Spectral Studies of Cobalt(II)- and Nickel(II)-Metallothionein[†]

Milan Vašák,* Jeremias H. R. Kägi, Barton Holmquist, and Bert L. Vallee

ABSTRACT: The zinc and cadmium of native rabbit metallothionein-1 were replaced stoichiometrically with either cobalt(II) or nickel(II). The electronic, magnetic circular dichroic (MCD), and electron spin resonance spectra of Co-(II)–metallothionein reflect distorted tetrahedral coordination of the cobalt atoms. Both the d–d and charge-transfer spectral regions closely resemble those of simple cobalt–tetrathiolate complexes, implying that their coordination chemistry is analogous. Ni(II) complex ions and Ni(II)–metallothionein similarly exhibit analogous MCD bands in the d–d region. The circular dichroic bands associated with ligand–metal charge-transfer transitions in the non-d–d region of Co(II)– and Ni(II)–metallothionein afford additional evidence for the similarity in tetrahedral microsymmetry of the two metal derivatives. The known ratio of 20 thiolate ligands to 7 metal ions, in conjunction with the spectral evidence for tetrathiolate coordination in metallothionein, represents good evidence that these metal thiolates are organized in clusters.

The elucidation of the structure of the metal binding sites in metallothionein, a metal- and sulfur-rich protein widely distributed in nature, is of considerable interest in view of its presumed involvement in metabolism, homeostasis, and detoxification (Kägi et al., 1974; Richards & Cousins, 1975; Webb & Magos, 1976; Kojima & Kägi, 1978). This protein was first isolated from equine renal cortex by Margoshes & Vallee (1957) in their search for a tissue constituent responsible for the accumulation of cadmium in animals. Subsequently, proteins with similar characteristics were also isolated from kidney, liver, and intestines of a wide variety of animal species and recently also from eukaryotic microorganisms (Kojima & Kägi, 1978; Lerch, 1979). All mammalian forms characterized to date contain a single polypeptide chain containing 20 cysteinyl residues among a total of 61 amino acid residues with a molecular weight of 6100 (Kojima et at., 1976). Significantly, there are seven atoms of Zn and/or Cd per molecule. Metallothionein contains neither aromatic amino acids nor histidine (Kägi & Vallee, 1961; Kojima et al., 1976). In contrast to most other metal–thiolate proteins for which Cys-X-Y-Cys metal binding sequences are typical (Croft, 1973; Eklund et al., 1974), where X and Y stand for amino acid residues other than Cys, mammalian metallothioneins contain seven Cys-X-Cys sequences (Kojima et al., 1976). All 20 cysteinyl residues participate in metal binding via mercaptide linkages (Kägi et al., 1974; Weser et al., 1973). On the basis of the cysteine/Me^{2+} ratio, complexometric measurements of proton release upon metal binding, and charge measurements, it has been postulated that generally the minimum metal binding unit consists of three thiolate ligands yielding negatively charged trimercaptide complexes (Kojima et al., 1976). Recent ^{113}Cd NMR studies have given strong evidence that these units are juxtaposed, forming polynuclear metal clusters with some mercaptides serving as bridging ligands (Otvos & Armitage, 1979; Sadler et al., 1978).

Despite ample documentation of the involvement of sulfhydryl groups in metal binding of metallothioneins, there is only limited information regarding the coordination geometry. On the basis of a comparative, semiempirical analysis of the charge-transfer spectra of metallothionein containing zinc, cadmium, or mercury, we have recently proposed tetrahedral coordination for the metals (Vašák et al., 1981). The aim of the present work was to examine this proposal by substituting the naturally occurring group-2B metal ions of metallothionein, i.e., cadmium and zinc, with cobalt(II) and nickel(II). Cobalt(II) has been employed commonly as a probe of zinc metalloenzymes and metalloproteins owing to its chromophoric characteristics, its coordination geometries which are similar to those of zinc, its paramagnetism, and the sensitivity of its d–d transitions to changes in coordination geometry. It is well documented that such substitution does not measurably alter the overall protein structure and preserves the catalytic function of many zinc enzymes (Vallee & Williams, 1968; Vallee & Wacker, 1970; Garbett et al., 1972; Vallee & Holmquist, 1980). Ni(II) ion substitution has not been used as widely, owing to its propensity for square-planar complexes and the existence of a limited number of model complexes suitable for study. However, recent studies on Ni(II)–aspartate transcarbamylase (Johnson & Schachman, 1980), Ni(II)–azurin (Tennent & McMillin, 1979), and Ni(II)–carboxypeptidase (Rosenberg et al., 1973, 1975; B. Holmquist and B. L. Vallee, unpublished experiments) have documented its potential to serve as a paramagnetic probe. Further, when spectra of both Co(II) and Ni(II) derivatives are available, the charge-transfer region can be analyzed in greater detail.

The present study examines the coordination geometry of rabbit Co(II)– and Ni(II)–metallothionein. It also presents a comparative analysis of the charge-transfer spectra of these derivatives of metallothionein.

Materials and Methods

Rabbit liver metallothionein-1 was isolated from rabbits injected subcutaneously 20 times with 1 mg of Cd/kg of body weight in the form of cadmium chloride in 2–3-day intervals (Kimura et al., 1979) and purified by the procedure of Kägi et al. (1974). The purity of each preparation was examined by amino acid and metal analyses, using amino acid analysis (Durrum D-500) and atomic absorption spectrometry (Instrumentation Laboratory, Model IL 157), respectively. Thionein, the metal-free protein, was prepared by dialyzing metallothionein against three changes of 0.1 M HCl. The molecular weight cutoff of the dialysis tubing employed was 3500 (Spectrapor Co). Protein concentration was determined spectrophotometrically by measuring the absorbance of thionein at 220 nm in 0.1 M HCl, using an absorption coefficient, A_{220}, of 7.9 mg cm^{-1} mL^{-1} (Bühler & Kägi, 1979). All

Table I: Absorption, CD, and MCD Spectral Data for Co(II)- and Ni(II)-Metallothioneins

Co(II)-metallothionein			Ni(II)-metallothionein		
absorption [nm (ϵ)]ᵃ	CD [nm ([θ] × 10⁻³)]ᵃ	MCD [nm ([θ]_M)]ᵃ	absorption [nm (ϵ)]	CD [nm ([θ] × 10⁻³)]	MCD [nm ([θ]_M)]
743 (435)	775 (−0.11)	757 (−0.42)			
	730 (+0.07)		750 sh (100)	675 sh (−1.49)	710 (−0.012)
690 (420)	700 (−0.015)	690 sh (−0.17)			
	675 sh (+0.04)	628 (+0.01)			
600 (245)	610 (+0.07)	580 (−0.015)			
400 sh (2170)	415 (−2.58)	425 (+0.2)	560 sh (580)	560 (−1.65)	
	370 sh (+0.71)	373 sh (−0.17)		480 (+1.01)	490 (−0.014)
	345 (+4.21)	342 (−0.30)	430 sh (2000)	440 (−1.06)	
320 (2845)	320 sh		340 sh (6560)	355 (+6.27)	370 (+0.13)
	300 (−1.09)	300 sh (−0.14)	290 (6990)	300 sh (−2.35)	320 (−0.29)
	275 (+0.57)			273 (−6.32)	
		250 (+0.22)			250 (−0.12)
	235 (−5.04)			230 (−12.14)	

ᵃ Values are based on the concentration of the metal; for definition, see Materials and Methods.

inorganic chemicals were of reagent grade or better. For removal of traces of metal contaminants, buffer solutions were extracted with dithizone in CCl_4 or passed over a column of Chelex-100 (Bio-Rad Corp.). Absorption spectra were recorded with Cary 15 and Perkin-Elmer model 340 spectrometers using 0.2-, 1.0-, and 2.0-cm path-length quartz cells. The spectra in the near-infrared region were obtained in solutions of D_2O (99.5%). Both natural circular dichroism (CD)[1] and magnetic circular dichroism (MCD) were recorded at room temperature with a Cary 61 spectropolarimeter equipped with a Varian superconducting solenoid. A magnetic field of 40 kG was applied. All MCD spectra are corrected for the CD components. Molar absorptivities, ϵ, are based on the concentration of cobalt(II) and nickel(II) in the sample and are designated as $\epsilon_{Co(II)}$ and $\epsilon_{Ni(II)}$, respectively, with units of M^{-1} cm^{-1}. CD and MCD are both expressed in terms of molar ellipticity, [θ], and are referred to metal concentrations with units of deg cm^2 $dmol^{-1}$. For the MCD measurements, the ellipticity was normalized to 1 G. The ESR spectra were recorded at a temperature of 4 K in a Varian E112 spectrometer operating in the X-band mode.

Preparation and Characterization of Co(II)- and Ni-(II)-Metallothionein. (a) Co(II)-Metallothionein. Thionein, 4 mg/mL in 0.1 M HCl, was mixed with a freshly prepared aqueous solution of cobalt(II) sulfate or chloride, yielding a metal ion concentration twice-that of the metal-binding sites. Prior to use, both the mixture and all other solutions used in the preparation of Co(II)-metallothionein were degassed by means of a vacuum line. The sample containing thionein was adjusted to pH 7 in an argon-purged glovebox by addition of 0.5 M Trizma base. Excess cobalt was removed from Co-(II)-metallothionein samples by adding moist Chelex-100 resin (30% by volume) and stirring for 5 min. The green-colored Co(II)-metallothionein derivative contained 6–7 mol of Co-(II)/mol. This complex is very sensitive to air, and exposure results in a light yellow product, a decomposition common to a number of sulfur-containing reconstituted proteins and model compounds (Davison & Switkes, 1971; Anglin & Davison, 1975; Lane et al., 1977; May & Kuo, 1978).

(b) Ni(II)-Metallothionein. Solid nickel chloride was added to 4 mg/mL thionein in 0.1 M HCl in an amount twice that of the metal-binding sites. Subsequently, the mixture was adjusted to pH 7.6 by addition of 0.5 M sodium N-(2-
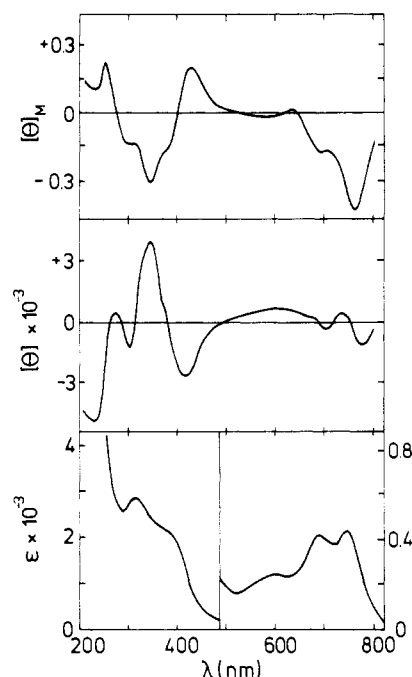

FIGURE 1: MCD (top), CD (middle), and absorption (bottom) spectra of Co(II)-metallothionein in 0.05 M Tris-HCl, pH 7.0, at 23 °C. For definition of the units employed, see Materials and Methods.

hydroxyethyl)piperazine-N'-2-ethanesulfonate. For removal of excess of Ni(II) from Ni(II)-metallothionein, the sample was passed through a Sephadex G-25 column preequilibrated with 0.05 M Hepes at pH 7.6. The yellow Ni(II)-metallothionein derivative contained 6–7 mol of Ni(II)/mol. Regardless of whether or not oxygen was present, the sample was stable at room temperature for about 2 h; thereafter, the color faded slowly.[2]

Results and Discussion

The metal/protein stoichiometry of Co(II)- and Ni(II)-substituted metallothionein, 6–7 mol/mol, is the same as that of native Zn and/or Zn and Cd containing forms. The addition of Zn(II) readily displaces either Co(II) or Ni(II), indicating that Zn binds more firmly than either one of them, probably at the same thiolate binding sites. This inference is supported by the correspondence between the non-d–d

[1] Abbreviations used: CD, circular dichroism; MCD, magnetic circular dichroism; ESR, electron spin resonance; LMCT, ligand–metal charge transfer; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid.

[2] This slow change in color is accompanied by transformation to a derivative with four Ni(II) atoms. The absorption maximum shifts from 290 to 280 nm while a new shoulder emerges at 320 nm.
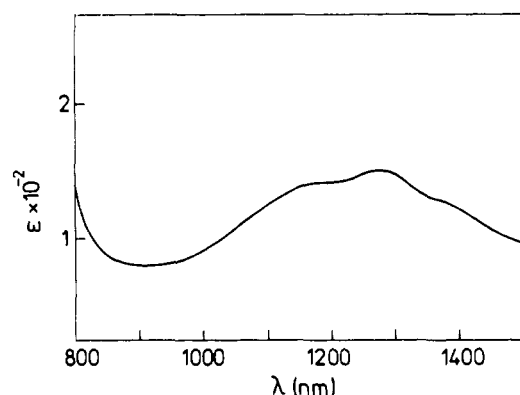
FIGURE 2: Near-infrared absorption spectrum of Co(II)—metallothionein in D_2O (0.05 M Tris-DCl, pD 7.4).

spectroscopic features of Co(II)— and Ni(II)—metallothionein, which like those of Zn(II)— and Co(II)—metallothionein (Vašák et al., 1981) are dominated by thiolate LMCT transitions (vide infra).

Low-Energy Region of the Co(II)-Metallothionein Spectrum. The electronic absorption spectrum of Co(II)—metallothionein is shown in Figure 1 bottom spectrum. There are broad bands in the d—d region with maxima at 600, 690, and 743 nm with molar absorptivities of 245, 420, and 435 M^{-1} cm^{-1}, respectively (Table I). These features, typical of tetrahedral coordination, closely resemble those reported for inorganic tetrathiolate complexes (Davison & Switkes, 1971; McMillin et al., 1974; Anglin & Davison, 1975; Lane et al., 1977; Dance, 1979) and for cobalt derivatives of crystallographically defined metalloproteins with four cysteine ligands e.g., cobalt(II) rubredoxin(May & Kuo, 1978) and horse liver alcohol dehydrogenase with cobalt at the noncatalytic metal-binding sites (Drum & Vallee, 1970; Sytkowski & Vallee, 1976; Maret et al., 1979). The resolved band pattern can be assigned to the spin-allowed $\nu_3[^4A_2 \rightarrow {}^4T_1(P)]$ transition. Splitting of this transition into three components with an energy separation of 2175 and 1146 cm^{-1} is larger than that to be expected from spin—orbit coupling alone. This is most likely due to the distortion of T_d symmetry which results in removal of the degeneracy of the upper-state $^4T_1(P)$. The molar absorptivity, of the most intense maximum at 743 nm, 435 M^{-1} cm^{-1}, is also compatible with pseudotetrahedral symmetry ($\epsilon > 250$ M^{-1} cm^{-1}) (Carlin, 1965; Rosenberg et al., 1975). A spin-allowed $\nu_2[^4A_2 \rightarrow {}^4T_1(F)]$ transition in the near-infrared region (Figure 2) also indicates T_d microsymmetry (Lever, 1968). It manifests as a broad band with a maximum at 1275 nm ($\epsilon = 165$ M^{-1} cm^{-1}) and with a shoulder at 1150 nm ($\epsilon = 155$ M^{-1} cm^{-1}).

Neglecting spin—orbit coupling interactions, it is possible to evaluate the strength of the ligand field Δ_t in T_d symmetry from the following equation (Lane et al., 1977):

$$\Delta_t^2 - 0.529(\nu_2 + \nu_3)\Delta_t + 0.294\nu_2\nu_3 = 0 \qquad (1)$$

and by employing the intensity-weighted maxima of the ν_2 and ν_3 bands, 1.54 and 0.83 μm^{-1}, respectively. The calculated value for Δ_t of 0.478 μm^{-1} indicates the weak ligand-field nature of the cysteinate groups and agrees fully with values found for the mercaptide and cysteinate model complexes (Lane et al., 1977).

These results differ from those obtained for equine Co(II)—metallothionein (Vašák, 1980). Despite the virtual identity of the high-energy region (vide infra), the absorptivities of the maxima in the d—d region are about 30% higher. In addition, the two low-energy bands undergo bathochromic shifts with an energy separation of 2175 cm^{-1}, as compared
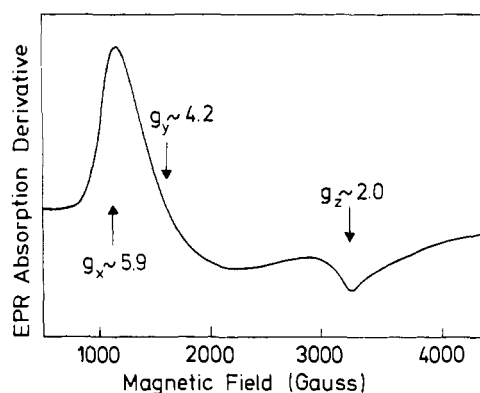

FIGURE 3: EPR spectrum of Co(II)—metallothionein. Conditions were the following: 1.14 × 10^{-3} M Co(II)—metallothionein in 0.05 M Tris-HCl, pH 7.0; microwave power 10 mW; microwave frequency 9.25 GHz; 4 K.

to 2000 cm^{-1} in equine Co(II)—metallothionein. Both features are compatible with a somewhat higher degree of distortion in the Co(II) derivative of rabbit metallothioneins that could arise from slight differences in the primary structures of the two proteins (Kojima et al., 1976; Kimura et al., 1979).

The MCD spectrum of Co(II)—metallothionein also provides convincing evidence for tetrahedral metal coordination. The low-energy region of the MCD spectrum exhibits a strong negative band at 757 nm with a pronounced shoulder at 690 nm (Figure 1, top spectrum). In addition, there is one weak positive and one weak negative MCD band at 628 and 580 nm, respectively (Table I). The same overall pattern was both predicted and observed in a number of inorganic tetrahedral and pseudotetrahedral complexes (Lin & Eyring, 1965; Stephens, 1965; Denning, 1966; Katô & Akimoto, 1974; Kaden et al., 1974) and in Co(II) proteins with this coordination geometry (Coleman & Coleman, 1972; Holmquist et al., 1975). Like the absorption spectra, the MCD spectra of rabbit and equine Co(II)—metallothionein differ also (Vašák, 1980). The maxima of the individual MCD bands vary slightly, and in rabbit Co(II)—metallothionein, there is an additional weak band at 580 nm (Figure 1, top spectrum).

The CD spectrum of Co(II)—metallothionein also displays several characteristic spectral features (Figure 1, middle spectrum). Not less than four extrema and one shoulder can be discerned in the region of the ligand field transition $\nu_3[^4A_2 \rightarrow {}^4T_1(P)]$. This optical asymmetry probably arises both from dissymmetric coordination geometry of distorted tetrahedral sites and from the intrinsic chirality of the cysteinate ligands.

Electron Spin Resonance (ESR) of Co(II)-Metallothionein. The ESR spectrum of a frozen solution of Co(II)—metallothionein (Figure 3) resembles that of a powder spectrum typical of a rhombically distorted high-spin Co(II) complex (Kennedy et al., 1972; Aasa et al., 1976) with parameters of $g_x \approx 5.9$, $g_y \approx 4.2$, and $g_z \approx 2.0$. The Co(II) signal is strongly temperature dependent and is not saturated at 20-mW incident microwave power. Above 16 K, no ESR signal could be detected; however, the signal is weak, about 5% of that of the high-spin $[(CoCl_4)^{2-}]$.

Low-Energy Region of the Ni(II)-Metallothionein Spectrum. The replacement of native Zn(II) and/or Cd(II) of the respective metallothioneins with Ni^{2+} ions results in a poorly resolved low-intensity electronic absorption spectrum that starts at about 1150 nm and gradually increases in intensity at lower wavelength (Figure 4, bottom spectrum). In the visible region, shoulders at 750, 560, and 430 nm are the only distinctive features (Table I). A crystallographically defined, tetrahedral, inorganic complex containing a NiS_4 core with maxima at 714,
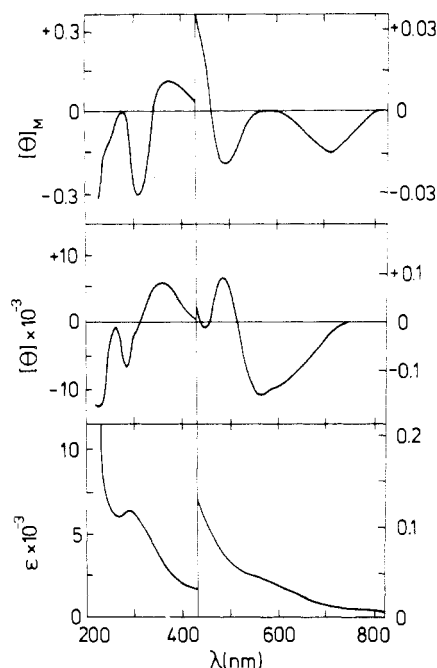
FIGURE 4: MCD (top), CD (middle), and absorption (bottom) spectra of Ni(II)–metallothionein in 0.05 M Hepes buffer, pH 7.6, at 23 °C. For definition of the units employed, see Materials and Methods.

785, 935, and 1150 nm has been reported (Davison & Switkes, 1971; Churchill et al., 1971). The persistence of a substantial absorption at 750 nm ($\epsilon = 100$ M^{-1} cm^{-1}) suggests that Ni(II) in Ni(II)–metallothionein is bound in nonplanar geometry (Ballhausen, 1962; Carlin, 1965; Lever, 1968). The absence of distinct d–d absorption bands can be attributed in part to the superimposition of the red edge of low-energy charge-transfer bands which extend into this spectral region. A similar masking of the d–d bands has also been noted for the Ni(II) derivative of azurin, a protein whose metal is known to be bound tetrahedrally (Adman et al., 1978). While more spectral detail can be discerned in the CD spectrum which exhibits massive ellipticity bands throughout the visible and the ultraviolet region (Figure 4, middle spectrum), the composite negative ellipticity band in the low-energy region remains unresolved. However, the MCD spectrum of Ni(II)–metallothionein gives unambiguous evidence for the existence of specific d–d transitions (Figure 4, top spectrum). The MCD band at 710 nm can be assigned to the $\nu_3[^3T_1(F) \rightarrow {}^3T_1(P)]$ ligand-field transition and is diagnostic of tetrahedral nickel (Churchill et al., 1971; Davison & Switkes, 1971; Holah & Coucouvanis, 1975). Almost in the same position (720 nm), an absorption band was recently observed also in a Ni(II) derivative of aspartate transcarbamylase. In this enzyme, the metal is bound by four cysteinyl residues arranged in T_d symmetry (Johnson & Schachman, 1980).

Charge-Transfer Spectra of Co(II)– and Ni(II)–Metallothionein. Since thionein does not absorb appreciably above 220 nm (Kägi & Vallee, 1961; Vašák et al., 1981), all transitions of Ni(II)– and Co(II)-substituted protein must arise from metal coordination sites. Thus, except for the low-energy d–d bands discussed already (vide supra), the remainder of the spectral bands must be attributed either to charge-transfer excitations or to ligand internal transitions (Mastropaolo et al., 1977) affected by the interaction with the metal ion. These non-d–d transitions which are resolved only partially become increasingly intense on going toward higher energy (Figures 1 and 4). The maxima at 320 and 290 nm are the most conspicuous features of the near-UV absorption spectra of the Co(II) and Ni(II) derivatives, respectively. The 320-nm
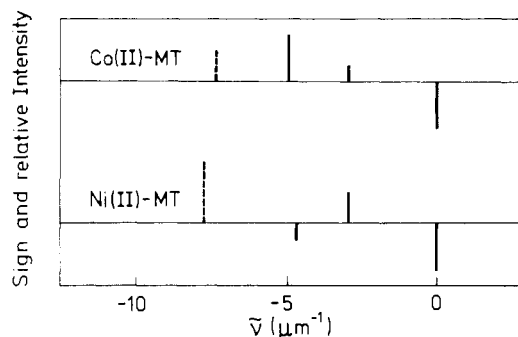

FIGURE 5: Bar diagram representation of the relevant part of the CD spectra of Co(II)–metallothionein and Ni(II)–metallothionein. The first charge-transfer transition of each spectrum has been adjusted to 0.0 on the energy scale.

charge-transfer band of Co(II)–metallotionein with a molar absorptivity of 980 M^{-1} cm^{-1} per Co(II) thiolate bond is also seen in cobalt-substituted forms of rubredoxin (May & Kuo, 1978), liver alcohol dehydrogenase (Drum & Vallee, 1970; Sytkowski & Vallee, 1976; Maret et al., 1979), carboxypeptidase A, and thermolysin, in the latter two instances when mercaptan-bearing inhibitors are bound (Holmquist & Vallee, 1979). The band is diagnostic of thiolate charge-transfer excitation [Cys S → Co(II)].

The CD and/or MCD spectra provide substantially more detail about this spectral region (Figures 1 and 4). Table I summarizes the spectral data of Co(II)– and Ni(II)–metallothionein. There are at least eight distinct bands or shoulders, leaving aside the features associated with d–d absorption.

In this spectral region, the CD spectrum provides the best resolution, and the MCD spectrum gives little additional information. Hence, further analysis will be restricted to the CD data. The CD spectra of the two metallothionein derivatives are quite similar with respect to the shape and sign of the bands (Figures 1 and 4, middle spectra), but the visible and near-UV charge-transfer bands of Ni(II)–metallothionein undergo a significant bathochromic shift. Thus, as judged by the location of the first absorption shoulder and the accompanying negative ellipticity bands, the first LMCT band of Co(II)– and Ni(II)–metallothionein is near 415 and 560 nm, respectively. When the CD spectra are alligned on an energy scale such that the positions of the first bands of the Co(II)– and Ni(II)–metallothionein coincide, the subsequent CD bands correspond very well as shown in Figure 5, allowing them to be classified as LMCT transitions also. The similarity in the spacing of the bands strongly implies their homologous origin from LMCT excitations involving corresponding molecular orbitals, which suggest closely similar geometries of the metal complexes in Co(II)– and Ni(II)–metallothioneins.

The spectra of a number of crystallographically defined Co(II) complex ions and of Co(II)-substituted metalloproteins in which the ion has been documented to be coordinated tetrahedrally to sulfur ligands exhibit sets of LMCT bands which are closely comparable to those occurring in Co(II)–metallothionein (Table II). These bands can be assigned tentatively to two π and one σ excitation of cysteine–sulfur to Co(II). In contrast, there is little information regarding the charge-transfer region of Ni(II)–sulfur complexes. Ni(II)–azurin, whose tetrahedral metal-binding site consists of two nitrogen and two sulfur ligands, is the only well-documented case (Adman et al., 1978; Tennent & McMillin, 1979). The bands at 561, 500, 438, 390, and 350 nm in its CD spectrum are in remarkable agreement with those of Ni(II)–metallothionein (Table I and Figure 4, middle spectrum).

Table II: High Energy CD and Absorption Bands for Crystallographically Defined Tetrahedral Co(II) Derivatives of Proteins and Inorganic Models with Sulfur Donor Atoms

metallothionein (nm)	azurin[a] (nm)	Co$_2$Zn$_2$-LADH[a] (nm)	rubredoxin[a] (nm)	compound[b]			
				I	II	III	IV
415	408	387	460[c]		440	476	416
370	373	355	375	370		375	
345	333	335	348		350		
(320)		323	325				
300							
275		d		280		d	
235							

[a] CD bands of proteins of known X-ray structure, i.e., azurin (Tennent & McMillin, 1979), which has binding ligands 2N (His), S (Met), and S (Cys); LADH (liver alcohol dehydrogenase) (Drum & Vallee, 1970; Sytkowski & Vallee, 1976), which has structural site binding ligands 4S (Cys); and rubredoxin (May & Kuo, 1978), which has binding ligands 4S (Cys). [b] Model compounds with coordination via the mercaptide sulfur. Compound I is a mull spectrum of Co[SC(CH$_3$)$_2$CH$_2$NH$_2$]$_2$ (Mastropaola et al., 1977). Compounds II, III, and IV (Dance, 1979) represent reflection spectra of (Me$_4$N)$_2$Co(SPh)$_{10}$, (Me$_4$N)$_2$Co$_4$(SPh)$_8$Cl$_2$, and (Me$_4$N)$_2$Co(SPh)$_4$, respectively. [c] Absorption band. [d] No data available.

All five bands are thought to arise from excitation of π and σ molecular orbitals of the S–Ni(II) bonds (Tennent & McMillin, 1979). The origin of the three high-energy CD bands at 300, 273, and 235 nm of Ni(II)–metallothionein (Table I) remains to be established. Since the aromatic amino acid residues of most proteins and the ligands of Co(II) complex ions generate intense background in the region below 300 nm, few reference spectra exist (Sytkowski & Vallee, 1976; Mastropaolo et al., 1977; May & Kuo, 1978; Dance, 1979). The very strong negative CD bands of Co(II)– and Ni(II)– metallothioneins at 235 and 230 nm, respectively, could arise from ligand- internal transitions. Alkaline solutions of cysteine and 2-mercaptoethanol exhibit absorption maxima at 237 and 233 nm, respectively (Benesch & Benesch, 1955; Donovan, 1969).

Conclusions

The spectroscopic data obtained in this first detailed study of Co(II)– and Ni(II)–metallothioneins permit a number of inferences about the structure of the metal-binding site in this protein. Analysis of the d–d region shows that Co(II) is bound to four thiolate ligands in distorted tetrahedral geometry. Moreover, in metallothionein containing seven Co(II) ions, the existence of substantial fine structure in the $\nu_3[^4A_2 \rightarrow {}^4T_1(P)]$ transition suggests that the pseudotetrahedral geometry of different binding sites must be quite similar. The recent finding that the lowest energy LMCT absorption bands of Cd(II)– and Zn(II)–metallothioneins correspond well with those predicted for tetrahedral tetrathiolate model complexes of these metals also supports these conclusions (Vašák et al., 1981). Jointly, the considerations indicate that—in spite of the absence of a truly periodic polypeptide structure—there must be a very high degree of regularity in the organization of the metal-binding sites. Most spectral features also indicate preservation of this tetrahedral type of geometry in Ni(II)–metallothionein. Yet, in contrast to the Co(II) derivatives, the electronic absorption spectrum fails to show a clearly resolved $\nu_3[^3T_1(F) \rightarrow {}^3T_1(P)]$ transition.

Ni(II) ions are known to have a propensity for square-planar coordination which would introduce strain into a presumed tetrahedral coordination site, causing some irregularity which might explain the above observations. Our observation that at room temperature Ni(II)–metallothionein is less stable than the corresponding Co(II) derivatives in the absence of oxygen (see Materials and Methods) would tend to support the conclusion.

The present data provide evidence that each of the seven metal ions is bound tetrahedrally to four thiolate ligands. This structural organization is compatible with the known 20 cysteinyl residues per polypeptide chain only if the oligonuclear clusters share some thiolate ligands of neighboring metal ions. Recent ^{113}Cd NMR measurements have given evidence of metal–metal coupling, indicating the existence of such clusters in metallothioneins (Otvos & Armitage, 1979). Similarly, the ESR signals of Co(II)–metallothionein are abnormally weak (vide supra) due to antiferromagnetic coupling through bridging thiolate ligands (M. Vašák, unpublished experiments).

Dark-field electron microscopy favors the existence of well-separated metal domains (Fiskin et al., 1977). The tetrathiolate complexes could form clusters in a number of ways. In the simplest model, metal ions could be joined through either one or two bridging cysteine–sulfur ligands. In the latter case, the resultant structure would closely resemble that found in the two Fe clusters of ferredoxins (Lovenberg, 1977). An adamantane-type structure would constitute another possibility.[3] This very regular cluster consisting of tetrahedral units was recently observed in crystallographically defined model compounds of cobalt(II) and zinc(II) and was suggested to occur in metallothionein (Dance, 1979, 1980). The number of bridging and terminal cysteine ligands constitute the major difference between the models. In adamantane clusters, about 70% of all cysteinyl residues would participate in bridging as contrasted with about 40% in the ferredoxin-type clusters and even less in the single bridged model.

Acknowledgments

References

Aasa, R., Manson, M., & Lindskog, S. (1976) Biochim. Biophys. Acta 453, 211.

Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) J. Mol. Biol. 123, 35.

Anglin, J. R., & Davison, A. (1975) Inorg. Chem. 14, 234.

Ballhausen, C. J. (1962) Introduction to Ligand Field Theory, p 258, McGraw-Hill, New York.

Benesch, R. E., & Benesch, R. (1955) J. Am. Chem. Soc. 77, 5877.

[3] Very recently homonuclear ^{113}Cd NMR decoupling studies have suggested the presence of two metal clusters with a total of 12 terminal sulfur ligands (Otvos & Armitage, 1980). One cluster of four metals contains nearly 50% bridging sulfur atoms to resemble the adamantane structure. Another cluster of three metals contains 33% bridging sulfur atoms, indicating a single bridged type.

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

Carlin, R. L. (1965) in *Transition Metal Chemistry* (Carlin, R. L., Ed.) Vol. 1, pp 1–33, Marcel Dekker, New York.

Churchill, M. R., Cooke, J., Fennessey, J. P., & Wormald, J. (1971) *Inorg. Chem. 10*, 1031.

Coleman, J. E., & Coleman, R. V. (1972) *J. Biol. Chem. 247*, 4718.

Croft, L. R. (1973) *Handbook of Protein Sequences*, pp 65–70, Joynson-Bruvvers Ltd., Oxford.

Dance, G. I. (1979) *J. Am. Chem. Soc. 101*, 6264.

Dance, G. I. (1980) *J. Am. Chem. Soc. 102*, 3445.

Davison, A., & Switkes, E. S. (1971) *Inorg. Chem. 10*, 837.

Denning, R. G. (1966) *J. Chem. Phys. 45*, 1307.

Donovan, J. W. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part A, p 101, Academic Press, New York.

Drum, D. D., & Vallee, B. L. (1970) *Biochem. Biophys. Res. Commun. 41*, 33.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, J., Boiwe, T., & Bränden, C.-I. (1974) *FEBS Lett. 44*, 200.

Fiskin, A. M., Peterson, G., & Brady, F. O. (1977) *Ultramicroscopy 2*, 389.

Garbett, K., Partrige, G. W., & Williams, R. J. P. (1972) *Bioinorg. Chem. 1*, 309.

Holah, D. G., & Coucouvanis, D. (1975) *J. Am. Chem. Soc. 97*, 6917.

Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216.

Holmquist, B., Kaden, T. A., & Vallee, B. L. (1975) *Biochemistry 14*, 1454.

Johnson, R. S., & Schachman, H. K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1995.

Kaden, T. A., Holmquist, B., & Vallee, B. L. (1974) *Inorg. Chem. 13*, 2585.

Kägi, J. H. R., & Vallee, B. L. (1961) *J. Biol. Chem. 236*, 2435.

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

Katô, H., & Akimoto, K. (1974) *J. Am. Chem. Soc. 96*, 1351.

Kennedy, F. S., Hill, H. A. O., Kaden, T. A., & Vallee, B. L. (1972) *Biochem. Biophys. Res. Commun. 48*, 1533.

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

Kojima, Y., & Kägi, J. H. R. (1978) *Trends Biochem. Sci. 3*, 90.

Kojima, Y., Berger, C., Vallee, B. L., & Kägi, J. H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3413.

Lane, R. W., Ibers, J. A., Frankel, R. B., Papaefthymiou, G. C., & Holm, R. H. (1977) *J. Am. Chem. Soc. 99*, 84.

Lerch, K. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) p 173, Birkhäuser, Basel.

Lever, A. B. P. (1968) *Inorganic Electronic Spectroscopy*, Chapter 9, Elsevier, Amsterdam.

Lin, S. H., & Eyring, H. (1965) *J. Chem. Phys. 42*, 1780.

Lovenberg, W., Ed. (1977) *Iron-Sulfur Proteins*, Vol. III, Academic Press, New York.

Maret, W., Anderson, I., Dietrich, H., Schneider-Bernlöhr, H., Einarsson, R., & Zeppezauer, M. (1979) *Eur. J. Biochem. 98*, 501.

Margoshes, M., & Vallee, B. L. (1957) *J. Am. Chem. Soc. 79*, 4813.

Mastropaolo, D., Thick, J. A., Potenza, J. A., & Schugar, H. J. (1977) *J. Am. Chem. Soc. 99*, 124.

May, S. W., & Kuo, J.-Y. (1978) *Biochemistry 17*, 3333.

McMillin, D. R., Holwerda, R. A., & Gray, H. B.(1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1339.

Otvos, J. D., & Armitage, I. M. (1979) *J. Am. Chem. Soc. 101*, 7734.

Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1094.

Richards, M. P., & Cousins, R. J. (1975) *Bioinorg. Chem. 4*, 215.

Rosenberg, R. C., Root, C. A., Wang, R. H., Cerdonio, M., & Gray, H. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 161.

Rosenberg, R. C., Root, C. A., & Gray, H. B. (1975) *J. Am. Chem. Soc. 97*, 21.

Sadler, P. J., Bakka, A., & Beynon, P. J. (1978) *FEBS Lett. 94*, 315.

Stephens, P. J. (1965) *J. Chem. Phys. 43*, 4444.

Sytkowski, A. J., & Vallee, B. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 344.

Tennent, D. L., & McMillin, D. R. (1979) *J. Am. Chem. Soc. 101*, 2307.

Vallee, B. L., & Williams, R. J. P. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 498.

Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins, 2nd Ed. 5*, 94.

Vallee, B. L., & Holmquist, B. (1980) *Methods for Determining Metal Ion Environments in Proteins* (Darnall, D. W., & Wilkins, R. & G., Eds.) pp 27–74, Elsevier/ North-Holland, New York.

Vašák, M. (1980) *J. Am. Chem. Soc. 102*, 3953.

Vašák, M., Kägi, J. H. R., & Hill, H. A. O. (1981) *Biochemistry 20*, 2852.

Webb, M., & Magos, L. (1976) *Chem.-Biol. Interact. 14*, 357.

Weser, U., Rupp, H., Donay, F., Linnenmann, F., Voelter, W., Voetsch, W., & Jung, G. (1973) *Eur. J. Biochem. 39*, 127.