curves for $\bar{n}_M$ = 0–9 resulted in 11.6, 17.7, 11.2, 10.6, and 5.4 metal-displaced protons at pH 4.5, 6, 7, 8.25, and 9.5, respectively. The estimated uncertainty in the above integrations is less than ±0.5.

Holoproteins Titrations. Metal binding in MT commences at pH as low as 2.5, as evidenced by changes in pCd as a function of pH in titration 10. Significant pH and pCd buffering takes place in the pH 4.5–5.8 region, where slightly over 20 protons are titrated. In a similar pH range, the metal-free protein has two titratable protons.

(37) Valentine, J. S., private communication, 1982.

Table III. Refined Cadmium Binding Constants of Rat Liver Apometallothionein 2

equil expression (K form)	log K^a	log $K_{app}{}^b$	log $\beta \pm$ SD^c	GOF^d	N_0	ΔpCd^e	ΔpH^f
		pH 6					
$5Cd^{2+} + LH_{24}{}^{4+} = Cd_5LH_{13}{}^{3+} + 11H^+$	−19	47	191 ± 2	1.5	40	0.08	0.05
$7Cd^{2+} + LH_{24}{}^{4+} = Cd_7LH_4{}^{2-} + 20H^+$	−54	66	156 ± 2				
$7Cd^{2+} + LH_{24}{}^{4+} = Cd_7LH_7{}^{+} + 17H^+$	−37	65	173 ± 2				
		pH 7					
$2Cd^{2+} + LH_{22}{}^{2+} = Cd_2LH_{16} + 6H^+$	−23	19	174 ± 3	2.1	42	0.08	0.11
$5Cd^{2+} + LH_{22}{}^{2+} = Cd_5LH_9{}^{-} + 13H^+$	−37	54	160 ± 1				
$5Cd^{2+} + LH_{22}{}^{2+} = Cd_5LH_{11}{}^{+} + 11H^+$	−21	56	176 ± 1				

^{a} log K = log β − log $\beta(LH_{24}{}^{4+})$ (pH 6), log β − log $\beta(LH_{22}{}^{2+})$ (pH 7), where log $\beta(LH_n)$ = $[LH_n]/[L^{20-}][H^+]^n$ = 209.7 for n = 24 and 196.8 for n = 22, as deduced from the constants in Table II. ^{b} log K_{app} is the apparent binding constant at a particular pH, defined as log K + npH, where n is the number of H^+ released on the right side of the equilibrium expression. ^{c} The actual constants refined by least squares (see text). The standard deviation, SD, was estimated to be half of the difference between the values of the constants determined from refinement of individual data sets in duplicate titrations. ^{d} Goodness of fit (see text). ^{e} ΔpCd is the average absolute difference between measured and calculated values of pCd. ^{f} ΔpH is the average absolute difference between measured and calculated values of pH.

Alkalimetric titration 9 (thionein with 8.8 equiv of Cd^{2+}) qualitatively suggests the unusually low pK_a's of some of the metal binding residues. In the domain pH 7.0–9.3, four protons titrate with $^1/_2\bar{n}_H$ pH values 7.7, 8.9, 9.0, and 9.2. These protons are likely dissociated from lysine groups (and possibly from the few sulfhydryl residues not coordinated to cadmium during pH 7 metal ion addition). The metal-free thionein (titration 8) has closely matching values 7.7, 8.7, 8.9, and 9.2. In addition to these values, thionein also exhibits $^1/_2\bar{n}_H$ pH values 7.2, 7.5, 7.9, 8.1, 8.3, 8.4, 8.5, 8.8, 9.0, and 9.3 (pH 7.0–9.3 region). There are no correspondences to these values in the holoprotein (titration 9), suggesting that the 10 "missing" apparent pK_a's are those of metal binding residues. Since 11.2 protons are liberated at pH 7 (vide supra), at least 1–2 metal binding residues have pK_a's >9.3. These arguments are necessarily very approximate: in this paragraph we have been treating the constants as if they were microconstants. Since they are macroconstants, we cannot with certainty assign an apparent pK_a to a specific residue. Qualitatively, however, the above comparisons do suggest that some of the metal binding residue pK_a's are low. Since expected sulfhydryl pK_a's are seldom below 9 (vide infra), the low $^1/_2\bar{n}_H$ values are surprising observations.

Least-Squares Refinement of Metal Binding Constants. After a laborious trial and error model testing process, the "best" models were obtained. (By "best" we mean the simplest schemes consistent with all of the data in a particular constant-pH titration.) Each of the constant-pH titration curves (Figure 2) was fitted to within ±0.07–0.35 pCd unit, on the average. In addition, the constancy of pH was predicted to within ±0.07–0.25. Table III summarizes the results of the refinement for pH 6 and 7.

The least-squares refinement procedure used "stability" macroconstants to represent equilibrium reactions. For example, log $\beta(Cd_7LH_4{}^{2-})$ = 156 (Table III) refers specifically to the expression

$$7Cd^{2+} + L^{20-} + 4H^+ = Cd_7LH_4{}^{2-}$$

The component L^{20-} represents the pH 11 form of thionein (that is, the "free" form), with the charge deduced from the published amino acid analysis.[24] (The choice of the "free" form for the macroligand does not preclude the possibility of residue pK_a's >11.) The use of such cumulative constants is convenient from computational considerations. One can combine these constants and the thionein pK_a's in any number of ways to produce constants corresponding to equilibrium expressions of a form more familiar than suggested by the stability constants. One such scheme (log K form) is presented in Table III. The "apparent" constants listed in Table III refer to constant-pH conditions.

Reproducibility of pCd and pH Measurements. At pH 6 and 7, the titration experiments were repeated to determine reproducibility of the cadmium activity measurements. Ten months had elapsed between the original and the replicate experiments. No substantial differences were found between the titration curves: seven metal ions were still being strongly bound at pH 6; only five metal ions were still being bound at pH 7. The pCd measurements were reproduced to within ±0.4 log unit. The deprotonation curves in the original titrations (Figure 3) were virtually identical with those in the repeated experiments.

Discussion

Apothionein. This study has resulted in several surprising observations. Metallothionein (specifically rat liver MT-2) appears to have extraordinarily low metal binding residue pK_a's. This had not been suspected in the literature.[38]

Nozaki and Tanford[39] have suggested that the pH-metric formation curve of a protein, such as the one shown in Figure 1, can usually be divided into three sigmoid regions, the transitions being at about pH 6 and 9. Below pH 6 is a region where carboxyl groups are titrated usually; imidazole and α-amino groups fall into the middle zone; the high-pH segment describes the titration of phenolic, ϵ-amino, and thiol residues. Metallothionein has no imidazole or phenolic residues; also, the α-amino group is acetylated. Thus MT should be expected to show just two sigmoid sections: one for carboxyl groups and the other for sulfhydryl and lysyl groups. This is precisely what Figure 1 shows, with a pH 5.6 boundary.

Since cadmium has poor binding affinity for primary amine groups in neutral solutions, based on model studies (vide infra), and since thionein exhibits metal-binding groups with pK_a's as low as 7, it follows that some sulfhydryl groups may have pK_a's as low as 7. These groups are 2–5 orders of magnitude more acidic than simple monosulfhydryl ligands, such as mercaptoacetic acid (pK_a = 10.7[40]), mercaptoethanol (pK_a = 9.5[41]), and mercaptoethylamine (pK_a = 8.3[3]). This increased acidity in thionein may be due to electrostatic interactions of sulfhydryl groups with the numerous positively charged lysyl groups, favoring the formation of zwitterionic groupings. Such low pK_a's are consistent with the reported extreme sensitivity of apothionein to air oxidation, even in solutions with pH as low as 3.[31]

The ionization of sulfhydryl groups in proteins under conditions of low ionic strength (0.15 M) has not been extensively studied.[22,39] Often sulfhydryl groups are "buried" in the protein and require denaturing salt conditions to reveal them in titration experiments. Abnormally low intrinsic pK_a's of 8.7 have been reported for rabbit muscle aldolase in 4 M urea.[42] The typically expected intrinsic value is 10.2,[39,43] under conditions of high salt concentration. One must keep in mind that intrinsic constants and macroconstants cannot always be directly compared.

Thionein Proton Release Effected by Cadmium Addition. The complicated patterns in Figure 3 had not been anticipated in the literature of metallothionein. Consider the proton loss curve for

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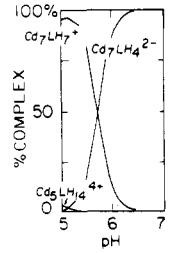

Figure 4. Calculated distribution-of-species curves as a function of pH. Total apometallothionein concentration is 0.1 mM; total cadmium ion concentration is 0.7 mM.

pH 7 (Figure 3). Kagi and Vallee[44] observed 2.9 H^+ released/Cd^{2+} added at pH 7. We observe a similar value, *but only for* $\bar{n}_M < 1$. It is not possible to tell whether the literature value applies to the entire addition of seven cadmium ions or the initial addition of metal ions. In our study, at pH 7, thionein evidently liberated only 2 H^+/Cd^{2+}, after the first two cadmium ions had been added. Such observations guided us in the choice of the species for the pH 7 model (Table III).

Implication of Apothionein Conformational Changes. After the intra-pH models were developed (Table III), it was of interest to test inter-pH consistency. It is quite possible that a species observed to form at a particular pH may be predicted to be present at another pH on the basis of the equilibrium constants yet not be observed to form at the other pH. This may be the case, for example, when the apoprotein undergoes a conformational change with changing pH such that a particular metal binding site becomes inaccessible. It may be thermodynamically the most stable state, but the means to its occupation may not be present, within the time frame of the equilibrium measurements. Apparently such a circumstance takes place with metallothionein. (Although apothionein generally has a random coil structure in acidic solutions, short-range order cannot be excluded.[45])

A simulation calculation was performed with use of all of the species listed in Tables II and III. The total metal and protein concentrations were set to be 0.7 and 0.1 mM, respectively. The hypothetical solution was then "titrated" with KOH from pH 4.5 to 9.5. The resulting distribution-of-species vs. pH curve is shown in Figure 4. The dianionic species $Cd_7LH_4^{2-}$ was predicted to form at pH 6 and to persist beyond pH 7. However, in the pH 7 titration there is no experimental evidence for its presence. The simulation calculations suggest that if metallothionein is formed by the addition of metal ions to the apothionein at pH 6, the seven-metal complex so formed should also be present at pH 7. However, if the pH is *first* raised to 7, then only five-cadmium complexes form by the addition of cadmium ions to apothionein. It is interesting to note that Bethune et al.[38] observed only a single dianionic metallothionein complex in the region pH 7.4–9.6 in electrophoretic mobility measurements.

The apoprotein formation curves (Figure 1) indicate that two protons are titrated in the domain pH 6–7. From considerations cited in other sections, the two protons may be originating from sulfhydryl centers. If apothionein undergoes a conformational change in the above pH interval, it may be due to the electrostatic consequences of the formation of two anionic thiolate centers.

The above inter-pH discordance is a significant observation. It implies that the metallothionein complex formed at a particular pH depends very much on the protocol of its preparation. The

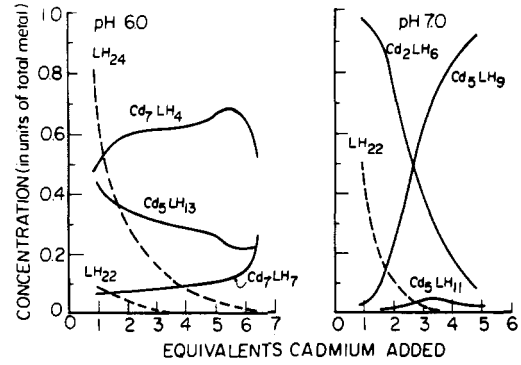

Figure 5. Calculated distribution-of-species curves as a function of added equivalents of cadmium ions at pH 6 and pH 7.

hysteresis effects observed by Law and Stillman[46] in circular dichroism titrations and by Galdes et al.[45] in proton NMR studies may be related to this phenomenon, although the comparison is complicated by the zinc ions present in the latter studies. It is particularly interesting to note that Boulanger and Armitage[13] determined the cadmium content in metal-exchanged human metallothionein to be 4.2–4.9 mol/mol of protein (The expected value was 7 mol/mol of protein.) This partial metal ion deficiency was confirmed by ^{113}Cd NMR. Similarly, the total metal content in the calf liver metallothionein used by Briggs and Armitage[14] in their ^{113}Cd NMR study was 6.4–6.6 at pH 9. Also the three-cadmium (B) cluster in the rabbit metallothionein NMR experiments[12] was only partially occupied, perhaps indicating that the metal-exchanged protein contained less than seven metal ions. (The NMR experiments were performed with solutions nearly 100 times more concentrated than those used in the present study.)

Metal Binding Cooperativity. At pH 6 it was not possible to fit the titration data with complexes possessing fewer than five metal ions, which suggests that the higher order aggregates are more stable than those of lower order. Furthermore, a simulation calculation at pH 6 reveals the simultaneous presence of the seven-metal complex and entirely *uncomplexed* protein, as shown in Figure 5. A similar effect has been reported for a two-copper form of superoxide dismutase for pH >7.[47] In both proteins metal binding appears to be highly cooperative. In metallothionein the effect seems to maximize at pH 6, as suggested by the characteristic relative flatness of the titration curve (Figure 2) in the interval $\bar{n}_M = 1$–5.

Possible Lysyl Coordination. Unlike the cases of pH 6 and 7, the pH 9.5 titration indicates that protons are released by cadmium ions well past the strong-binding saturation end points. This proton release may be associated with weak binding of cadmium by lysine residues in thionein. We have observed relatively strong cadmium binding by amino groups at this pH in model compounds cysteamine,[3] penicillamine,[2] and *N,N'*-dimethyl-*N,N'*-bis(2-mercaptoethyl)ethylenediamine.[48] Alternatively, the pH dependence may be due to (a) formation of hydroxy mixed-ligand complexes or (b) metal-induced protein unfolding, exposing ionizable nonligand sites.

Acknowledgment. Support from National Institutes of Health Grant ESO 1629 to J.S.G. is gratefully acknowledged.

Registry No. Cd, 7440-43-9.

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