Cadmium–Thiolate Clusters in Metallothionein: Spectrophotometric and Spectropolarimetric Features†

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ABSTRACT: Cd–thiolate cluster formation in rabbit liver metallothionein 1 (MT) has been followed at pH 8.4 by monitoring spectroscopic features below 300 nm as a function of increasing Cd-to-apometallothionein (apoMT) ratio. The emerging absorption profiles form a family of closely similar spectra attributable to tetrahedral Cd–tetrathiolate coordination previously established for Cd_7-MT [Vašák, M., Kägi, J. H. R., & Hill, H. A. O. (1981) *Biochemistry, 20*, 2852–2856]. However, there is a 6-nm red shift of the unresolved lowest energy absorption band when >3 equiv of Cd(II) is incorporated. This shift is paralleled by a changeover in the circular dichroism (CD) features of MT from a broad monophasic positive CD profile with ellipticity bands near 240 and 220 nm to a biphasic CD spectrum characterized by positive ellipticity bands at 260 and 224 nm and an interposed negative band at 240 nm. Both features can be attributed to a changeover from separate Cd–tetrathiolate units formed at low metal-to-apoMT ratio to Cd–thiolate clusters when the supply of cysteine ligands becomes limiting. A comparable red shift signaling the transition from the mononuclear to a trinuclear tetrahedral Cd–tetrathiolate complex is also observed upon titration of the synthetic tetrathiol dodecapeptide *N*-Ac-Pro-Cys-Orn-Cys-Pro-Glu-Cys-Glu-Cys-Arg-Arg-Val with Cd(II). The latter studies also provide evidence for the predominantly ligand (sulfur) character of the lowest energy Cd–tetrathiolate ligand–metal charge-transfer transition. As a corollary it is inferred that the biphasic CD profile arises from excitonic coupling of these sulfur-centered transition dipole moments dissymmetrically oriented within the Cd(II)–thiolate clusters.

Metallothioneins (MTs)[1] exhibit spectroscopic features that have long been known to be characteristic of their mode of metal binding. Thus, studies of the absorption properties of the native Cd- and Zn-containing forms from equine and human kidney have given the earliest clues that the metal ions are bound to the protein through mercaptide bonds forming thiolate complexes (Kägi & Vallee, 1961; Pulido et al., 1966; Weser et al., 1973). Recently, it was shown by ^{113}Cd NMR[1] spectroscopy that these complexes are joined to form oligonuclear metal–thiolate clusters (Otvos & Armitage, 1980), yielding structures that have been fully confirmed by X-ray diffraction (Furey et al., 1986) and 2D NMR[1] spectroscopy (Braun et al., 1986).

In a previous study we have shown that the broad absorption shoulder near 250 nm characteristic of Cd-MT[1] arises from the superimposition of at least three incompletely resolved Cd–thiolate transitions upon the featureless absorption spectrum of the apoprotein (Vašák et al., 1981). By the application of Jørgensen's semiempirical electronegativity theory for charge-transfer excitations and by use of tetrahalide spectra as a reference, the lowest energy band located at 249 nm was tentatively identified as the first ligand–metal charge-transfer transition of the tetrahedral Cd–tetrathiolate complex.

The Cd–thiolate absorption bands are also associated with highly characteristic optical rotatory dispersion (Ulmer et al., 1962; Pulido et al., 1966) and Cd[1] features (Weser et al., 1973; Rupp & Weser, 1978; Bühler & Kägi, 1979; Law et al., 1984). In all vertebrate MTs examined the CD spectra of the Cd complex are characterized by a biphasic ellipticity profile with maxima near 257 and 224 nm and an interspaced minimum near 240 nm (Nordberg & Kojima, 1979). Superimposed upon the Cd spectrum of the polypeptide moiety, these features have long been thought to be an indication of structural dissymmetry of the Cd–thiolate complexes (Ulmer et al., 1962; Bühler & Kägi, 1979).

In the present titration study of apoMT[1] with Cd(II) we found that both the absorptive and the chiroptical features are altered profoundly as a function of metal-binding site occupation and that these changes are related to Cd–thiolate cluster formation. The data also confirm our previous finding that under the conditions employed the formation of the metal–thiolate clusters in MT is a stepwise process in which the first few metal equivalents are coordinated to separate sets of cysteine ligands and where the oligonuclear aggregates make their appearance only when the supply of ligands becomes limiting (Vašák & Kägi, 1981; Bernhard et al., 1986).

Materials and Methods

Rabbit liver MT was isolated from rabbits submitted to a total of 20 subcutaneous injections of 1.6 mg of $CdCl_2$/kg of body weight at intervals of 2–3 days. MT was purified by a combination of the procedures of Kägi et al. (1974) and Kimura et al. (1979). The purity of the preparation was examined by amino acid analysis (Durrum D-500) and by metal analysis using atomic absorption spectrometry (Instrumentation Laboratory IL 157). All studies reported in this paper were performed on the charge-separable isoform MT-2.

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[1] Abbreviations: MT, metallothionein; Cd-MT, cadmium-containing metallothionein; apoMT, metal-free metallothionein (apometallothionein); ^{113}Cd NMR, nuclear magnetic resonance of ^{113}Cd nuclei; 2D NMR, two-dimensional nuclear magnetic resonance; CD, circular dichroism; EPR, electron paramagnetic resonance; P-12, synthetic dodecapeptide *N*-Ac-Pro-Cys-Orn-Cys-Pro-Glu-Cys-Glu-Cys-Arg-Arg-Val; HPLC, high-pressure liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; ^{111m}Cd, metastable nuclide of Cd.

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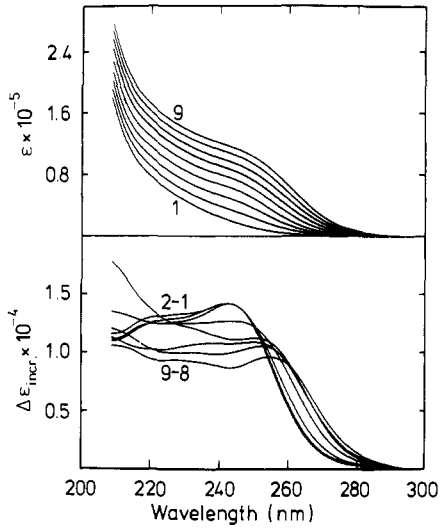

FIGURE 1: Effects of Cd(II) binding on the absorption spectrum of apoMT. Top: Absorption spectra of 8 μM apoMT in 10 mM Tris-HCl, pH 8.4, to which $CdCl_2$ was added successively by using a microsyringe device. At each step the concentration of Cd(II) was increased by 7 μM. The lowest spectrum (1) was recorded prior to the first metal addition. The highest spectrum (9) was attained after 7 equiv of Cd(II) was added. Further increments of Cd(II) produced no appreciable additional spectral changes. Bottom: Increment difference absorption spectra obtained by subtracting the successive spectra of Figure 1, top. The first (spectrum 2 – spectrum 1; 2–1) and last (spectrum 9 – spectrum 8; 9–8) increment difference spectra are labeled. The intermediate increment difference spectra are identifiable on the basis of their progressively decreasing amplitude near 230 nm. ϵ and $\Delta\epsilon_{incr}$ refer to molar protein concentration.

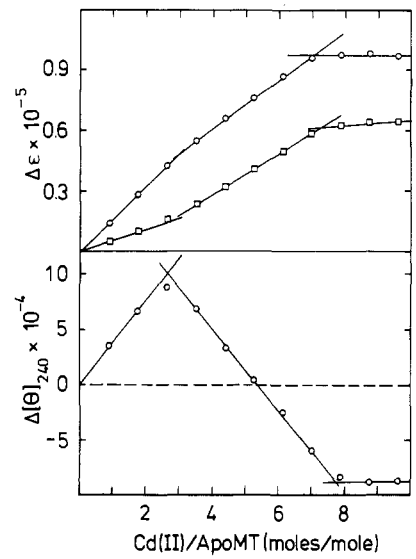

FIGURE 2: Spectrophotometric and spectropolarimetric titration of apoMT with Cd(II). Top: The Cd-induced contribution to the absorbancy of 245 (○) and 260 nm (□) is plotted as a function of Cd-to-apoMT ratio. Data were taken in part from the experiments shown in Figure 1. From the absorbancy of the complexes the absorbancy of apoMT is subtracted. The molar difference absorbancy $\Delta\epsilon$ refers to protein concentration. Bottom: The Cd-induced contribution to the ellipticity at 240 nm $\Delta[\theta]_{240}$ is plotted as a function of Cd-to-apoMT ratio. The data are taken in part from the experiment shown in Figure 4, bottom.

ApoMT was prepared by passing native MT over Sephadex G-25 equilibrated with 0.01 M HCl (Vašák & Kägi, 1983). The tetrathiol dodecapeptide P-12[1] was synthesized by the solid-phase method according to standard procedures (Willner, 1987). Its purity was checked by HPLC[1] and amino acid analysis. ApoMT and peptide concentrations of stock solutions were determined by amino acid analysis and by titration of sulfhydryl groups with DTNB[1] (Birchmeier & Christen, 1973).

All solutions employed in the titration studies were degassed. Adjustments to the required pH were made in an argon-purged glovebox by the addition of 0.5 M Trizma base (Sigma) to calibrated solutions of apoMT and of the dodecapeptide P-12, in 0.01 M HCl. Following transfer of the sample to a quartz cell covered with a septum cap and removal from the glovebox, titrations with Cd(II) were performed under air exclusion employing a microsyringe arrangement (Willner, 1987). All experiments were performed at least in duplicate. Absorption spectra were recorded on a Hitachi Perkin-Elmer Model 340 spectrophotometer connected on-line with an Epson personal computer. CD spectra were obtained on a Jasco 500 spectropolarimeter. Molar absorbancy and molecular ellipticity have units of M^{-1} cm^{-1} and deg cm^2 $dmol^{-1}$, respectively.

RESULTS

Figure 1 (top) shows the changes in electronic absorption brought about by the incremental addition of Cd(II) to apoMT at pH 8.4. The absortion envelope resulting from the formation of the tetrahedral Cd–thiolate complexes (Vašák et al., 1981) increases monotonically until saturation with Cd(II) is reached. However, these changes in absorbance are not fully proportional to the increase in metal content (Figure 2, top). Thus, when measured at the center of the broad Cd–thiolate band (245 nm), the absorbance increases at first with a slope equivalent to a molar absorbancy per Cd(II) of 16 100. Above 3 equiv of Cd(II) bound the increment per Cd(II) is reduced to a molar absorbancy of 12 600. Above 7 equiv no significant increase in absorbance occurs.

Besides the overall rise in absorbance attending the stepwise incorporation of Cd(II) into apoMT there is also a marked broadening of the profile of the absorption envelope. This feature is manifested conspicuously by the change in profile of the increment difference spectra with increasing binding site occupation (Figure 1, bottom). Thus, the first 3 equiv of Cd(II) produce closely similar increment difference spectra with a difference maximum at about 243 nm whereas binding of the remaining 4 equiv generates spectra with a difference maximum at about 256 nm, signaling a red shift of the Cd–thiolate absorption edge. This shift is also evident from the upward deflection of the spectrophotometric titration curve when absorption is monitored at 260 nm (Figure 2, top).

The actual extent of the bathochromic displacement of the Cd–thiolate absorption bands is discerned most clearly in the spectra from which the contribution of the protein moiety is subtracted and where the measured difference absorbancy is referred to the concentration of the thiolate ligands involved in Cd(II) coordination. In Figure 3 (top) such difference profiles are shown for forms of MT reconstituted with 3 and 7 equiv of Cd(II), respectively. In Cd_3-MT [where Cys is present in nearly 7-fold excess over Cd(II)], each metal ion is assumed to be coordinated to separate sets of four thiolate ligands. In Cd_7-MT the ligand-to-metal ratio is 2.86 thiolates per Cd(II) allowed for by the amino acid composition and in accord with the observed metal–thiolate cluster structure (Otvos & Armitage, 1980).

On going from Cd_3- to Cd_7-MT, there is a 6-nm red shift of the absorption edge. However, there are no changes in the overall features of the absorbance profile which was attributed to tetrahedral tetrathiolate–Cd coordination (Vašák et al., 1981). In Cd_3-MT the maxima of the two previously resolved lowest energy Cd–thiolate absorption bands can be discerned near 240 and 220 nm. In Cd_7-MT these bands are broadened and shifted toward the red. Significantly, the amplitudes of the difference absorption profiles of Cd_3- and Cd_7-MT remain nearly the same, indicating that they are not affected by the

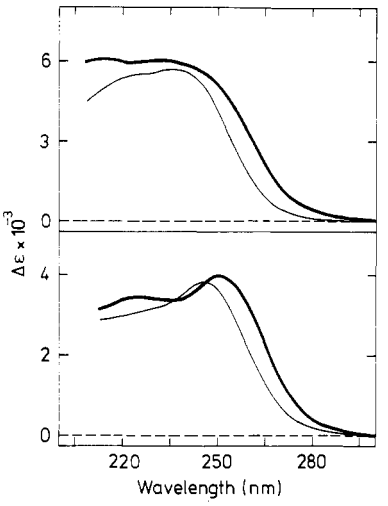

FIGURE 3: Influence of metal-to-ligand stoichiometry on Cd–thiolate absorption profiles. Top: Difference absorption spectra of Cd_3-MT (thin line) and Cd_7-MT (thick line) vs. apoMT. Samples were prepared by adding appropriate amounts of $CdCl_2$ to a 7.3 μM solution of apoMT in 10 mM sodium phosphate, pH 7.0. The molar difference absorbancy, $\Delta\epsilon$, refers to the concentration of Cd-bound Cys (see text). Bottom: Difference absorption spectra of mononuclear (thin line) and trinuclear (thick line) complexes of the synthetic tetrathiol dodecapeptide P-12 with Cd(II) vs. the metal-free peptide.[2] The mononuclear complex Cd(P-12) was prepared by adding an equimolar amount of $CdCl_2$ to 6 μM peptide, in 10 mM Tris-HCl, pH 8.4. The trinuclear complex $Cd_3(P\text{-}12)_2$ was prepared by adding a 1.5-fold or higher molar amount of $CdCl_2$ to the peptide in 10 mM Tris-HCl, pH 8.4. The molar difference absorbancy, $\Delta\epsilon$, refers to the concentration of Cys. The 30% lower amplitude of the difference absorbance profiles compared to those of Cd-MT (Figure 3, top) is the result of the greater deprotonation of Cys at pH 8.4 which entails a larger absorption of free thiolate ligands in the subtracted metal-free peptide.

30% change in thiolate-to-Cd ratio.

Figure 3 (bottom) shows that very comparable spectral differences are also displayed by two tetrahedral complexes of the synthetic tetrathiol peptide P-12 with Cd(II) that differ in metal-to-peptide ratio. As with Cd-reconstituted MT, there is a red shift of the absorption envelope by about 6 nm when the P-12-to-Cd stoichiometry in the complex is reduced from 1 to 0.66, corresponding to a lowering of the thiolate-to-Cd ratio from 4 to 2.66. Again, the general features of the absorbancy profile are not affected appreciably by the change in stoichiometry and there is no difference in the overall intensity.

Dramatic changes in function of metal-binding site occupation are also displayed in the CD spectra (Figure 4). As shown in Figure 4 (top), binding of the first 3 equiv of Cd(II) generates a broad CD band which is superimposed upon the CD spectrum of the polypeptide chain of the apoprotein. As displayed by the difference CD spectra (Figure 4, bottom), its profile is made up of at least two only partly resolved positive ellipticity bands with maxima near 240 nm and near 220 nm. Beyond 3 equiv of Cd(II) bound, upon further addition a completely novel CD profile emerges, leading to the well-known biphasic CD spectrum of native Cd-containing and fully Cd-reconstituted MT (Rupp & Weser, 1978; Vašák & Kägi, 1983; Law et al., 1984). Its distinguishing features are the positive difference ellipticity bands at 259 nm and at 224 nm and the interspaced sharply negative band at 240 nm. Very notably, in the formation of these bands isodichroic points emerge at 249 and 233 nm. The opposing changes in ellipticity at 240 nm attending titration of the apoprotein with Cd(II) are summarized in Figure 2, bottom. They parallel those observed in absorption (Figure 2, top).

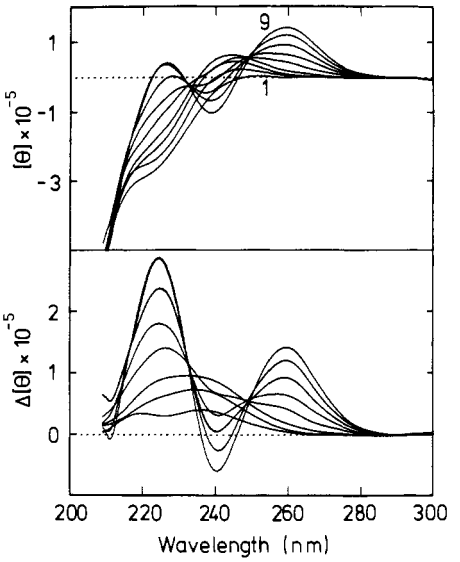

FIGURE 4: Effects of Cd(II) binding on the circular dichroism spectrum of apoMT. Top: Circular dichroism spectra of 8 μM apoMT in 10 mM Tris-HCl, pH 8.4, to which $CdCl_2$ was added in successive steps. Same conditions as in Figure 1. The lowest spectrum (1) was recorded prior to the first Cd(II) addition. The highest spectrum (9) was attained when about 7 equiv of Cd(II) was added. The molecular ellipticity $[\theta]$ refers to protein concentration. Bottom: Cd-induced difference circular dichroism, $\Delta[\theta]$, obtained by subtracting in the top panel the contribution of apoMT (spectrum 1) from the spectra of partially and fully reconstituted Cd-MT.

Discussion

From their homology to the absorption spectra of tetrahedral halide complexes of Cd(II), we have previously inferred that the characteristic and intense absorption shoulder of Cd-MT near 250 nm is a manifestation of tetrahedral coordination of Cd(II) to thiolate ligands (Vašák et al., 1981). The same coordination geometry has also been suggested by perturbed angular correlation of γ-ray spectroscopy (PAC) of the fully and partially metal occupied, ^{111m}Cd-substituted derivatives (Vašák & Bauer, 1982) and by extended X-ray absorption fine structure (EXAFS) measurements (Abrahams et al., 1985) and has recently been confirmed by X-ray crystallography (Furey et al., 1986).

The present spectrophotometric titration experiments with Cd(II) indicate that the overall features of tetrahedral tetrathiolate coordination are maintained at all stages of reconstitution of the metalloprotein (Figure 1, top). However, the minor qualitative spectral changes manifesting themselves with metal addition indicate that these Cd–thiolate absorption bands are also influenced by the degree of saturation of the apoprotein with Cd(II) (Figure 1, bottom). Thus, the red shift developing upon addition of more than 3 Cd(II) equiv to apoMT is reminiscent of the changes in the electronic absorption and in the magnetic and EPR[1] properties observed in analogous titration studies of apoMT with Co(II) (Vašák & Kägi, 1981). In these studies the incorporation of the first 3–4 equiv of Co(II) into tetrahedral sites was shown to lead to the generation of EPR signals typical of high-spin Co(II) and proportional in amplitude to the number of metal ions bound. These features were gradually abolished again, however, when additional Co(II) equivalents were bound. These stoichiometry-dependent changes were interpreted to indicate the preferential formation of noninteracting high-spin complexes of Co(II) with four separate thiolate groups under conditions of ligand excess and their subsequent transformation to antiferromagnetically coupled thiolate-bridged Co(II) clusters when the number of ligands becomes limiting. The

sharp transition of the optical features observed in the present study after addition of the first 3 equiv of Cd(II) (Figure 2) allows the inference that under the conditions employed binding of Cd(II) to apoMT follows the same pattern. At low Cd-to-thiolate ratios mononuclear complexes with four separate thiolate ligands per Cd(II) are formed which are characterized by an absorption maximum at 243 nm (Figure 1, bottom). The bathochromic shift of about 6 nm developing upon further addition of Cd(II) is to be viewed as a manifestation of the transformation of the initially mononuclear tetrahedral tetrathiolate complexes to the Cd–thiolate clusters. In this process the ensemble of cysteine ligands is partitioned into terminal thiolates coordinated to only one Cd(II) and into bridging thiolate ligands coordinated with two vicinal Cd(II). The full extent of the red shift is reached when 7 metal ions are bound to 12 terminal and 8 bridging ligands in Cd_7-MT (Otvos & Armitage, 1980), consistent with the limiting ratio of 2.86 equiv of thiolate per Cd(II). Entirely analogous bathochromic shifts of the UV absorption bands attributable to metal–thiolate cluster formation are also observed when Hg(II) (Bernhard, 1986), Co(II) (Vašák & Kägi, 1981), and Fe(II) (Good & Vašák, 1986) are bound to apoMT.

That the red shift observed with increasing Cd content reflects the appearance of bridging thiolate ligands in the protein is also supported by the comparison of the spectra of the complexes of the tetrathiol dodecapeptide P-12 with equivalent and larger amounts of Cd(II) (Figure 3, bottom). The results from spectrophotometric titration strongly imply that up to a 1:1 Cd-to-peptide ratio this peptide forms exclusively a monomeric mononuclear tetrahedral complex with four terminal thiolate ligands.[2] When more Cd(II) is added, two monomeric complexes are thought to join to form a dimer with a 2:3 peptide-to-Cd stoichiometry equivalent to a thiolate-to-Cd ratio of 2.66. This lowered ligand-to-metal ratio is compatible with the formation of a trinuclear Cd–thiolate cluster in which the extra Cd(II) forms a third tetrahedral thiolate complex interposed between the two monomeric Cd–thiolate complexes, thereby changing four of the eight terminal thiolate ligands into bridging ligands. The comparable extent of the red shifts of about 6 nm in the titration of apoMT and of P-12 with Cd(II) acknowledges the fact that the proportions of bridging cysteine residues with 40% in Cd_7-MT and 50% in $Cd_3(P\text{-}12)_2$, respectively, are similar.

The inference that the observed spectral shifts are the result of the transformation of terminal thiolate ligands into bridging ligands is in line with our earlier proposition that the Cd–thiolate transitions retain a strong ligand-specific character (Bühler & Kägi, 1979). This conjecture was supported by the observation that the absorption intensities per ligand are closely similar to those of the free ligand, i.e., of the deprotonated mercaptan. In the present titration data this strong ligand contribution to the excitations of the complex is also documented by the observation that the absorbtivity of the oligonuclear Cd–thiolate complexes is related to the number of thiolate ligands rather than to the number of Cd(II) in these complexes. Thus, as shown in Figure 3, the maximum extinction coefficient of the first transition of the Cd-bound thiolate ligand remains essentially unchanged when clusters are formed, i.e., when about 30% more Cd(II) is accommodated per ligand.

[2] Titration of the tetrathiol dodecapeptide P-12 with Cd(II) yields sharp breaking points at 1:1 and 1:1.5 molar peptide-to-Cd ratios consistent with the formation of a monomeric mononuclear Cd(P-12) complex and a dimeric trinuclear $Cd_3(P\text{-}12)_2$ complex, respectively. As judged from studies with space-filling models, both complexes allow for tetrahedral tetrathiolate coordination of Cd(II) (Willner, 1987).

The proposition that the Cd–thiolate transitions are allocable mainly to the ligand also has bearing on the interpretation of the CD features of Cd-MT and its changes upon Cd–thiolate cluster formation. The complex CD profiles and the highly conspicuous qualitative changes occurring in the CD spectrum upon titration of apoMT with Cd(II) testify to the generally more complex and composite origin of the spectropolarimetric features (Figure 4, top). The spectra that contain contributions both from Cd–thiolate and from polypeptide transitions are simplified somewhat, however, by the subtraction of the CD spectrum of apoMT (Figure 4, bottom). This subtraction is justified since between 245 and 215 nm the apoprotein displays only a broad and featureless negative CD shoulder (Bühler & Kägi, 1979) and since there is no indication that the transition from random coil to β-turn type conformation thought to occur in some segments on metal binding to apoMT (Wagner et al., 1985; Pande et al., 1986) introduces in this spectral region changes comparable in magnitude to the ellipticity contributions of the Cd–thiolate transitions (Woody, 1974).

Although some of the chiroptical features of the Cd–thiolate chromophore of MT have been reported a quarter of a century ago (Ulmer et al., 1962) and a number of studies have followed (Pulido et al., 1966; Weser et al., 1973; Rupp & Weser, 1978; Law et al., 1984), their molecular origin has remained unexplained. On the above proposition that the lowest energy Cd–thiolate band represents predominantly an excitation located at the sulfur atom of the thiolate ligands, it is a reasonable assumption that the ellipticity also arises primarily from dissymmetric interaction of these ligands with their environment. Potential sources of rotatory power of such ligand-centered transitions are the configurational asymmetry of the cysteine side chain and the "coordinational" asymmetry conditioned by the geometry of the multidentate metal–thiolate complexes. A significant contribution to CD from the stereoconfiguration of the cysteine residue is anticipated, considering that the liganding sulfur atom is separated from the chiral α-carbon by only one carbon atom. In fact, such configurational or static CD is observed in the region of the thiolate absorption bands of fully deprotonated cysteine and of other chiral mercaptans (Jung et al., 1973). In general, CD spectra of such origin are characterized by the coincidence of their bands with the spectral location of the underlying absorption bands. Indeed, in the present data such correspondence is evident between the positive difference CD profiles generated upon addition of the first 3 equiv of Cd(II) (Figure 4, bottom) and the difference absorption spectrum of the same complex (Figure 3, top). Both spectra show maxima or shoulders at approximately 240 and 220 nm. By contrast, the radical change in CD profile that sets in when the fourth equivalent of Cd(II) is bound and when bridging thiolate ligands make their appearance has no easily recognizable correlate in the absorption spectrum. Moreover, these changes are difficult to explain on the basis of the configurational asymmetry of the cysteine ligands alone since to judge from the preservation of the absorption profile neither the coordination geometry nor the physical character of the Cd–thiolate transitions is altered drastically with additional Cd(II) incorporation (vide supra). It appears, therefore, most likely that the new chiroptical features differ in physical origin by being conditioned by "coordinational" dissymmetry of the Cd–thiolate complexes. In principle, such optical activity can be generated by the interaction of dissymmetrically coordinated chromophoric ligands located either, as suggested earlier, within the coordination sphere of a single metal ion (Ulmer

et al., 1962; Bühler & Kägi, 1979) or, as postulated here, within the greater framework of the cluster structure. In systems composed of spatially separated chromophoric groups with noncoplanar transition moment dipoles of sufficiently high oscillatory strength, optical activity can arise by a dynamic coupling mechanism (Schellman, 1968; Bosnich, 1969). In such interactions between identical or energetically closely lying transitions the resulting spectrum often exhibits features of biphasic or conservative CD in which the ellipticity displays a "derivative-shaped" profile with its inflection point centered about the absorption maximum (Guéron et al., 1974). That such conservative CD signals contribute indeed to the CD spectrum of Cd-MT when Cd–thiolate complexes are formed has been suggested earlier (Bühler & Kägi, 1979) and is strongly supported by the change to a profile with alternating positive, negative, and positive ellipticity bands separated by inflection points near 233 and 249 nm (Figure 4, bottom). The biphasic character of the new signals is especially obvious at the lowest energy absorption band which is shifted upon cluster formation to about 250 nm (Figure 3, top) and which is flanked by a positive and a negative ellipticity band at its low- and high-energy side, respectively. The biphasic character of this signal fully explains the shape and the location of the conspicuous 257–259-nm positive ellipticity band found in all Cd-containing mammalian MTs (Rupp & Weser, 1978; Law et al., 1984). Additional support for this assignment comes from our recent demonstration that the application of a classical formalism (DeVoe, 1965) to excitonic coupling of the transition dipole moments located at the bridging thiolates of the known Cd–thiolate clusters of mammalian MT (Furey et al., 1986) closely predicts sign, size, and spectral location of the first two low-energy CD bands (Willner, 1987). The origin of the cluster-induced second positive CD band located near 224 nm is less clear (Figure 4, bottom). Conceivably, it is part of a second unresolved biphasic CD signal associated with a second higher energy Cd–thiolate transition (Vašák et al., 1981).

On the evidence that the spectral changes attending the titration of apoMT with Cd(II) reflect the transformation of nonbridged into bridged metal–thiolate complexes, the present data offer proof that at the conditions of these experiments, i.e., at pH 8.4, the formation of the Cd–thiolate clusters, like that of the Co(II)–thiolate clusters (Vašák & Kägi, 1981), is a stepwise process in which the first metal ions are coordinated to separate sets of thiolate ligands and in which oligonuclear aggregates only emerge when the options for separate metal binding become restricted. The same conclusions were recently drawn from a study in which the cysteine residues of partially and completely reconstituted Cd-MT were chemically modified with [^{14}C]iodoacetamide (Bernhard et al., 1986). In these experiments, too, the emergence of the stable clusters was shown to be preceded by the formation of separate complexes of the first 2–3 equiv of Cd(II) with cysteine residues selected at random along the polypeptide chain.

In conclusion, these data show that the absorption and CD features of the Cd–thiolate complexes with apoMT and related model peptides are conditioned by the organization of the metal ions in thiolate clusters. Both a 6-nm bathochromic shift of the first absorption band and the transition from monophasic to biphasic CD can be correlated with the transformation of singly coordinated terminal thiolate ligands in separate Cd–tetrathiolate complexes into doubly coordinated bridging thiolate ligands in the Cd–thiolate clusters. The characteristic chiroptical features of Cd_7-MT are proposed to arise from excitonic coupling of the lowest energy ligand–metal charge-transfer transitions centered at the newly formed bridging thiolate ligands of the clusters. The presence of such signals in vertebrate (Weser et al., 1973), invertebrate (Law et al., 1984), and plant (Rauser et al., 1983) MTs may constitute, in fact, the most direct indication of clustered Cd–thiolate complexes in these proteins.

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Registry No. L-Cys, 52-90-4; tetrathiol dodecapeptide, 109686-76-2; Cd, 7440-43-9.

REFERENCES

Abrahams, I. L., Garner, C. D., Bremner, I., Diakun, G. P., & Hasnain, S. S. (1985) *J. Am. Chem. Soc. 107*, 4596–4597.

Bernhard, W. R. (1986) Ph.D. Thesis, University of Zürich, Zürich, Switzerland.

Bernhard, W R., Vašák, M., & Kägi, J. H. R. (1986) *Biochemistry 25*, 1975–1980.

Birchmeier, W., & Christen, P. (1971) *FEBS Lett. 18*, 209–213.

Bosnich, B. (1969) *Acc. Chem. Res. 2*, 266–273.

Braun, W., Wagner, G., Wörgötter, E., Vašák, M., Kägi, J. H. R., & Wüthrich, K. (1986) *J. Mol. Biol. 187*, 125–129.

Bühler, R. H. O., & Kägi, J. H. R. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) pp 211–220, Birkhäuser, Basel.

DeVoe, H. (1965) *J. Chem. Phys. 43*, 3199–3208.

Furey, W. F., Robbins, A. H., Clancy, L. L., Winge, D. R., Wang, B. C., & Stout, C. D. (1986) *Science (Washington, D.C.) 231*, 704–710.

Good, M., & Vašák, M. (1986) *Biochemistry 25*, 8353–8356.

Guéron, M., Eisinger, J., & Lamola, A. A. (1974) in *Basic Principles in Nucleic Acid Chemistry* (Ts'o, P. O. P., Ed.) Vol. 1, pp 311–398, Academic, New York and London.

Jung, G., Ottnad, M., & Rimpler, M. (1973) *Eur. J. Biochem. 35*, 436–444.

Kägi, J. H. R., & Vallee, B. L. (1961) *J. Biol. Chem. 235*, 2435–2442.

Kägi, J. H. R., Himmelhoch, S. R., Whanger, P. D., Bethune, J. L., & Vallee, B. L. (1974) *J. Biol. Chem. 249*, 3537–3542.

Kimura, M., Otaki, N., & Imano, M. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) pp 163–168, Birkhäuser, Basel.

Law, A. Y. C., Cherian, M. G., & Stillman, M. J. (1984) *Biochim. Biophys. Acta 784*, 53–61.

Nordberg, M., & Kojima, Y. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) pp 41–124, Birkhäuser, Basel.

Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094–7098.

Pande, J., Vašák, M., & Kägi, J. H. R. (1985) *Biochemistry 24*, 6717–6722.

Pulido, P., Kägi, J. H. R., & Vallee, B. L. (1966) *Biochemistry 5*, 1768–1777.

Rauser, W. E., Hartmann, H.-J., & Weser, U. (1983) *FEBS Lett. 164*, 102–104.

Rupp, H., & Weser, U. (1978) *Biochim. Biophys. Acta 533*, 209–226.

Schellman, J. A. (1968) *Acc. Chem. Res. 1*, 144–151.

Ulmer, D. D., Kägi, J. H. R., & Vallee, B. L. (1962) *Biochem. Biophys. Res. Commun. 8*, 327–330.

Vašák, M., & Kägi, J. H. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6709–6713.
Vašák, M., & Bauer, R. (1982) *J. Am. Chem. Soc. 104*, 3236–3238.
Vašák, M., & Kägi, J. H. R. (1983) *Met. Ions Biol. Syst. 15*, 213–273.
Vašák, M., Kägi, J. H. R., & Hill, H. A. O. (1981) *Biochemistry 20*, 2852–2856.
Weser, U., Rupp, H., Fonay, F., Linnemann, F., Voelter, W., Voetsch, W., & Jung, G. (1973) *Eur. J. Biochem. 39*, 127–140.
Willner, H. (1987) Ph.D. Thesis, University of Zürich, Zürich, Switzerland.
Woody, R. W. (1974) in *Peptides, Polypeptides and Proteins* (Blout, E. R., Bovey, F. A., Goodman, M., & Lotan, N., Eds.) pp 338–350, Wiley, New York.

Calcium Binding to Mixed Cardiolipin–Phosphatidylcholine Bilayers As Studied by Deuterium Nuclear Magnetic Resonance†

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ABSTRACT: Calcium binding to bilayer membranes containing cardiolipin (CDL) mixed with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was investigated by using phosphorus-31 and deuterium nuclear magnetic resonance (NMR) spectroscopy. The destabilizing effect of Ca^{2+} on CDL bilayers, including the formation of hexagonal H_{II} and isotropic phases, was eliminated when CDL was mixed with sufficiently large proportion of POPC. Thus, for the mixture CDL–POPC (1:9 M/M), ^{31}P NMR spectra retained a line shape typical of fluid bilayer lipids even in the presence of 1.0 M Ca^{2+}. Specifically head-group-deuteriated CDL or POPC showed in this mixture ^2H NMR spectra indicating that both lipids remained in a fluidlike bilayer at Ca^{2+} concentrations up to 1.0 M. Any phase separation of Ca^2–CDL clusters could be excluded. The residence time of Ca^{2+} at an individual head group binding site was shorter than 10^{-6} s. The deuterium quadrupole splitting, $\Delta\nu_Q$, of POPC deuteriated at the α-methylene segment of the choline head group was found to be linearly related to the number of bound calcium ions, X_2, for the CDL–POPC (1:9 M/M) mixture. The effective surface charge density, σ, could be determined from the measured amount of bound Ca^{2+}. Subsequently, the surface potential, ψ_0, and the concentration of free Ca^{2+} ions at the plane of ion binding were calculated by employing the Gouy–Chapman theory. Various possible models of the equilibrium binding of Ca^{2+} could then be tested. The Langmuir adsorption isotherm with a Ca^{2+} binding constant of 15.5 M^{-1} gave the best fit to the experimental data. Sodium binding was comparatively weak with a binding constant of 0.75 M^{-1}. A comparison of Ca^{2+} binding constants for different membrane lipid compositions revealed that the increase in Ca^{2+} binding observed in the presence of negatively charged lipids was predominantly an electrostatic effect rather than being due to differences in the intrinsic Ca^{2+} affinity. Ca^{2+} was able to reduce the surface potential by binding and neutralizing negative surface charges in addition to having a screening effect.

Cardiolipin, by virtue of its negative charge, should enhance the membrane surface binding of cationic ligands. The binding to the membrane surface of metal ions such as calcium can alter the physical properties of membrane lipids (Tilcock, 1986) and influence the electrical and functional properties of the membrane itself (McLaughlin, 1977). For other ligands, such as anaesthetics and particular signal peptides and proteins, binding to the membrane surface is intrinsic to their mode of functioning. Clearly, given the biological relevance of these various surface binding events, it is desirable to obtain a quantitative understanding of the properties of the lipid membrane surface, how these properties are influenced by the lipid composition, and how their alteration can affect ligand binding to the membrane surface.

While numerous techniques have been employed to study the binding of metal ions (and other ligands) to membrane lipids, ^2H nuclear magnetic resonance (NMR)[1] of specifically deuteriated lipid polar head groups offers certain advantages. The measurements are performed on large multilamellar vesicles so that the lipid configuration is not strained as it would be in the case of small sonicated vesicles. The binding data can be obtained even at quite high ion concentrations so that the complete ion binding isotherm can be described. This allows a distinction to be made between various possible modes of binding. ^2H NMR is sensitive to the charge at the surface of the membrane rather than at an idealized plane of shear some distance from the surface as with measurements of ζ potentials via electrophoretic mobility. Finally, ^2H NMR not only provides a quantitative measure of the thermodynamics of ion binding but also simultaneously monitors the molecular conformation of the membrane lipids.

Using ^2H NMR, we have been able to describe quantitatively the binding of calcium to neutral phosphatidylcholine membranes (Altenbach & Seelig, 1984) and to negatively

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[1] Abbreviations: NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycerol 3-phosphate; CDL, cardiolipin; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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