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Magnetic Circular Dichroic Spectra of Cobalt(II) Substituted Metalloenzymes†

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ABSTRACT: The magnetic circular dichroic (MCD) spectra of cobalt(II) substituted metalloenzymes have been studied and compared to a series of four-, five-, and six-coordinate cobalt(II) model complexes previously examined (T. A. Kaden et al. (1974), *Inorg. Chem. 13, 2585*). The MCD spectra of cobalt substituted carboxypeptidase A, procarboxypeptidase A, and thermolysin are consistent with earlier deductions of tetrahedral coordination from absorption spectra and also with X-ray structure analysis. Inhibitors fail to alter their MCD spectra significantly. The MCD spectra of cobalt alkaline phosphatase and carbonic anhydrase are more complex and their pH dependence and alteration by inhibitors are discussed in terms of known cobalt(II) models.

The substitution of metal atoms at the active sites of metalloenzymes is one of the mildest and most selective procedures currently available for the chemical modification of enzymes. Generally, such substitutions do not measurably alter overall protein conformation or structure and often preserve catalytic function (Vallee and Wacker, 1970; Davies et al., 1968; Holmquist and Vallee, 1974). When such substitutions replace a colorless, diamagnetic atom, e.g., zinc, with one which is chromophoric and paramagnetic, e.g., Co(II), considerable experimental advantages can accrue in correlating coordination geometries at the active sites with functional properties of metalloenzymes. Thus, the absorption spectra of a number of Co(II) substituted zinc metalloenzymes are unusual when compared to those of Co(II) complex ions of known structure (Vallee and Williams, 1968). Such spectra permit deductions both regarding the detailed and overall characteristics of active site geometry and the function of metals in metalloenzymes. Both electronic and magnetic resonance spectra constitute an important resource to enlarge upon deductions from X-ray crystallographic structure analysis and to extend its potential to the study of conformation and structure of enzymes in solutions.

Among the spectral approaches now feasible, the potential of magnetic circular dichroism (MCD)[1] remains relatively unexplored in defining Co(II) coordination in metalloenzymes though it would likely reveal information not previously accessible by other spectroscopic techniques presently in common use. Thus, while molar absorptivity

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[1] Abbreviations used are: MCD, magnetic circular dichroism; Me_6tren, tris(2-dimethylaminoethyl)amine; POA, *N*-2-picolyloxamide; Et_4dien, *N,N,N',N'*-tetraethyldiethylenetriamine; terpy, 2,2';6'2''-terpyridine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

depends on the electronic dipole moment of a transition, the intensity of the MCD signal is proportional to the product of the electronic and magnetic dipole. In general d-d* transitions of Co(II) transitions are electronically forbidden but magnetically allowed, and, hence, are particularly attractive for detailed examination. Inspection of the Faraday effect of a series of Co(II) complexes suggested that studies of Co(II) substituted enzymes might significantly enlarge understanding of the geometry, symmetry, and function of metals at metalloenzyme active sites (Kaden et al., 1972, 1974).

We here present MCD spectra of a number of enzymatically active, Co(II) substituted zinc metalloenzymes. Interpretation of the spectra are based on comparisons with Co(II) complex ions of known coordination geometry and symmetry. Compared with absorption or natural circular dichroic spectra, Co(II) MCD spectra are relatively insensitive to distortions of symmetry in the coordination sphere of the metal and seem to reflect primarily the *overall* geometry of the metal ion at the active site. However, addition of inhibitors and substrates, or alterations of pH, significantly alter the MCD spectra of Co(II) metalloenzymes and provide a means of relating structure of metal coordination in the metalloenzyme to its function.

Materials and Methods

MCD measurements were performed using a Cary 61 circular dichroic spectrophotometer equipped with a Varian Model V4145 superconducting magnet, energized by a Varian V4106 superconducting magnet power supply. Magnetic field strengths were varied from 0 to 47 kG using the current-field calibration constant supplied with the magnet, 0.6 A/kG. Measurements were made routinely at a field of 40 kG. In general, scans were performed at a speed of 0.2 nm/sec with a spectral band width of 2 nm. Appropriate attention to the effects of scan speeds, spectral bandwidths, and instrument pen period time constants ensured optimal spectral resolution.

Natural CD was measured either with the Cary 61 or a Cary 60 spectropolarimeter equipped with a Cary 6001 CD attachment. Cylindrical sample cells, constructed of Suprasil of 1- and 2-cm pathlengths with a volume of from 1 to 4 ml were used for all studies. Absorption of all samples was maintained at less than 2.0 A. Absorption spectra were obtained with a Cary 14 spectrophotometer equipped with a Cary tungsten-iodine high intensity light source. Unless otherwise stated, all measurements were conducted at room temperature (23–25°). Absorptivities, ϵ, are given in units of M^{-1} cm^{-1}. Values of the molar magnetic ellipticity $[\theta]_M$ in units of deg cm^2 dmol^{-1} G^{-1} were calculated from the difference between the ellipticity in the presence and absence of the magnetic field and normalized to 1 G. All MCD spectra were recorded with the magnetic field parallel to and in the direction of the light beam.

The CD was calibrated with an aqueous solution of *d*-10-camphorsulfonic acid, $\theta_{289\ nm}^{1\ mg/ml} = 0.308°$ (Cassim and Yang, 1969). The MCD was calibrated with an aqueous solution of Johnson-Matthey "Specpure" $CoSO_4 \cdot 7H_2O$, 0.05 *M*, $[\theta]_M$, 510 nm = -6.2×10^{-3} deg cm^2 dmol^{-1} G^{-1}, in good agreement with the value determined previously for this salt (McCaffery et al., 1967). Calibrations of MCD using this standard were performed periodically after current ramp down into persistent mode operation to ensure no loss of field during this process.

The experimental absorption and MCD curves were analyzed by means of two computer programs written for an IBM 360 computer. The first program, based on the method of moments (Henry et al., 1965), uses relations 1 and 2,

$$\langle\epsilon\rangle_n = \int \frac{\epsilon}{\nu} (\nu - \nu_0)^n d\nu \quad (1)$$

$$\langle[\theta]_M\rangle_n = \int ([\theta]_M/\nu)(\nu - \nu_0)^n d\nu \quad (2)$$

where $n = 0$ or 1 for the zeroth and first moments, respectively, and ν_0 is defined as the frequency which makes $\langle\epsilon\rangle_1 = 0$. IBM subroutine QSF is the central part of this program. The second program is a version of Marquad's nonlinear least-squares program (Middleton and Hamilton, 1970) which was modified to fit the absorption and MCD spectra with gaussian curves using eq 3 for absorption and

$$\epsilon = \sum_i \epsilon_{max,i} \exp[-(\nu - \nu_{0,i})^2/\Delta\nu_i^2] \quad (3)$$

$$[\theta]_M = \sum_j R_j \nu(\nu_{0,j} - \nu)/\Delta_j^3 \exp\left[\frac{-(\nu - \nu_{0,j})^2}{\Delta_j^2}\right] + \sum_l R_l' \nu \exp\left[\frac{-(\nu - \nu_{0,l})^2}{\Delta_l^2}\right] \quad (4)$$

eq 4 for the MCD spectra (Stephens, 1970), where *i*, *j*, and *l* are the index numbers of absorption bands, biphasic and monophasic MCD bands, respectively, $\epsilon_{max,i}$ is the maximal molar absorptivity at the frequency $\nu_{0,i}$, $\Delta\nu_i$ the bandwidth, and R, R', and Δ are the corresponding constants defining the MCD bands. Complete MCD spectra could not be obtained in some cases, precluding direct integration. In these instances eq 3 and 4 were used to fit the spectra to full curves to allow integration and determination of zeroth and first moments according to eq 1 and 2. While these equations are of the same basic form as those used in the rigid shift approximation (Stephens et al., 1966), this method of analysis is impractical since the Co(II) spectra are composed of many overlapping electronic transitions.

Deionized glass distilled water was used throughout. Special precautions necessary in working with metalloenzymes were taken to exclude adventitious metal ions (Thiers, 1959). All buffer and reagent solutions used in the experiments employing proteins were rendered free of transition metals by extraction with 0.01% dithizone, and all glassware was washed with 50% nitric acid–sulfuric acid. "Specpure" cobalt sulfate (Johnson-Matthey) was used to reconstitute the metalloenzymes from the respective apoenzymes. Phenylphosphonic acid was recrystallized from water. All other materials were reagent grade or the highest grade available.

Alkaline phosphatase was isolated from *Escherichia coli* by the osmotic shock pressure method and had an activity of 23 μmol of *p*-nitrophenyl phosphate hydrolyzed per min per mg of protein (Simpson et al., 1968). Apoalkaline phosphatase, the zinc-free protein, was prepared by extensive dialysis of enzyme, 2–3 $\times$ 10^{-4} *M* vs. three changes of EDTA, 10^{-2} *M*, in Tris, 0.01 *M*, pH 8.0, over a period of 5 days. The apoenzyme was reconstituted with either 2 or 4 g-atoms of Co^{2+}/mol to result in Co_2-phosphatase or Co_4-phosphatase, respectively, as measured by atomic absorption (Fuwa and Vallee, 1963). Perturbation of the absorption and MCD spectra with phosphate, arsenate, and phenyl phosphonate was accomplished by addition of small volumes of concentrated solutions of these ions to the cobalt enzymes.

[2] Units of magnetic molar ellipticity, $[\theta]_M$, where not indicated are deg cm^2 dmol^{-1} G^{-1}.

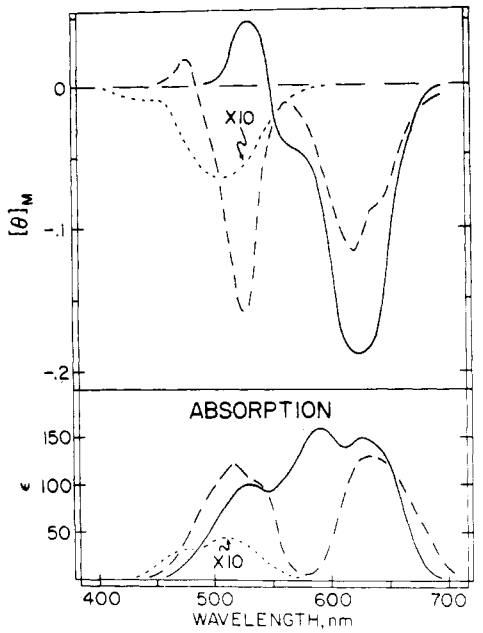

FIGURE 1: MCD spectra (above) and absorption spectra (below) of Co(II) model complexes; $Co(H_2O)_6^{2+}$ (···); $Co(Me_6tren)Br_2$ (- - -) in acetonitrile; $Co(OH)_4^{2-}$ (—) in 50% NaOH.

Thermolysin (Calbiochem, Los Angeles) was obtained as a lyophylized, three times recrystallized material containing 30% calcium acetate. The enzyme was recrystallized further to remove peptide fragments, by dissolving the enzyme, 50 mg/ml, in NaBr, 5 *M*, Tris, 0.05 *M*, $CaCl_2$, 0.01 *M* at pH 7.5 followed by dialysis to low ionic strength against the same buffer but without NaBr (Latt et al., 1969). The zinc content of this material was 1 ± 0.1 g-atom/mol as measured by atomic absorption and exhibited an activity of 1.2×10^6 M^{-1} sec^{-1}, when measured as described by Latt et al., 1969.

Zinc was removed from thermolysin by washing crystalline suspensions with 1,10-phenanthroline, 0.01 *M*, pH 7.5, over a period of 10 hr, 0°, followed by extensive washing of the crystals for 10 hr with six changes of water. The Co(II) substituted enzyme (Holmquist and Vallee, 1974) was prepared by addition of 1.1 equiv of aqueous $CoSO_4$ to the apoenzyme, $2-4 \times 10^{-4}$ *M*, dissolved in NaBr, 3 *M*, containing Tris, 0.05 *M*, pH 7.5, and $CaCl_2$, 0.01 *M*. Absorption, CD, and MCD perturbation spectra with the inhibitor β-phenylpropionyl-L-phenylalanine were obtained after direct addition of aliquots of concentrated solutions of inhibitor in buffer directly to the cobalt enzyme.

Cobalt(II) substituted bovine carbonic anhydrase (Sigma) was prepared by the method of Lindskog and Malmström (1962). Spectral perturbation studies with substituted sulfonamides were performed as described for alkaline phosphatase and thermolysin.

The preparation of cobalt(II) and carboxypeptidase A (Cox) and cobalt(II) procarboxypeptidase A and the methods for spectral perturbation studies have been described (Latt and Vallee, 1971; Behnke and Vallee, 1972).

Results

Interpretation of the MCD spectra of Co(II) substituted metalloenzymes is greatly facilitated by comparing their spectra to those of suitable Co(II) complexes. The MCD spectra of three such Co(II) complexes are presented in Figure 1. They are among a series of Co(II) complex ions studied earlier (Kaden et al., 1972, 1974) which exemplify the characteristic MCD spectra observed for octahedral, tetrahedral, and pentacoordinate geometries commonly observed for Co(II) complex ions.

The intensity of the absorption spectra of octahedral Co(II) complexes such as $Co(H_2O)_6^{2+}$ are generally low, $\epsilon = 4-10$. Their MCD spectra are characterized by a slightly structured, negative MCD band centered near 510 nm with a range of the magnetic molar ellipticity, $[\theta]_M$, of from -6.0×10^{-3} to -2.8×10^{-2}.[2] The absorption spectra of

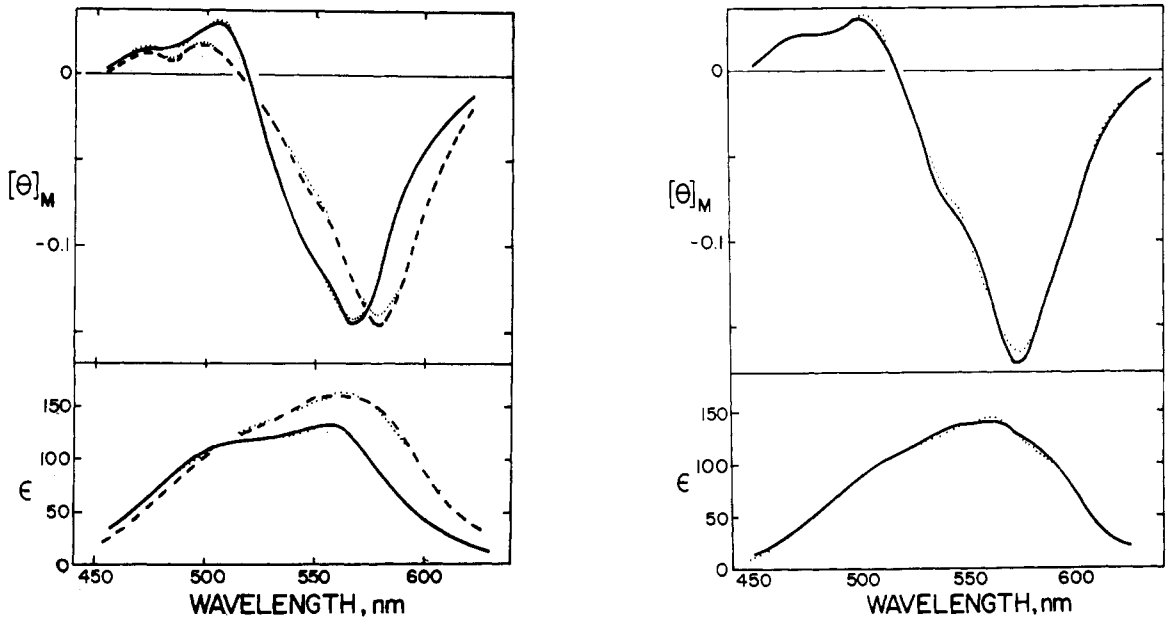

FIGURE 2: (Left) MCD spectra (above) and absorption spectra (below) of cobalt carboxypeptidase, pH 7.1, 1 *M* NaCl, 5 m*M* Tris, in the absence (- - -) or presence (—) of 15 m*M* Gly-L-Tyr. The dotted curves are simulated spectra calculated from parameters based on the computed best fit to the spectral measurements. (Right) MCD spectra (above) and absorption spectra (below) of cobalt procarboxypeptidase. Conditions are identical with those used for cobalt carboxypeptidase. Dotted curves adjacent to the spectra are calculated from parameters based on the computed best fit.

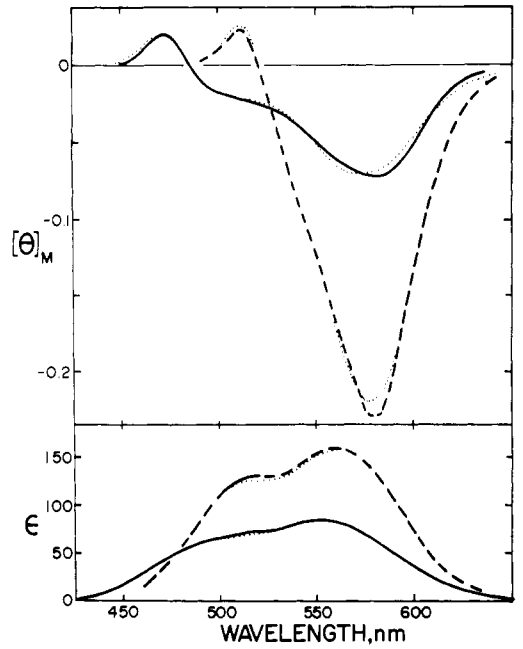

FIGURE 3: MCD spectra (above) and absorption spectra (below) of cobalt thermolysin, 2.2×10^{-4} *M*, at pH 6.8, in 10 m*M* Hepes, 3 *M* NaBr, 10 m*M* $CaCl_2$, in the absence (—) and presence (- - -) of the competitive inhibitor β-phenylpropionyl-L-phenylalanine, 10 m*M*.

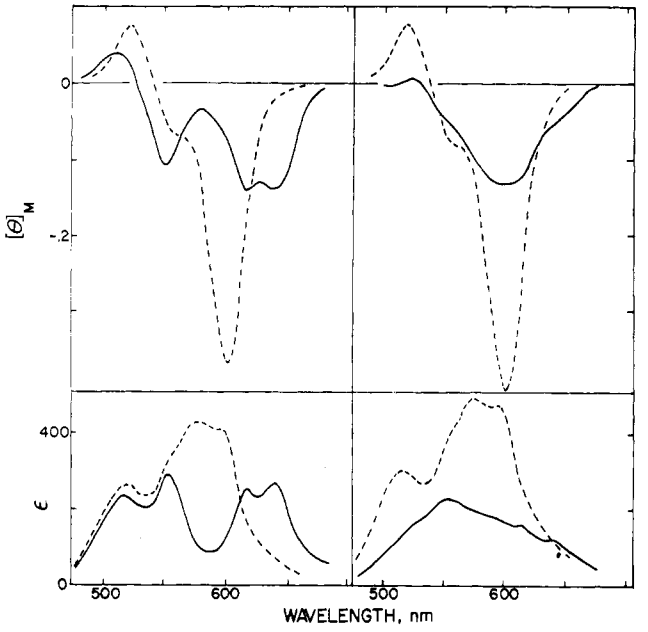

FIGURE 4: Effect of pH and acetazolamide on the MCD spectra (above) and absorption spectra (below) of bovine cobalt carbonic anhydrase. (Left) pH 8.95 in the absence (—) and presence (- - -) of 1.5 equiv of acetazolamide; 2.4×10^{-4} *M* enzyme in 10 m*M* Mes buffer. (Right) pH 5.9 in the absence (—) and presence (- - -) of 1.5 equiv of acetazolamide; 2.5×10^{-4} *M* enzyme in 0.01 *M* Mes buffer. MCD spectra are all measured at 40 kG.

tetrahedral Co(II) complexes such as $Co(OH)_4^{2-}$ are more intense and exhibit an MCD spectrum consisting of an intense negative band near ~600 nm, with values of $[\theta]_M$ ranging from −0.02 to −3.0 and a weaker, positive band at shorter wavelength, ~530 nm. The visible absorption spectrum of pentacoordinate Co(II), e.g., $Co(Me_6tren)Br_2$, is composed of two structured bands at wavelengths corresponding to two major negative MCD bands maximal near 600 and 500 nm. Complexes with this coordination geometry exhibit molar magnetic ellipticities varying from -6×10^{-2} to -2×10^{-1}. In some pentacoordinate Co(II) complexes an additional, weak positive MCD band may be observed at shorter wavelengths.

The MCD spectra of cobalt carboxypeptidase (Latt and Vallee, 1971) and cobalt procarboxypeptidase, Figure 2 (Behnke and Vallee, 1972), are very similar, each characterized by a pronounced, negative and a smaller, positive MCD band. The slowly hydrolyzed substrate of cobalt carboxypeptidase, Gly-L-Tyr, is known to alter the CD spectrum and its sign significantly (Latt and Vallee, 1971). Binding of this dipeptide gives rise to a small hypsochromic shift both in the absorption and MCD spectrum (Figure 2). However, it neither alters the overall shape nor the sign of the MCD signal.

The MCD and absorption spectra of cobalt thermolysin (Figure 3) closely resembles those of cobalt carboxypeptidase and that of its zymogen with maxima at similar wavelengths. Both the MCD and absorption spectra increase in intensity upon addition of either β-phenylpropionyl-L-phenylalanine or carbobenzoxy-L-phenylalanyl-L-leucine, competitive inhibitors of the enzyme (Figure 3).

The MCD spectrum of Co(II) substituted bovine carbonic anhydrase is considerably more complex than those of the proteins considered thus far, being highly sensitive to inhibitors and pH. In slightly acidic solutions, pH 5.8, a negative and a smaller positive band are observed (Figure 4), resembling the MCD spectra of cobalt carboxypeptidase, cobalt procarboxypeptidase, and cobalt thermolysin, whereas at pH 8 and above the spectrum is more complex consisting of a small positive and three negative bands (Figure 4). These MCD spectra correlate with and reflect the acidic and alkaline absorption spectra described by Lindskog (1963). Addition of the potent inhibitor, acetazolamide, to the alkaline form of cobalt carbonic anhydrase transforms and markedly simplifies the MCD spectrum (Figure 4). Inhibitors, *p*-carboxybenzenesulfonamide, *p*-nitrobenzenesulfonamide, and sulfanilamide, perturb the MCD spectrum in a closely similar manner. The resultant spectra resemble those of the acid pH form of this protein as well as those of cobalt carboxypeptidase, procarboxypeptidase, and of cobalt thermolysin (Figures 2 and 3).

The MCD and absorption spectra of various forms of cobalt alkaline phosphatase are shown in Figure 5. Co_2-phosphatase, reconstituted from apoalkaline phosphatase and 2 g-atoms of Co^{2+}/mol, exhibits very low enzymatic activity (Simpson and Vallee, 1968). Both the absorption and MCD spectra are weak, each consisting of a slightly structured envelope centered near 520 nm. The absorption and MCD spectra of Co_4-phosphatase—the functionally active enzyme which contains four cobalt atoms—is considerably more complex and intense than the enzyme containing two cobalt atoms. The absorption spectrum reveals at least four peaks corresponding to a single positive and several negative MCD bands. Addition of arsenate or phenyl phosphonate to Co_4-phosphatase markedly enhances both the absorption and MCD spectra. In the case of phenyl phosphonate, the MCD spectrum closely resembles that of 4-coordinate Co(II) complexes. Addition of phosphate, the simplest substrate of the enzyme, also markedly alters the absorption and MCD spectra which now exhibit three negative bands (Figure 5).

The relevant spectral and MCD parameters have been obtained by a curve fitting procedure using eq 1-4 and are listed in Table I. The accuracy obtained and the assump-

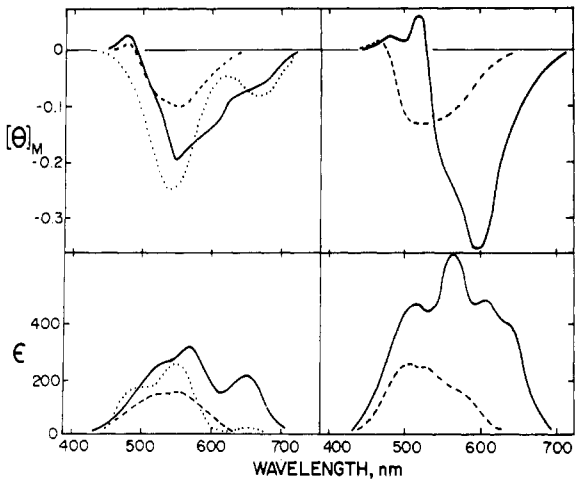

FIGURE 5: MCD spectra (above) and absorption spectra (below) of cobalt substituted alkaline phosphatases. (Left) Co_2-phosphatase (- - -); Co_4-phosphatase (—), and Co_4-phosphatase in the presence of phosphate, 1 mM (· · ·). Enzyme concentration is 4.5×10^{-4} M in 10 mM Tris (pH 8.0). (Right) Co_4-phosphatase (4.5×10^{-4} M) at pH 8.0, 10 mM Tris, in the presence of arsenate, 1 mM (- - -), or phenyl phosphonate, 1 mM (—).

tions used in applying these procedures have been discussed previously (Kaden et al., 1974). In Figures 2 and 3 examples of the calculated spectra and their fit to the experimental data obtained in the curve fitting procedures are shown as the dotted lines adjacent to the experimental curves.

On the basis of the structure of their MCD spectra and the associated moments, the Co(II) substituted enzymes examined can be divided into three groups. (1) Cobalt carbonic anhydrase at pH 8.95 and Co_4-phosphatase exhibit at least two major negative bands whose wavelength maxima and moments are very similar to those of the five-coordinate models. (2) Cobalt carboxypeptidase and procarboxypeptidase in the absence and presence of Gly-L-Tyr, cobalt thermolysin with and without the inhibitor, β-phenylpropionyl-L-phenylalanine, cobalt carbonic anhydrase in the presence of acetazolamide and other sulfonamide inhibitors or at low pH, and Co_4-phosphatase in the presence of arsenate or phenyl phosphonate all exhibit one major transition and closely resemble tetrahedral models. (3) Co_4-phosphatase in the presence of phosphate, the simplest substrate of the enzyme, is unique so far. Three major negative bands are required to fit the experimental results.

Discussion

The absorption spectra of Co(II) substituted metalloenzymes have been studied extensively to discern the geometry of Co(II)- and pari passu Zn(II) coordination and the presumable geometry of its ligands (Vallee and Wacker, 1970). However, assignment of specific coordination geometries from absorption and CD spectra alone has been difficult. Magnetic circular dichroism in conjunction with absorption spectra are proving very effective in approximating the overall coordination geometries of Co(II) complex ions more accurately, X-ray diffraction analysis serving as the primary standard of reference (Kaden et al., 1972). Such studies suggested an analogous approach to the examination of Co(II) substituted enzymes by MCD.

Magnetically induced optical activity results from the interaction of the magnetic field with degenerate energy levels and from the mixing of energy levels in the presence of the field. Due to this interaction, a given transition absorbs left and right circular polarized light unequally when examined in a magnetic field, giving rise to magnetic circular dichroism. Though natural and magnetic CD are measured experimentally by nearly identical means, the information derived differs fundamentally. Natural optical activity reflects inherent molecular asymmetry of the metal environment in metal complex ions, whereas the Faraday effect signals the nature of the energy levels underlying the observed transition and the overall coordination of the metal chromophore. Importantly, the magnetic field can render optically inactive chromophores optically active and the resultant signal provides information regarding the coordination geometry of such metal complex ions.

Assuming gaussian band shapes, well-resolved magnetic circular dichroic spectra can be separated into A, B, and C terms which serve to characterize the observed transitions (Schatz and McCaffery, 1969). The A term is biphasic and results from the Zeeman splitting of a degenerate excited state. The B term arises from the mixing of energy levels which have a nonzero magnetic dipole transition moment; it is either positive or negative having its maximum intensity at the absorption maximum of frequency ν. The C term originates from lifting the degeneracy in the ground state

Table I

	ν_0 (cm^{-1})	$\langle\epsilon\rangle_0 \times 10^{-1}$	$-\langle[\theta]_M\rangle_0/\langle\epsilon\rangle_0$ ($\times 10^4$, β cm)	$-\langle[\theta]_M\rangle_1/\langle\beta\epsilon\rangle_0$
Cobalt procarboxypeptidase	18,800	3.0	4.6	7.7
Cobalt carboxypeptidase	18,500	3.5	3.6	7.2
Cobalt carboxypeptidase + Gly-L-Tyr	18,900	3.0	3.8	6.5
Cobalt thermolysin	18,900	2.0	4.5	7.9
Cobalt thermolysin + β-phenylpropionyl-L-phenylalanine	18,500	3.1	7.2	12.3
Cobalt carbonic anhydrase (pH 5.85)	17,500	1.9	8.5	14.3
Cobalt carbonic anhydrase (pH 8.95)	16,000	2.6	11.7	18.7
	19,100	5.5	0.9	8.75
Cobalt carbonic anhydrase + acetazolamide (pH 8)	18,300	9.6	3.5	5.8
Co_2-phosphatase	18,800	3.7	4.1	7.6
Co_4-phosphatase	15,700	1.5	4.8	7.6
	18,600	6.3	4.3	7.6
Co_4-phosphatase + arsenate	19,200	5.2	4.6	8.6
Co_4-phosphatase + phenyl phosphonate	18,100	16.8	2.6	4.3
Co_4-phosphatase + phosphate	15,700	1.9	3.0	4.7
	19,300	4.3	9.2	17.3

by the magnetic field; the Boltzmann distribution of the electrons populating the resulting energy levels produces differential absorption in the left and right circularly polarized light beams. Both B and C terms are gaussian shaped but the C term is temperature dependent while the B term is not, allowing for their experimental differentiation.

As indicated earlier, complexity of the Co(II) spectra precludes separation of the MCD spectra into individual transitions and thus assignment of term type. Consequently use was made of the method of moments to analyze the various spectra analogously to that employed in the study of the MCD of Co(II) model complexes, in order to permit direct comparisons.

Examination by MCD of a series of octahedral, tetrahedral, and pentacoordinate Co(II) complexes allows an inference regarding overall geometries, which has proven more difficult by other spectral methods. Spectra characteristic for the three common coordination numbers of Co(II) show that the octahedral complexes, e.g., $Co(H_2O)_6^{2+}$, exhibit a single major negative MCD band of low intensity centered at the wavelength of their absorption maxima, ~500 nm. Tetrahedral complexes, e.g., $Co(OH)_4^{2-}$, all display MCD spectra consisting of a strong negative and a weak positive band. Five-coordinate complexes, e.g., $Co(Me_6Tren)Br_2$, are more complex and consist of several negative and, in some instances, a small positive band. In addition to the identification of the various coordination numbers by their MCD band shape, signs, and positions, the values of moments discriminate between six coordinate and four or five coordinate Co(II). The former generally have greater first moments than the latter, and thus allow a more complete comparison of the metalloenzyme spectra with those of Co(II) complex ions which have proven to be relatively insensitive to distortions of the coordination sphere of the metal ion.

Cobalt Substituted Procarboxypeptidase, Carboxypeptidase, and Thermolysin. Sequence analysis (Bradshaw et al., 1969) together with spectral (Latt and Vallee, 1971) and X-ray structure studies (Lipscomb et al., 1968) of native carboxypeptidase A have shown that the active site zinc is located in an irregular, tetrahedral environment coordinated to imidazole nitrogens of His residues 69 and 196, and to the carboxyl group of Glu-72. The fourth coordination site is thought to be occupied either by a water molecule or perhaps a hydroxide ion (Lipscomb et al., 1968). The zinc atom at the active site of crystalline thermolysin has recently been found to be in a similar coordination geometry with ligands identical with those of carboxypeptidase, i.e., His-142, His-146, Glu-166 and a water or hydroxide ion forming a distorted tetrahedron (Titani et al., 1972; Colman et al., 1972). Detailed knowledge of the coordination geometry of the metal in these two enzymes presents an exceptional opportunity to compare the results of spectral studies with those of X-ray structure analysis.

Spectral data of cobalt carboxypeptidase (Latt and Vallee, 1972; Vallee and Latt, 1970) led to the conclusion that the metal ion is situated in an irregular tetrahedral ligand field, consistent with interpretation of data from X-ray crystallography. Its MCD spectrum, consisting of a negative band at high wavelength, and a smaller, positive band at low wavelength, closely resembles those of tetrahedrally coordinated Co(II) complex ions (Figure 2) supporting this view. Addition of Gly-L-Tyr, a substrate of this enzyme with a low turnover number, shifts the MCD spectrum to but a small degree. The binding of peptide substrates, such as Gly-L-Tyr, to carboxypeptidase is thought to occur by coordination of the amide carbonyl oxygen to the active site zinc atom through displacement of either water or hydroxide, of the fourth coordination site (Vallee et al., 1963, 1970; Lipscomb et al., 1969). The MCD of cobalt carboxypeptidase is consistent with such a displacement and suggests that the metal coordination number remains unchanged; the MCD retains the characteristics of overall tetrahedral geometry.

The absorption and MCD spectra cobalt carboxypeptidase and procarboxypeptidase (Figure 2) are virtually identical based on the shape of the signals, their intensities, and the values of their moments. The inference of similar geometries of Co(II) in the zymogen and the enzyme, based on these spectral properties, led to the prediction that procarboxypeptidase would exhibit peptidase activity, as verified subsequently (Behnke and Vallee, 1972).

The substitution of Co(II) for Zn(II) at the active site of thermolysin doubles enzymatic activity (Holmquist, 1970) and generates characteristic absorption bands in the visible region (Holmquist and Vallee, 1974). Although of lower absolute intensity, the MCD spectrum of cobalt thermolysin (Figure 3) resembles those of cobalt procarboxypeptidase and cobalt carboxypeptidase. The near identity of the zeroth and first moments of thermolysin to those of carboxypeptidase and procarboxypeptidase is consistent with similar active site geometries and ligands in each of these three proteins. Preliminary spectral studies suggested tetrahedral coordination of Co(II) in thermolysin (Vallee and Latt, 1970; Kaden et al., 1972) as confirmed subsequently by X-ray structure analysis (Colman et al., 1972). Addition of the inhibitors β-phenylpropionyl-L-phenylalanine (Figure 3) or Cbz-L-phenylalanyl-L-leucine increases the intensity but does not significantly change the pattern of the MCD signal. Thus, peptide inhibitors or substrates may displace and substitute for one of the ligands of the active site cobalt of carboxypeptidase and thermolysin but without inducing a change in the coordination number of the metal ion.

Both X-ray crystallography and the MCD spectrum indicate tetracoordination of the active site metal of thermolysin, and the effects of inhibitors suggest distortion from regular geometry. This is apparent both from the intensification of the MCD and absorption spectra and from the marked decrease of the intensity of the CD spectrum in the presence of inhibitors (Holmquist and Vallee, 1974) indicative of a loss in asymmetry about the cobalt atom. Similar distortion has been described and analyzed earlier in detail for cobalt carboxypeptidase (Latt and Vallee, 1971).

Cobalt Carbonic Anhydrase. At pH 5.8, the MCD spectrum of cobalt carbonic anhydrase (Figure 4) is quite analogous to those of tetrahedral Co(II) complex ions (Kaden et al., 1974) such as $Co(OH)_4^{2-}$, and to the tetrahedral enzymes already discussed. However, the MCD spectrum generated at alkaline pH differs from all of these and more nearly resembles that of pentacoordinate Co(II) complexes suggesting a pH-dependent change in cobalt coordination geometry. Remarkably, addition of inhibitors such as acetazolamide, sulfanilamide, *p*-carboxybenzenesulfonamide, and *p*-nitrobenzenesulfonamide to the alkaline form of the enzyme modifies the MCD spectrum to resemble the acid form and the tetrahedral Co(II) complexes. The geometry of Co(II) in the alkaline form of carbonic anhydrase has been thought to be tetrahedral (Coleman and Coleman, 1972) or pentacoordinated (Dennard and Williams, 1966). X-Ray crystallographic analysis of one form of carbonic

anhydrase C, at pH 8, finds zinc bound to three histidyl residues, i.e., 93, 95, and 117, and a water or hydroxyl ion in a highly distorted tetrahedron which is not altered significantly by a sulfonamide inhibitor binding to the zinc atom (Kannan et al., 1972).

The alkaline form of cobalt carbonic anhydrase most closely resembles the five-coordinate complexes, Co(Et$_4$dien)Cl$_2$, Co(Me$_6$tren)Br$_2$, Co(terpy)Cl$_2$, and Co(POAH$_{-1}$)$_2$ suggesting the existence of pentacoordination at high pH (Kaden et al., 1972). Thus, while it appears that at acid pH values and at both acid and alkaline pH in the presence of sulfonamide inhibitors the coordination about the active site cobalt atom is four coordinate, the exact nature of the coordination geometry and number in solution at alkaline pH remains in question. It must be remembered that the five-coordinate cobalt model compounds differ from the cobalt binding site(s) of the enzyme both with regard to the ligand atoms and the microscopic detail of their interactions with the metal. The change in coordination number as a function of the active site inhibitor complexes suggested by the MCD spectra would require considerable flexibility of the metal ligands and of adjacent residues. An apparent shift in coordination also occurs when cobalt carbonic anhydrase solutions, inhibited by cyanide, are frozen resulting in both the formation of five-coordinate geometry as well as a low spin Co(II) dicyanide complex (Haffner and Coleman, 1973).

Co$_2$ and Co$_4$ Substituted Alkaline Phosphatases. Addition of 2 equiv of Co(II) to *E. coli* apoalkaline phosphatase results in a protein with an absorption spectrum of low intensity and an MCD spectrum resembling that of octahedral Co(II) complex ions (Figures 1 and 5) consistent with the results of electron paramagnetic resonance studies (Kennedy et al., 1972). Addition of 2 further equiv of Co^{2+} yields an active enzyme which exhibits an intense, complex, absorption spectrum (Simpson and Vallee, 1968) (Figure 5). The origins of these spectra have been related to these two sets of Co(II) atoms, though their interrelationships are not as yet entirely clear. However, while not identical, the MCD spectrum of Co$_4$-phosphatase resembles those observed for pentacoordinated Co(II) complexes. Addition of arsenate or phenyl phosphonate alters the MCD spectrum to one characteristic of Co(II) complex ions of known tetrahedral geometry. The MCD spectrum of the enzyme in the presence of inorganic phosphate, however, differs from that of any of the model compounds studied so far, though the basis for this difference is unknown. Yet, in alkaline phosphatase as in carbonic anhydrase, binding of inhibitor and/or substrate, at or near the active site metal, transforms their MCD spectra into one closely resembling tetrahedral geometry.

While the interpretation of absorption spectra of Co(II) complexes and Co(II) enzymes has often proven difficult, MCD spectra, being less sensitive to distortions of the coordination sphere of the metal ion, would appear especially useful in predicting the overall geometry of active Co(II) substituted metalloenzymes and their susceptibility to alterations in coordination number upon binding or catalysis. In addition to qualitative comparisons of band shapes and signs of the MCD spectra, the values of the Faraday parameters reveal distinct similarities. Thus far the tetrahedral-like Co(II) enzymes and tetrahedral Co(II) models are characterized by values for the zeroth moment near $-3.5 \times 10^{-4}\ \beta$ cm. It is doubtful that this could reflect the chemical nature of the ligands which can differ greatly between the Co(II) enzymes and cobalt complexes.

Work at different temperatures will be required to differentiate the various components of the Faraday effect and thereby to detail further relationships between the various MCD spectra and the information available relative to metal coordination. However, variations of temperature on the order of 100° are required to adequately differentiate B from C terms, necessitating solvents other than water. Further efforts are needed to establish conditions suitable for this purpose.

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Circular Dichroism Changes in Galactosyltransferase upon Substrate Binding†

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ABSTRACT: Circular dichroism studies with the galactosyltransferase isolated from bovine skim milk are described. Addition of UDP-galactose to the galactosyltransferase-Mn^{2+} complex causes a decrease in the negative mean residue ellipticity in the 205–220-nm range and positive increases in the 265- and 275–290-nm ellipticity. These data are consistent with the view that a conformation change involving aromatic amino acid residues occurs upon the binding of UDP-galactose to the galactosyltransferase-Mn^{2+} complex. No effects in the near-ultraviolet circular dichroism spectrum were observed upon the addition of UDP or glucose to the galactosyltransferase-Mn^{2+} complex.

Bovine skim milk galactosyltransferase (UDPgalactose: D-glucose 1-galactosyltransferase, EC 2.4.1.22) catalyzes the transfer of galactose from UDP-galactose to form β-1,4 linkages with either glucose (Fitzgerald et al., 1970a) or *N*-acetylglucosamine (Brew et al., 1968). α-Lactalbumin inhibits the reaction with *N*-acetylglucosamine but is required to obtain significant rates with glucose (Fitzgerald et al., 1970a). Magee and Ebner (1974) have shown that UDP-galactose and other uridine nucleotides give protection of a critical sulfhydryl residue in galactosyltransferase when Mn^{2+} is present. These same compounds also protect to varying degrees against inactivation of galactosyltransferase by trypsin (Magee and Ebner, 1974). These data indicated the possibility of a conformational change in the galactosyltransferase-Mn^{2+} complex upon addition of UDP-galactose.

Circular dichroism measurements have been used (D'Souza and Freisheim, 1972; Teichberg et al., 1970; Fretto and Strickland, 1971; Omenn et al., 1969) to investigate conformational changes in enzymes upon addition of substrates and/or inhibitors. Circular dichroism measurements were made on galactosyltransferase in the presence and absence of the substrates, UDP-galactose and glucose, and the product, UDP. The results showed that there was a change in the circular dichroism spectra only upon the addition of UDP-galactose.

Experimental Procedure

Tris, 2-mercaptoethanol, uridine diphosphate, and glucose were from Sigma; $MnCl_2$ and KCl were from J. T. Baker; UDP-galactose was from Calbiochem; and dioxane was from Fisher.

Galactosyltransferase was isolated from bovine milk by the procedure described by Magee et al. (1973). The specific activity of the enzyme as assayed by the method of Fitzgerald et al. (1970b) was 5.1 units/mg of protein. Protein was estimated by absorbance at 280 nm assuming an extinction coefficient of 1.0 for 1 mg ml^{-1} cm^{-1}.

Circular dichroism spectra were measured at 24° in a Cary Model 61 spectropolarimeter; 2-cm path-length cuvets were used in obtaining near-ultraviolet (250–310 nm) circular dichroism spectra while 1-mm cuvets were used for far-ultraviolet (200–250 nm) spectra. The buffer for all determinations was 10 m*M* Tris–100 m*M* KCl–0.5 m*M* 2-mercaptoethanol adjusted to pH 7.5. The mean residue weight of galactosyltransferase, 113, was calculated from the amino acid analysis of Trayer and Hill (1971). All spectra of galactosyltransferase represent the difference between solutions of enzyme, buffer, and added substrates and solutions of buffer and added substrates.

Results

No change is apparent in the far-ultraviolet circular di-

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