Table VIII. log Protonation Contants (K_1, K_2), Metal-Ligand Stability Constants (β_n), and Effective Stability Constants (β_{3eff}, pH 7.4) for the Equilibrium Reactions of Al, Ga, and In with Hppp at 25 and 37 °C in 0.15 M NaCl[a,b]

constant	metal	25 °C	37 °C
log K_1		9.40 (1)	9.61 (1)
log K_2		3.03 (1)	3.18 (1)
log β_1	Al	11.36 (3)	11.86 (10)
	Ga	17.5 (2)[c]	
	In	13.34 (1)	
log β_2	Al	21.78 (8)	23.13 (21)
	Ga	28.8 (2)[c]	
	In	22.66 (2)	
log β_3	Al	30.74 (11)	32.44 (23)
	Ga	36.3 (2)[c]	
	In	31.12 (3)	
log β_{3eff}	Al	24.74 (14)	25.81 (26)
	Ga	30.3 (2)[c]	
	In	25.12 (6)	

[a] Ga constants are reported for solutions containing ~0.2 M NaCl (see ref 7). [b] Numbers in parentheses represent standard deviations between successive runs. [c] Estimated by LFER.

by the high overall (β_3) and conditional ($\log \beta_{3eff} = \log \beta_3 - 3(\log K_2 - pH)$[28]) formation constants for $Ga(ppp)_3$ in Table VIII. The overall constant is lower than we found[7] for the Ga complexes of *N*-alkylated pyridinones ($\log \beta_3 = 36$ vs ~38); however, the lower K_2 value for this ligand means that the conditional stability constant at pH 7.4 is only slightly smaller than that for the *N*-alkylated ligands ($\log \beta_{3eff} = 30.3$ vs ~30.8). The same holds true for the Al and In complexes of ppp^-. The overall stability constants are lower than we found for Al[5] or In[7] with the *N*-alkylated pyridinones, but the conditional stability constants are only very slightly smaller for ppp^- (for Al $\log \beta_{3eff} = 24.7$ vs 24.9; for In $\log \beta_{3eff} = 25.1$ vs 25.6). Clearly, changes in the substituent on the ring nitrogen of the pyridinone ring make minor alterations in the overall formation constants ($\log \beta_3$), and these variations may be attributed to the changes in the pK_as of the ligands. The effective overall formation constant at pH 7.4 ($\log \beta_{3eff}$) demonstrates this thermodynamic indifference when β_3 is normalized to blood plasma conditions (pH 7.4 and 0.15 M NaCl). The predominance of the ML_3 species at physiological pH was demonstrated in the $\log \beta_{3eff}$ values in Table VIII.

The consistently high $\log \beta_{3eff}$ values for the tris(N-substituted-3-hydroxy-2-methyl-4-pyridinonato)gallium(III) complexes led us previously to study in detail the in vivo biodistribution of these compounds with $^{67}Ga^{3+}$.[7] The *N*-alkyl ligands were excreted quickly via the urinary pathway. We have prepared the compounds described herein in order to increase the lipophilicity and alter the biodistribution.

Acknowledgment is made to the British Columbia Health Care Research Foundation, the NSERC of Canada, and the U.S. Public Health Service (CA 48964) for operating grants, to the NSERC for a University Research Fellowship (C.O.), and to the UBC for a University Graduate Fellowship (Z.Z.). We are particularly indebted to Prof. David Clevette for help with the potentiometric measurements and to Prof. James Trotter for discussions and the use of his crystallographic facilities.

Supplementary Material Available: Complete crystallographic data (Table SI), hydrogen atom parameters (Tables SII and SIII), anisotropic thermal parameters (Tables SIV and SV), intramolecular bond distances involving hydrogen atoms (Tables SVI and SVII), intramolecular bond angles involving hydrogen atoms (Tables SVIII and SIX), torsion angles (Tables SX and SXI), intermolecular contacts (Tables SXII and SXIII), and least-squares planes (Tables SXIV and SXV) (24 pages); measured and calculated structure factor amplitudes (Tables SXVI and SXVII) (38 pages). Ordering information is given on any current masthead page.

(28) Martin, R. B. *Clin. Chem.* **1986**, *32*, 1797.

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Platinum(II)-Thiolate Cluster Formation in Heptaplatinum Metallothionein

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Received March 20, 1990

The binding of platinum(II) to metallothionein (MT) has been studied by spectrophotometric titrations of rat liver apometallothionein-1 (apoMT) with platinum(II) complexes at pH 7.4-7.5. A red shift of ~6000 cm^{-1} in the intense absorption maximum in the electronic spectra of the partially platinated apoMT adducts is observed upon the binding of ≥3 molar equiv of platinum(II). This shift is similar to red shifts of the lowest energy LMCT transitions observed for $[X_2Pt(\mu\text{-}X)_2PtX_2]^{2-}$ ions (X = Br, I) relative to the corresponding PtX_4^{2-} ions and can be attributed to a lowering in energy of $M\sigma^* \leftarrow L\pi$ transitions from the terminal thiolates due to the presence of bridging thiolates. The partially platinated apoMT solutions (0-7 molar equiv of bound platinum(II) ions) were also assayed for free thiolate contents by using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The DTNB studies showed that platinum equivalents 1-3 block 4 ± 1 cysteines/platinum and that equivalents 5-7 block a total of 3 ± 1 cysteines (an average of 1 cysteine/Pt). These experiments suggest that the platinum(II)-thiolate clusters in Pt_7MT form via the initial production of approximately three $Pt(cysteine)_4^{2-}$ centers followed by formation of μ-thiolato bridges.

Metallothionein (MT)[1-5] is a low molecular weight cytosolic protein with an unusually high cysteine content (20 cys in the mammalian proteins) that chelates a diversity of metal ions in vivo and in vitro. Native MT contains primarily Cd(II) and Zn(II) in two metal-thiolate clusters, and in vitro reconstitutions of the metal-free form of the protein (apoMT) to generate Cd_7MT and Zn_7MT have been widely studied by spectroscopic techniques.[6] Adducts of MT with a variety of other metal ions have also been studied by spectroscopic means, including Co(II),[7-9] Ni(II),[7] Fe(II),[10] and Hg(II) adducts.[11]

Available evidence strongly suggests that MT is a major in vivo binding site for metabolites of *cis*-diamminedichloroplatinum(II)

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(1) Margoshes, M.; Vallee, B. L. *J. Am. Chem. Soc.* **1957**, *79*, 4813.
(2) Vasák, M. *J. Mol. Catal.* **1984**, *23*, 293.
(3) Kägi, J. H. R., Nordberg, M., Eds. *Metallothionein*; Birkhauser-Verlag: Basel, 1979.
(4) Nordberg, M.; Nordberg, G. F. *Life Sci. Res. Rep.* **1987**, *37*, 53.
(5) Kägi, J. H. R., Kojima, Y., Eds. *Metallothionein-2*; Birkhauser-Verlag: Basel, 1988.
(6) Vasák, M.; Kägi, J. H. R. *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1983; Vol. 15, p 213.
(7) Vasák, M.; Kägi, J. H. R.; Holmquist, B.; Vallee, B. L. *Biochemistry* **1981**, *20*, 6659.
(8) Vasák, M. *J. Am. Chem. Soc.* **1980**, *102*, 3953.
(9) Vasák, M.; Kägi, J. H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6709.
(10) Good, M.; Vasák, M. *Biochemistry* **1986**, *25*, 8353.

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and other platinum anticancer drugs.[12] Induction of MT biosynthesis may be useful in mediating the dose-limiting renal toxicity of these agents,[13] but conversely, such increased in vivo platinum(II) binding to MT may make tumor cells more resistant to the anticancer effects of platinum drugs.[14-16] We have reported mixed-metal adducts from the reactions of platinum(II) with Cd_7MT and native MT and the reconstitution of apoMT with platinum(II) to produce Pt_7MT.[17] Stoichiometries of metal binding under aerobic and anaerobic conditions were presented, and the reactivity of the Pt_7MT adduct toward electrophiles indicated a significant kinetic inhibition of cysteine modification in comparison to reactions with apoMT, Cd_7MT, and Zn_7MT. The Pt_7MT adduct is formed at pH ~7 even in the presence of a large excess of platinum(II), and we suggested that this stable seven-metal adduct has Pt–cys clusters and that the single methionine of MT is also involved in platinum(II) binding.

We report here spectral changes that accompany the stepwise incorporation of platinum(II) into apoMT. These titrations in conjunction with thiolate assays of the partially platinated adducts with the colorimetric reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) show that a binding sequence originally suggested by Vasák and Kägi[9] for the binding of Co(II) to apoMT is evidently operative in the case of Pt_7MT.

Experimental Section

Reagents. Rat liver MT-1 and MT-2 were isolated and purified according to a literature procedure.[18] ApoMT was prepared by exhaustive dialysis of the native protein against 0.01 M HCl. ApoMT and Pt concentrations were obtained by ICP-AES spectrometric determinations of sulfur[19] (S I 180.73 nm) and Pt (Pt II 214.42 nm) performed on a Perkin-Elmer Plasma II Emission spectrometer. Water was purified and deionized (Barnstead Nanopure system) and passed through Chelex resin (obtained from Sigma) prior to use.

Titrations. A stock solution of K_2PtCl_4 in water was used for all studies. These stock solutions contain a mixture of $[Pt(Cl)_x(OH)_y(H_2O)_z]^{n+}$ complexes. A series of deaerated apoMT-1 solutions containing different molar equivalents of platinum(II) were prepared in a dinitrogen-purged glovebox as follows. Aliquots (40 μL) of 56 μM apoMT in 0.01 M HCl were adjusted to pH 7.4 with a predetermined amount of K_2HPO_4 solution. An appropriate volume of a 0.450 mM K_2PtCl_4 solution was added to each apoMT sample to produce solutions containing 0–8 molar equiv of platinum(II), and the samples were left standing for 3 h to equilibrate before dilution to a final volume of 0.65 mL (final apoMT concentrations in all experiments were 3.5 μM). Buffered 12 mM DTNB (pH 7.5) was also prepared in the glovebox.

In the glovebox, each sample was transferred to a 1-cm cuvette and sealed with a greased septum. A gastight syringe charged with 60 μL of DTNB solution was then mounted atop the cuvette with the needle protruding through the septum (a short length of tightly fitting rubber tubing was used to stabilize the assembly). After recording the UV–visible spectrum (IBM 9430 spectrophotometer), the DTNB solution in the syringe was discharged into the cuvette, and another spectrum was recorded 1 min after mixing. The spectra were stored for difference spectrum calculations. The cuvette and syringe were then cleaned and returned to the glovebox, and the above procedure was repeated for each of the remaining samples.

Spectra of blanks containing all reagents except protein and platinum(II) were recorded and digitally subtracted from corresponding sample spectra. Free thiolate concentrations were estimated by using the reported molar absorptivity of the dianion of TNB (2-nitro-5-thiobenzoic acid), 14150 M^{-1} cm^{-1} at 412 nm.[20] In control studies with other proteins and MT derivatives, the typical experimental errors (2σ) for thiol assay by this method were found to be ≤±0.5 SH groups.

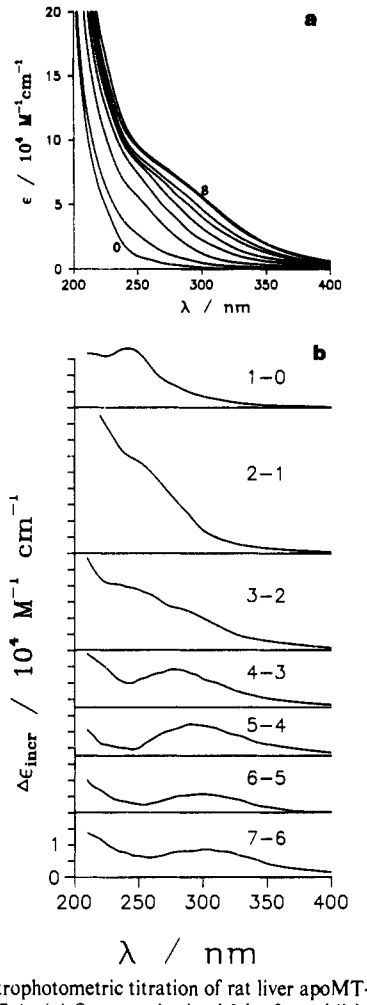

Figure 1. Spectrophotometric titration of rat liver apoMT-1 with K_2PtCl_4 solution at pH 7.4. (a) Spectra obtained 3 h after addition of 0–8 molar equiv of K_2PtCl_4 to apoMT-1, as described in the Experimental Section. "0" indicates the apoMT-1 spectrum (no added Pt); "8" indicates the spectrum obtained with 8 equiv of Pt. Spectra for equivalents 1–7 are in order between spectra 0 and 8. (b) Incremental difference spectra obtained by subtracting the preceding spectrum from each spectrum in a.

For circular dichroism studies, a series of deaerated 10 μM apoMT-1 solutions containing 0–10 molar equiv of platinum(II) were prepared in the glovebox. ApoMT solutions in 0.01 M HCl were adjusted to pH 7.5 with NaOH and K_2HPO_4 and then diluted to volume after addition of platinum(II) increments, as described for the UV–visible studies. CD spectra were recorded on a Jasco J-500 spectropolarimeter by using 1-cm cuvettes sealed with greased septa. Spectra of blanks containing all reagents except protein and platinum(II) were subtracted from the corresponding sample spectra taken in the same cuvette. Five replicate scans were recorded for each sample and blank, and the stored spectra were signal-averaged.

Binding Kinetics. In the glovebox, a syringe containing 80 μL of 4.95 mM platinum(II) stock solution was mounted on a sealed cuvette containing 720 μL of 11.1 μM apoMT buffered at pH 7.5 (50:1 Pt:apoMT molar ratio). This assembly was transferred from the glovebox to the spectrophotometer. The contents of the syringe were discharged into the cuvette, and the increase in absorbance at 280 nm versus time was recorded.

Results and Discussion

Spectrophotometric Titration. Figure 1a shows the electronic spectra recorded for a series of apoMT solutions equilibrated with 1–8 molar equiv of platinum(II) at pH 7.4 for 3 h. Previous experiments showed that the solutions reach apparent equilibrium after 3 h, with no further change in the UV–visible spectrum being

(11) Vasák, M.; Kägi, J. H. R.; Hill, H. A. O. *Biochemistry* **1981**, *20*, 2852.
(12) (a) Litterst, C. L. *Agents Actions* **1984**, *15*, 520. (b) Zelazowski, A. J.; Garvey, J. S.; Hoeschele, J. D. *Arch. Biochem. Biophys.* **1984**, *229*, 246.
(13) Naganuma, A.; Satoh, M.; Imura, N. *Cancer Res.* **1987**, *47*, 983.
(14) Bakka, A.; Endresen, L.; Johnsen, A. B. S.; Edminson, P. D.; Rugstad, H. E. *Toxicol. Appl. Pharmacol.* **1981**, *61*, 215.
(15) Endresen, L.; Schjerven, L.; Rugstad, H. E. *Acta Pharmacol. Toxicol.* **1984**, *55*, 183.
(16) Kelly, S. L.; Basu, A.; Teicher, B. A.; Hacker, M. P.; Hamer, D. H.; Lazo, J. S. *Science* **1988**, *241*, 1813.
(17) Bongers, J.; Bell, J. U.; Richardson, D. E. *J. Inorg. Biochem.* **1988**, *34*, 55.
(18) Sobocinski, P. Z.; Canterbury, W. J., Jr.; Mapes, C. A.; Dinterman, D. E. *Am. J. Physiol.* **1978**, *234*, E399.
(19) Bongers, J.; Walton, C. D.; Richardson, D. E.; Bell, J. U. *Anal. Chem.* **1988**, *60*, 2683.
(20) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Anal. Biochem.* **1979**, *94*, 75.

evident over several hours. The commonly used approach of adding increments of metal ion to a single solution of apoMT was not practical here, as the slow platinum(II) binding to apoMT at these low concentrations requires >1 h to reach equilibrium for each step. ApoMT is rapidly air-oxidized at neutral pH, and the method used here avoids any cumulative oxidation of MT as the titration progresses.

The spectrum of apoMT contains only an intense band at 190 nm arising from the strong π–π^* and weak n–π^* amide transitions of the polypeptide backbone and the lowest thiol transition of the cysteine side chains near 195 nm.[6] Because of the absence of aromatic residues in MT, the apoMT spectrum has no 280-nm peak. The spectrum of the yellow Pt_7MT adduct (7 molar equiv) contains a broad shoulder of absorption ($\epsilon_{280} \simeq 7 \times 10^4$ M^{-1} cm^{-1}) that gradually tails off into the visible range. The ultraviolet absorptions of Pt_7MT[17] and other metal MT adducts[6] have been assigned to ligand-to-metal charge transfer (LMCT). The spectra in Figure 1a confirm the earlier observation[17] that apoMT becomes saturated after binding 7 molar equiv of platinum(II) (the very slight increase in absorbance for >7 molar equiv of platinum(II) is due to the absorbance of the added stock solution).

An interesting pattern emerges in the incremental difference spectra (Figure 1b) obtained by subtracting the preceding spectrum from each spectrum in Figure 1a. Binding of the first molar equivalent of platinum(II) to apoMT results in an absorption peak centered at about 250 nm ($\epsilon \simeq 10^4$ M^{-1} cm^{-1}). An intense shoulder of absorption appears at 250 nm in the incremental difference spectrum for the second molar equivalent of platinum(II). A relatively large increase in absorbance at wavelengths <230 nm also appears upon binding of both the second and third molar equivalents of platinum(II). The difference spectrum for the third molar equivalent of platinum(II) contains two broad maxima at 250 and 300 nm. For molar equivalents 4–7, band maxima are observed in the 280–300-nm region, and the incremental increases in intensity in the difference spectra are less than those observed for the first three Pt equivalents.

Willner and co-workers observed a 6-nm red shift and diminishment in the intensities of incremental difference spectra for the addition of greater than 3 molar equiv of Cd(II) to apoMT.[21] Good and Vasák also report a red shift in the electronic spectra recorded during stepwise incorporation of Fe(II) into apoMT.[10] These red shifts as the titration of apoMT exceeds 3 molar equiv of metal ions have been attributed to the onset of thiolato bridging following the initial formation of three $M(cys)_4^{2-}$ units.[21] Bridging thiolates are supplied both by the remaining uncoordinated thiolates and by transformation of some terminal thiolates to bridging thiolates. Supporting evidence for this proposed mechanism was provided by similar red shifts that occur during titration of the synthetic tetrathiol dodecapeptide P-12 (Ac-P-C-Ornithine-C-P-E-C-E-C-R-R-V) with Cd(II) that are presumably due to the formation of a monomeric mononuclear complex and a thiolato-bridged dimeric trinuclear complex, respectively.[21] Further evidence for this mechanism of cluster formation comes from an earlier EPR study[9] in which an increase in EPR signal intensity occurs with titration of apoMT with up to 3–4 molar equiv of Co(II) ions followed by a decrease in intensity to zero with the addition of further ions to the saturation point of 7 molar equiv. The loss of paramagnetism is likely due to electronic coupling between thiolato-bridged Co(II) ions.[9]

The red shift in absorbance maximum (~6000 cm^{-1}) at later stages of the platinum(II) titration of apoMT is larger than that found for other metal ions mentioned above and is consistent with the onset of thiolate bridging with ~3 or more equiv of platinum(II). In assigning the origin of the red shift it is helpful to consider the electronic spectra of platinum(II) tetrahalides (PtX_4^{2-}) and their dihalo-bridged dimers ($Pt_2X_6^{2-}$) as models. The LMCT spectra of the square-planar tetrahalide complexes of Pt(II), Pd(II), and Au(III) consist of two intense bands separated by about 9000–12000 cm^{-1}.[22] The lowest energy band ($\epsilon \simeq 10^4$ M^{-1} cm^{-1}) originates from the two closely spaced $M\sigma^* \leftarrow L\pi$ transitions, and the second, more intense band ($\epsilon \simeq 4 \times 10^4$ M^{-1} cm^{-1}) originates from the dipole-allowed $M\sigma^* \leftarrow L\sigma$ transition.[22] For PtX_4^{2-}, the $M\sigma^* \leftarrow L\sigma$ band usually occurs at wavelengths too low to be experimentally observed. The dihalo-bridged dimers of Pt(II) and Pd(II), on the other hand, typically have intense absorption bands which lie 5000–7000 cm^{-1} below the first LMCT bands of the corresponding monomers.[22-26] Polarized crystal spectra[23] of $(Et_4N)_2Pt_2Br_6$ led to the assignment of the lowest energy intense LMCT band as arising from transfer of π electrons from the terminal ligands rather than the bridging ligands. Without repetition of the detailed molecular orbital treatment,[23] the occurrence the first $M\sigma^* \leftarrow L\pi$ LMCT band of $(Et_4N)_2Pt_2Br_6$ at a wavelength 49 nm longer than that of K_2PtBr_4[24] can be qualitatively explained by a very simple argument.[23] The Pt atoms in the dimer should be more electronegative than the Pt atom in the monomer because there are fewer bromides per Pt in the dimer to donate electronic charge to the metal centers. An independent ab initio molecular orbital calculation also yielded this same conclusion.[28] It is not surprising that the first LMCT transition in the dimer should occur at lower energy than in the monomer, as it is well-known, from the work of Jorgensen,[29] that the energy of this band can be correlated with the difference in electronegativities of metal and ligand.

By analogy, the red shift of the band maxima in the incremental difference spectra during the later stages of the spectrophotometric titration of apoMT with platinum(II) (Figure 1b) is ascribed to the lowering in energy of the $M\sigma^* \leftarrow L\pi$ LMCT transitions from the terminal thiolates due to the presence of bridging thiolates. In addition, the relatively lower intensities of the incremental difference spectra for the last four equivalents are consistent with the transformation of terminal thiolates to bridging thiolates. It does not appear likely that the red shift is a result of a change in the ligand geometry about the metal centers (e.g., from square planar to tetrahedral), since platinum(II) strongly prefers a square-planar geometry and only two distinct band maxima are observed at high and low platinum stoichiometries. A gradual change in the geometry around the various centers as more platinums are bound would probably result in broadened and gradually shifted charge-transfer bands.

DTNB Assays. We have obtained additional evidence that the above mechanism is operative in the binding of platinum(II) to apoMT by use of the colorimetric thiolate assay reagent, DTNB. As previously reported,[17] DTNB reacts many times slower with thiolates coordinated to platinum(II) in Pt_7MT than with free thiolates. This difference in reactivity makes it possible to determine the concentrations of both coordinated and free thiolates at each step in the titration of apoMT with platinum(II). This experiment is not amenable to the study of labile metal ion binding to apoMT, as bound ions such as Cd(II) or Zn(II) do not sufficiently inhibit the reaction of DTNB with coordinated thiolates.

Figure 2 shows the results obtained when each of the solutions used in the spectrophotometric titration was allowed to react for 1 min with a 315:1 molar excess of DTNB:MT under anaerobic conditions. A reaction time of 1 min is sufficient for the rapid reaction of the reagent with free thiols but is insufficient for significant modification of platinum-bound thiols.[17] The first three molar equivalents of platinum(II) block modification of 4 ± 1 cysteine thiolates/platinum(II). Binding of the remaining four molar equivalents of platinum(II) ions blocks modification of progressively fewer thiolate ligands per platinum(II), and the last three molar equivalents (5–7) protect a total of only 3 ± 1 thiols/MT (an average of ~1 cysteine/Pt). These observations

(21) Willner, H.; Vasák, M.; Kägi, J. H. R. *Biochemistry* **1987**, *26*, 6287.
(22) Gray, H. B. *Transition Met. Chem.* **1965**, *1*, 239.
(23) Martin, D. S., Jr.; Rush, R. M.; Peters, T. J. *Inorg. Chem.* **1976**, *15*, 669.
(24) Day, P.; Smith, M. J.; Williams, R. J. P. *J. Chem. Soc.* **1968**, 668.
(25) Mason, W. R., III; Gray, H. B. *J. Am. Chem. Soc.* **1968**, *90*, 5722.
(26) Olsson, L. F. *Inorg. Chem.* **1986**, *25*, 1697.
(27) Kroening, R. F.; Rush, R. F.; Martin, D. S.; Clardy, J. C. *Inorg. Chem.* **1974**, *13*, 1366.
(28) Baranovskii, V. I.; Davydova, M. K.; Panina, N. S.; Panin, A. I. *Koord. Khim.* **1976**, *2*, 409.
(29) Jorgensen, C. K. *Prog. Inorg. Chem.* **1970**, *12*, 101.

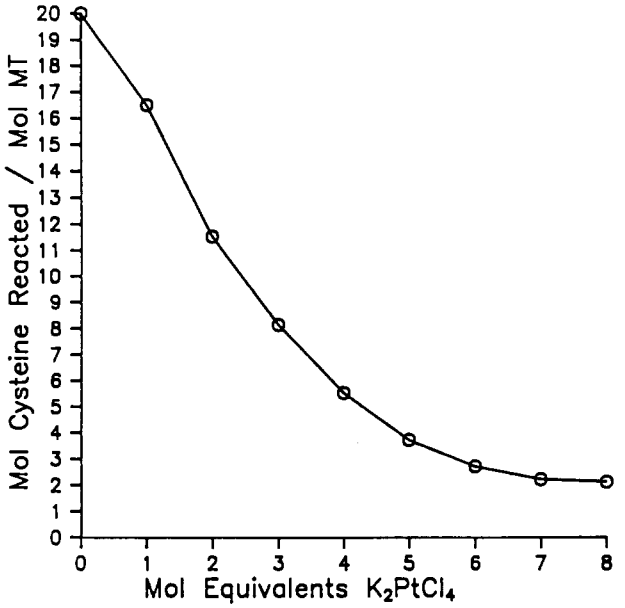

Figure 2. Results of the reaction of the partially platinated apoMT-1 adducts from each step of the spectrophotometric titration with the thiolate assay reagent, DTNB. DTNB addition was within 10 min after recording the corresponding spectrum in Figure 1. The number of reactive thiols determined after 1 min of DTNB reaction is shown as a function of the equivalents of Pt added to apoMT. Estimated experimental error (2σ) for each point is ±0.5 SH groups modified. At 7 equiv of Pt, 18 ● 1 of the 20 cysteines are blocked toward reaction with DTNB.

are consistent with the formation of approximately three $Pt(cys)_4$ centers for 1–3 equiv of Pt and formation of thiolato-bridged clusters at higher Pt:MT ratios. After the protein is saturated with the full complement of seven platinum(II) ions, 2 ± 1 thiolates react rapidly with DTNB. This residual reactivity could be due to either uncoordinated thiolates in Pt_7MT, relatively reactive coordinated thiolates, or both.

Spectropolarimetric Titration. It was anticipated that complementary spectral information for the intermediate Pt_xMT adducts could be obtained from circular dichroism (CD) spectra, and titration experiments were therefore carried out by using procedures similar to the UV–visible studies. Unfortunately, under the experimental conditions used here, the CD spectra of apoMT containing 0–7 molar equiv of bound platinum(II) are less useful than the corresponding electronic absorption spectra because of the weak molar ellipticities and rather featureless spectra observed in the LMCT region of Pt–MT adducts.[17] The CD spectrum of apoMT at neutral pH is similar to its electronic absorption spectrum in that it is nearly devoid of spectral information except for a sharply decreasing negative ellipticity band beginning at about 240 nm and extending into the far ultraviolet. Binding of 1–6 mol of platinum(II) leads to a positive ellipticity peak at ~250 nm, but the measured intensities do not follow a simple trend as a function of metal bound (the variability of intensities may be associated with the limits of instrumental reproducibility). Binding of the seventh and final molar equivalent of platinum(II) causes band broadening and an increase in the (+) intensity of the spectrum to yield a maximum at 250 nm ($\Delta\epsilon = +4 \times 10^4$ deg cm^2/dmol). Unlike the spectrum reported previously,[17] the strong negative ellipticity below ~240 nm is also observed for Pt_7MT.

Rate of Pt_7–MT Formation. The kinetics of formation of the stable Pt_7MT adduct have been studied in preliminary experiments with platinum(II) in large excess. Changes in the electronic absorption spectrum provide a convenient albeit crude measure of the extent of reaction of platinum(II) with apoMT. Absorbance was monitored versus time at 280 nm for a micromolar solution of apoMT mixed with a 50-fold molar excess of platinum(II) at room temperature. This wavelength was chosen because the absorption at 280 nm increases in a nearly linear fashion versus molar equivalent of bound platinum(II) (Figure 1). Under the conditions used in these experiments, the formation of Pt_7MT

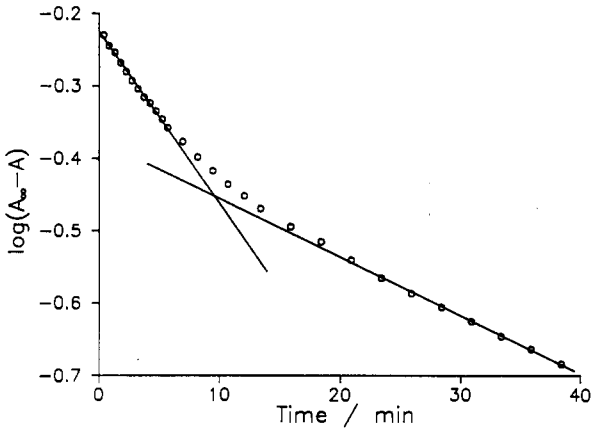

Figure 3. Reaction of 10 μM rat liver apoMT-1 with a 50-fold molar excess of K_2PtCl_4 solution in 15 mM phosphate buffer pH 7.5. The reaction was followed by the absorbance change at 280 nm, and experimental points are shown as circles. A_∞ is the absorbance at 280 nm estimated for $t = \infty$. The biphasic nature of the reaction is indicated by the two best fit lines for the initial rate constant (slope) of the reaction and that of the later, slower phase.

proceeds with biphasic kinetics and is half-saturated in ~20 min (Figure 3). In contrast, the formation of Cd_7MT and Zn_7MT under similar conditions is complete within seconds.

Platinum–MT adducts formed under the conditions of the kinetic study were isolated and characterized by inductively coupled plasma analysis, as described elsewhere.[17,19] The Pt_7MT stoichiometry found under similar reaction conditions in the earlier study[17] was also found for these samples.

Conclusions

The UV–visible difference spectra (Figure 1) and the DTNB thiol modification studies (Figure 2) are both consistent with formation of up to three $Pt(cys)_4$ centers per MT in the presence of 3 molar equiv of platinum(II). The UV–visible difference spectrum for the third molar equivalent of platinum(II) has two shoulders at ~250 and ~300 nm, suggesting that some degree of thiolate bridging is present. For higher molar equivalents of Pt per apoMT, the ~6000-cm^{-1} LMCT band red shift and the decreased numbers of thiols blocked per Pt are consistent with the formation of thiolato-bridged platinum(II) clusters in the protein.

With current experimental results, the degree of homogeneity of the Pt_xMT adducts is not established. The initial binding of platinum(II) to apoMT, which has many distinct free thiolates available for metal binding, is probably a random process under kinetic control. Although the spectra obtained in Figure 1 were stable after a prior equilibration time of 3 h, the complexes formed with <7 mol of Pt/mol MT are not necessarily homogeneous in structure, and intramolecular rearrangement to form more stable Pt_xMT derivatives might occur with longer, but experimentally inaccessible, equilibration times. In contrast, it is probable that the binding of seven labile cadmium(II) ions to apoMT at neutral pH is a random process producing a distribution of kinetic products followed by rapid intramolecular reorganization of the cadmium(II)–thiolate bonds to form a single homogeneous Cd_7MT as the thermodynamic product. ^{113}Cd NMR studies of Cd_7MT[30] and metal ion exchange between Cd_7MT and Zn_7MT[31] and a variable-temperature CD study of cadmium(II) binding to Zn_7MT[32] all suggest that metal ions are mobile within a cluster. The greater kinetic inertness of platinum(II) may inhibit the type of facile intramolecular rearrangement described for cadmium(II) and zinc(II). The initial binding process and formation of metal–thiolate clusters to form Pt_7MT appear quite similar to those

(30) Vasák, M.; Hawkes, G. E.; Nicholson, J. K.; Sadler, P. J. *Biochemistry* **1985**, *24*, 740.
(31) Nettesheim, D. G.; Engeseth, H. R.; Otvos, J. D. *Biochemistry* **1985**, *24*, 6744.
(32) Stillman, M. J.; Zelazowski, A. J. *J. Biol. Chem.* **1988**, *263*, 6128.

observed for adducts with cadmium(II) and other metal ions; however, it is possible that the arrangement of platinum(II) ions among the 20 cysteine residues in the equilibrium product at room temperature is heterogeneous.[33]

Platinum(II) is also different from the other metal ions studied in its strong preference for a square-planar stereochemistry. On the basis of the similar elongated Stokes' radii of Pt_7MT and Cd_7MT (1.8 and 1.6 nm, respectively) and other evidence, we suggested a two-domain structure for Pt_7MT with three platinum(II) ions in both the α and β domains with a seventh platinum(II) bound to the N-terminal methionine and further proposed a hypothetical $Pt_3(cys)_9^{3-}$ β cluster containing square-planar platinum(II) centers.[17] The results of the present study are consistent with the presence of platinum clusters in Pt_7MT, but no evidence for a specific Pt–cysteine connectivity has been found. The DTNB assays, which show two reactive thiols in Pt_7MT (Figure 2), do suggest the possibility of $Pt_3(cys)_9^{3-}$ clusters in both domains with two uncoordinated cysteines in the 11-cysteine α domain.

(33) It was hoped that ^1H NMR spectroscopy would yield evidence for possible homogeneity of the Pt_7MT derivative. The ^1H NMR spectrum of Pt_7MT is similar to the spectrum of native MT in its gross features, but individual resonances of the amino acids could not be resolved at 300 MHz under conditions that gave well-resolved spectra for Cd_7MT (Bongers, J. Ph.D. Dissertation, University of Florida, 1989). The broad, weak ^1H NMR signal may be a consequence of heterogeneity of the platinum clusters in the sample, unusual relaxation properties of Pt_7MT, or other unknown factors.

Acknowledgment. The Plasmall was purchased with a Biomedical Research Support grant funded by the National Institutes of Health and administered by the Division of Sponsored Research, University of Florida. Partial support of this project was provided by a Milheim Foundation grant and the Division of Sponsored Research, University of Florida. D.E.R. is an A. P. Sloan Foundation Research Fellow, 1988–1990. We thank Joanne Lopez for her expert technical assistance.

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Ferric Ion Sequestering Agents. 23. Synthesis of Tris(hydroxypyridinethione) Ligands and Their Ferric Complexes; X-ray Structure Analysis of *N,N',N''*-Tris((1,2-didehydro-1-hydroxy-2-thioxopyrid-6-yl)carbonyl)-2,2',2''-triaminotriethylaminato)iron(III)[1]

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Received May 7, 1990

Hexadentate thiohydroxamate ligands derived from 2-mercaptopyridine-6-carboxylic acid 1-oxide and triamines have been prepared by reaction of the amines with the active amide produced from the carboxylic acid and 1,1'-carbonyldiimidazole in DMF. The triamides isolated are *N,N',N''*-tris((1,2-didehydro-1-hydroxy-2-thioxopyrid-6-yl)carbonyl)-2,2',2''-triaminotriethylamine, *N,N',N''*-tris((1,2-didehydro-1-hydroxy-2-thioxopyrid-6-yl)carbonyl)-1,-5,10-triazadecane, and 1,3,5-tris((*N*-methyl-*N*-((1,2-didehydro-1-hydroxy-2-thioxopyrid-6-yl)carbonyl)amino)methyl)benzene. These ligands have been characterized by their elemental analyses and IR and NMR spectra, and the ferric complexes have also been prepared and characterized. The structure of the complex *N,N',N''*-tris((1,2-didehydro-1-hydroxy-2-thioxopyrid-6-yl)carbonyl)-2,2',2''-triaminotriethylaminato)iron(III)·0.5$CHCl_3$ has been determined by single-crystal automated-counter X-ray diffraction. The complex crystallizes in the triclinic space group $P\bar{1}$ with $z = 4$, $a = 11.073$ (1) Å, $b = 13.858$ (1) Å, $c = 22.401$ (2) Å, $\alpha = 70.216$ (8)°, $\beta = 71.847$ (8)°, $\gamma = 68.104$ (8)°. Two crystallographically independent molecules of the ferric complex and one disordered chloroform molecule are in the asymmetric unit. Full-matrix least-squares refinement using 6000 reflections with $F_o^2 > 3\sigma(F_o)^2$, with all non-hydrogen atoms given anisotropic temperature factors, converged to $R = 0.040$ and $R_w = 0.058$. The coordination polyhedron around the ferric ion is intermediate between octahedral and trigonal-prismatic geometries and is similar to that of the ferric complex of 1-hydroxy-2(1*H*)-pyridinone.

Introduction

As part of a program devoted to the synthesis and characterization of ligands that might be useful in the metal chelation therapy of iron overload,[2] we have taken a biomimetic approach to the synthesis of ferric ion specific sequestering agents based on the siderophores (low molecular weight sequestering agents produced by microorganisms to enable them to solubilize and uptake essential ferric ion in a physiological environment).[3-6] Two major functional groups found in siderophores are hydroxamic acids, as in ferrichromes[7] and ferrioxamines,[8] and catechol, as in enterobactin[9] (see Figure 1). Less common functional groups found in siderophores are hydroxypyridinone[10,11] and thiohydroxamic acid (in *N*-(methylthio)formohydroxamic acid).[12,13] Most siderophores contain three bidentate ligating subunits in order to accommodate the preferred hexacoordinate geometry of ferric ion.[3]

We previously reported the synthesis of iron sequestering agents having three bidentate ligating subunits; the subunits in these ligands were catecholate,[14-20] hydroxamate,[1] or hydroxypyridionone.[21] We also have prepared and determined the structures of ferric complexes with simple catecholate,[22] thiohydroxamate,[23] and hydroxypyridinonate[2] ligands, as well as the

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(1) For the previous paper in this series, see: Garrett, T. M.; McMurry, T. J.; Hosseini, M. W.; Reyes, Z. E.; Hahn, F. E.; Raymond, K. N. *J. Am. Chem. Soc.*, in press.
(2) Raymond, K. N.; Chung, T. D. Y.; Pecoraro, V. L.; Carrano, C. J. In *The Biochemistry and Physiology of Iron*; Saltman, P., Hegenauer, J., Eds.; Elsevier Biomedical: New York, 1982; p 649.
(3) Raymond, K. N.; Müller, G.; Matzanke, B. F. *Top. Curr. Chem.* **1984**, *123*, 49.
(4) Neilands, J. B. *Annu. Rev. Microbiol.* **1982**, *36*, 285.
(5) Neilands, J. B. *Microbiol. Sci.* **1985**, *1*, 9.
(6) Matzanke, B. F.; Müller-Matzanke, G.; Raymond, K. N. In *Iron Carriers and Iron Proteins*; Loehr, T. M., Ed.; VCH Publishers: New York, 1989; p 1.
(7) Neilands, J. B. *J. Am. Chem. Soc.* **1952**, *74*, 4846.
(8) Bickel, H.; Vischer, G. E.; Keller-Schierlein, W.; Prelog, V.; Vischer, E.; Wettstein, A. *Helv. Chim. Acta* **1960**, *43*, 2129.
(9) Pollack, J. R.; Neilands, J. B. *Biochem. Biophys. Res. Commun.* **1970**, *38*, 989.
(10) Show, E. *J. Am. Chem. Soc.* **1949**, *71*, 67.
(11) Bapat, J. B.; Black, D. S. C.; Brown, R. F. C. *Adv. Heterocycl. Chem.* **1969**, *10*, 199.
(12) Itoh, S.; Inuzuka, K.; Suzuki, T. *J. Antibiot.* **1970**, *23*, 542.
(13) Egawa, Y.; Umino, K.; Awataguchi, S.; Kawano, Y.; Okuda, T. *J. Antibiot.* **1970**, *23*, 267.

0020-1669/91/1330-0519$02.50/0