Ligand Perturbation Effects on a Pseudotetrahedral Co(II)(His)$_3$-Ligand Site

A MAGNETIC CIRCULAR DICHROISM STUDY OF THE Co(II)-SUBSTITUTED INSULIN HEXAMER*

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Magnetic circular dichroism (MCD) spectra of a series of adducts formed by the Co(II)-substituted R-state insulin hexamer are reported. The His-B10 residues in this hexamer form tris imidazole chelates in which pseudotetrahedral Co(II) centers are completed by an exogenous fourth ligand. This study investigates how the MCD signatures of the Co(II) center in this unit are influenced by the chemical and steric characteristics of the fourth ligand. The spectra obtained for the adducts formed with halides, pseudohalides, trichloroacetate, nitrate, imidazole, and 1-methylimidazole appear to be representative of near tetrahedral Co(II) geometries. With bulkier aromatic ligands, more structured spectra indicative of highly distorted Co(II) geometries are obtained. The MCD spectrum of the phenolate adduct is very similar to those of Co(II)-carbonic anhydrase (alkaline form) and Co(II)-β-lactamase. The MCD spectrum of the Co(II)-R$_6$-CN adduct is very similar to the CN adduct of Co(II)-carbonic anhydrase. The close similarity of the Co(II)-R$_6$-pentafluorophenolate and Co(II)-R$_{a}$-phenolate spectra demonstrates that the Co(II)-carbonic anhydrase-like spectral profile is preserved despite a substantial perturbation in the electron withdrawing nature of the coordinated phenolate oxygen atom. We conclude that this type of spectrum must arise from a specific Co(II) coordination geometry common to each of the Co(II) sites in the Co(II)-R$_6$-phenolate, Co(II)-R$_6$-pentafluorophenolate, Co(II)-β-lactamase, and the alkaline Co(II)-carbonic anhydrase species. These spectroscopic results are consistent with a trigonally distorted tetrahedral Co(II) geometry ($C_{3v}$), an interpretation supported by the pseudotetrahedral Zn(II)-(His)$_3$(phenolate) center identified in a Zn(II)-R$_6$ crystal structure (Smith, G. D., and Dodson, G. G. (1992) Biopolymers 32, 441–445).

Several structural features frequently characterize the catalytic sites of zinc enzymes. These features comprise a distorted tetrahedral geometry incorporating one or more histidine groups and a coordinated “activated” water molecule oriented out into a cavity or cleft which forms the substrate binding site on the enzyme surface (1, 2). The R-state$^1$ insulin hexamer represents a well characterized biomolecular analog of this type of coordination unit (3, 4). The metal chelate site in the R-state insulin hexamer comprises a pseudotetrahedral array of three histidines and one exogenous small molecule ligand as shown in Fig. 1. The tris imidazole-Zn(II) motif occurs at the active site of several structurally characterized zinc metalloenzymes, including the carbonic anhydrases (Zn(His)$_3$(H$_2$O), β-lactamase (Zn(His)$_3$S-lys), astacin (Zn(His)$_3$(Tyr)(H$_2$O)), α-carboxypeptidase (Zn(His)$_3$(H$_2$O)), and the metzincins (5–9). The analysis of spectroscopically congruent transition metal-substituted metalloprotein derivatives has provided revealing insights into the active site structure and mechanistic details of many functionally diverse metalloproteins (1, 10–12). Despite the importance of such derivatives and their small molecule synthetic analogs, to our knowledge, there are no well characterized examples of small molecule complexes that stabilize pseudotetrahedral tris imidazole coordination with Zn(II) as well as with the chromophoric metal ion probes Co(II) and Cu(II).

The R-state insulin hexamer is one system that has been shown to stabilize pseudotetrahedral M(His)$_3$L (M = Zn(II), Co(II), Cu(II)) coordination in which the fourth ligand site may be occupied by a wide range of exogenous small molecule ligands (L) (3). This hexamer thus represents a unique and highly pertinent spectrochemical model system for the investigation of the structural and spectroscopic interrelationships of this type of metal center. A major difficulty with biological chromophores is the fact that metalloproteins usually impose low symmetry ligation at the active sites which result in Co(II) derivatives that are difficult to model both theoretically and with small molecule Co(II) complexes. Furthermore, the high spin Co(II) ion in a low symmetry environment gives rise to a particularly complex splitting of the ligand field bands due to the low symmetry geometry in concert with several different electronic effects (13). The difficulty in accounting for the ligand field components observed experimentally has highlighted the importance of Co(II) model systems that incorporate biologically relevant ligands into nonclassical chelate geometries that may be perturbed in a systematic manner.

1 The abbreviations used are: T and R, designate insulin forms, respectively, with extended (T) and α-helical (R) conformations for residues 1–8 of the B-chain. The Trr Rrr and R$^3$ designations (15) correspond to three crystallographically identified forms of the insulin hexamer. Co(II)-R$_6$, the R$_6$ insulin hexamer with Co(II) substituted for Zn(II) at the His-B10 site; CA, carbonic anhydrase; Co(II)-carbonic anhydrase, the enzyme with Co(II) substituted for Zn(II) at the active site; M, metal ion bound to the His-B10 site of the insulin hexamer; L, small molecule ligands; PFP, pentafluorophenolate; MCD, magnetic circular dichroism; sh, shoulder; LMCT, ligand to metal charge transfer; T, tesla.

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The insulin hexamer consists of three dimeric units associated about a 3-fold symmetry axis thus forming a torus-shaped molecule (14). The hexamer has been shown to exhibit allosteric ligand binding characteristics that involve two classes of sites as follows: (i) the metal ion at the His-B10 site to which anionic ligands coordinate and (ii) six hydrophobic pockets formed between subunits to which neutral phenolics bind (Fig. 1). Three conformationally distinct allosteric forms of the zinc insulin hexamer have been characterized crystallographically (4, 19–21). These forms have been designated as $T_6$, $T_3R_3$, and $R_6$ where the $T$-state refers to a subunit conformation in which the $B$-chain residues B1–B9 exist in an extended conformation, whereas in the R-state these residues adopt an α-helical conformation (15). The hexamers bind Zn(II) ions at two sites; each site is formed by a set of three symmetry-related His-B10 residues. In the $T_6$ structure (19) and in the $T_3$ half of the $T_3R_3$ structure (20), the $T$-state subunits form an octahedral Zn(II) site comprising the three His-B10 nitrogens and three water molecules. In the $R_6$ structure (4, 21) and in the $R_3$ half of the $T_3R_3$ structure, the three His-B10 nitrogens form a pseudotetrahedral (C$_p$) Zn(II) site completed by a fourth ligand from solution (Fig. 1A). The three symmetry equivalent B1–B10 α-helices form a cylindrical channel to the metal ion along the central 3-fold axis of the $R_6$ hexamer.

Ligand binding studies of Co(II)-insulin hexamers in solution have established that saturating concentrations of certain phe- nolic compounds, in conjunction with the heterotropic effects of anionic ligands that bind to the Co(II) sites, displace the equil- ibrium strongly in favor of the R$_6$ conformation (16, 22). As part of our continuing studies to characterize the spectrochem- ical properties of transition metal-substituted insulin hexamers and the structures of the R-state species, we have measured the MCD induced in the visible absorption bands of a series of Co(II)-R$_6$ hexamer complexes.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The chemicals used in these studies were reagent grade or better and were used as supplied. Metal-free human insulin was supplied by the Novo Research Institute (Denmark).

**Instrumentation**—MCD spectra were measured with an instrument based on a Cary 14 double monochromator, a MgF$_2$ polarizer (Karl Lambrecht), a CaF$_2$ modulator (Hinds International), and a lock-in amplifier (SR 510, Stanford Research Systems) under the control of a PC-AT microcomputer. This instrumentation was used in conjunction with a superconducting magnet (Spectromag III, Oxford Instruments) producing a magnetic flux density of 4 T ($1 \text{T} = 10,000 \text{G}$) or an electromagnet producing a magnetic flux density of 1.2 T. Quartz cells with path lengths of 0.1 and 0.8 cm were used with the 1.2- and 4-T magnets, respectively. The MCD spectra have been corrected for natural optical activity. Electronic absorption spectra were recorded on a Perkin-Elmer l-17 spectrophotometer. All measurements were conducted at room temperature. MCD is reported as the molar magnetic ellipticity, $[\theta]_M$, and is expressed in units of degrees cm$^2$ dmol$^{-1}$ G$^{-1}$. The SI unit is m$^2$ mol$^{-1}$ T$^{-1}$; 1 deg cm$^2$ dmol$^{-1}$ G$^{-1} = 4 \pi \text{ m}^2 \text{ (18 m10)}^{-1}$ mol$^{-1}$ T$^{-1}$.

**RESULTS**

**Effects of the Exogenous Ligand on the MCD Spectrum, Phenolate Anions**—Previous work (22) has shown that the spectral profiles of the electronic transitions in the visible region are very sensitive to the nature of the small molecule ligand coordi- nated to the Co(II) ion of R$_6$ adducts. The MCD and electronic absorption spectra of the Co(II)-R$_6$-phenolate complex are presented in Fig. 2, A and B. The absorption spectrum exhibits four bands located respectively at 508, 543, 622, and 641(sh) nm. The MCD induced by a 4 T magnetic field resolves these features as a positive band at 498 nm accompanied by negative bands at 538, 613, and 629(sh) nm. An additional small positive band occurs at 465 nm. In view of the special significance of this spectrum as a spectroscopic model for the hydroxide ion adduct of Co(II)-substituted carbonic anhydrase (Co(II)-CA), the 4 T magnet was used in an attempt to identify possible fine structure in the MCD bands. However, the MCD induced by the 4 T magnetic induction did not resolve any additional spectral features when compared with the 1.2 T experiment. The remaining MCD spectra reported herein were recorded with a 1.2 T magnetic flux density. The spectrum of the Co(II)-R$_6$-pentafluorophenolate complex (Fig. 2, C and D) displays profiles analogous to those of the Co(II)-R$_6$-phenolate complex. In each case, the absorption spectra comprise envelopes split into two high energy bands and two lower energy bands. In the electronic absorption spectrum of the Co(II)-R$_6$-pentafluorophenolate complex, bands occur at 524(sh), 543, 606 and 629(sh) nm. These are distinguishable in the MCD spectrum as a positive band at 503 nm and negative bands at 538, 595 nm, and 621(sh) nm with an additional small positive band at 465 nm. The structure and steric bulk of the phenol and pentafluoro- phenol molecules are very similar; therefore, it can be reasoned that the differences in the spectra of Fig. 2, A and B and C and D, originate from the different donor properties of the respective coordinated O atoms.

**Halides and Pseudohalides**—The absorption spectrum of the Co(II)-R$_6$-CN$^-$ adduct (Fig. 3, A and B) is clearly quite different from that of the phenolic and pentafluorophenolate adducts. It is narrower, more intense, and exhibits only two absorption features. The MCD spectrum resolves these features into a positive band at 529 nm and a negative band at 575 nm. The qualitative appearance of this spectrum exemplifies many of the adducts reported in Table I for which the actual spectra are not shown (viz. imidazole, 1-methylimidazole, chloride, bro-
The Co(II) geometry in this complex is distorted tetrahedral with two additional long Co(II)-O bonds. The electronic structure in metalloproteins for which Co(II)-substituted derivatives can be prepared. Octahedral Co(II) complexes possess weakly intense bands at approximately 500 nm (€ > 100 M$^{-1}$ cm$^{-1}$), whereas the visible absorption bands of tetrahedral complexes occur at lower energy and are considerably more intense (€ > 300 M$^{-1}$ cm$^{-1}$). The spectral features of five-coordinate Co(II) centers are less diagnostic but are generally observed to be less intense than those of tetrahedral complexes (150 < € < 300 M$^{-1}$ cm$^{-1}$). Three ligand field transitions are expected for Co(II) ions possessing tetrahedral symmetry. The lowest energy transition, $^{4}T_{2}(F) \rightarrow {^{4}A_{2}}$, occurs in the 3000–5000 cm$^{-1}$ region and
is usually not observed. The second transition, $^4T_1(F) \leftrightarrow ^4A_2$, occurs in the near-infrared region, and the third transition, $^4T_1(P) \leftrightarrow ^4A_2$, occurs in the visible region, 715–500 nm. Our MCD experiments were restricted to the visible spectral region incorporating the $^4T_1(P) \leftrightarrow ^4A_2$ transition. In distorted tetrahedral environments, the low symmetry splitting of the $^4T_1(P)$ level results in a splitting of the $^4T_1(P) \leftrightarrow ^4A_2$ absorption envelope that provides a qualitative indication of the deviation from tetrahedral symmetry. Detailed interpretations of Co(II) electronic spectra are complicated by the effects of the low symmetry ligand field in addition to spin-orbit coupling, Jahn-Teller effects, and the possibility of spin forbidden transitions. Definitive correlations of the electronic and geometric structures of Co(II) chromophores require single crystal spectral measurements to be made directly on crystallographically defined Co(II) sites. For metalloproteins, such studies are inconveniently labor intensive and are generally technically infeasible. Consequently, much emphasis has been placed on making qualitative comparisons with small molecule Co(II) complexes, particularly those containing biologically relevant ligands such as thiolates, carboxylates, and imidazoles. The value of the Co(II)-R$_6$ hexamer as a spectroscopic model arises because (i) pseudotetrahedral Co(II)His$_3$L systems are rare, (ii) the fourth exogenous ligand, L, may be varied extensively, and (iii) several crystal structures have been reported defining the pseudotetrahedral Zn(II)His$_3$L sites of Zn(II)-R$_6$ or Zn(II)-T$_3$R$_3$ hexamer complexes (4, 20, 21, 27–29).

Coordination Geometries Range from Near-tetrahedral to Highly Distorted Tetrahedral—The MCD spectra recorded for the Co(II)-R$_6$ complexes formed with halides and pseudohalides exhibit two major bands, one positive at higher energy and the other negative at lower energy. This type of spectrum is exemplified by that of the Co(II)-R$_6$-CN$_2$ adduct (Fig. 3, A and B) that corresponds to a Co(II) geometry close to regular tetrahedral symmetry. This conclusion is supported by the MCD spectra observed for structurally characterized tetrahedral Co(II) complexes, such as Co(py)$_2$Br$_4$ (where py is pyridine), [CoCl$_4$]$^{2-}$, and [Co(SCN)$_4$]$^{2-}$ (30). In contrast, the Co(II)-R$_6$ complexes formed with phenolate, pentafluorophenolate, and benzenethiolate possess spectra that exhibit three or four clearly resolved major bands, as shown in Fig. 2, A–D, and Fig. 3, G and H, respectively. These spectra may arise from Co(II) centers that possess coordination geometries appreciably distorted from tetrahedral. Several structurally characterized small molecule
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Table I

| Ligand                  | Absorbance maximum | MCD maxima | i         | ii        | $\langle\theta\rangle_{\text{max}}$ |
|------------------------|--------------------|------------|----------|----------|-------------------------------|
| Phenolate              | 622 (500)          | (+, +)     | 538 (−) | 613 (−)  | 629 (−)sh                      | 3,800 | 18,200 | −0.25 |
| Pentfluoro phenolate   | 606 (480)          | (+, +)     | 538 (−) | 595 (−)  | 621 (−)sh                      | 3,200 | 18,400 | −0.38 |
| Benzenthiole            | 610 (700)          | (+, +)     | 575 (−) | 617 (−)  | 568 (−)                        | 2,800 | 17,800 | −0.73 |
| Sorbate                | 535 (310)          | (+, +)     | 555 (−) | 578 (−)  | 578 (−)                        | 2,600 | 18,900 | −0.24 |
| Acetate                | 535 (310)          | (+, +)     | 532 (−) | 578 (−)  | 578 (−)                        | 2,400 | 18,600 | −0.46 |
| Trichloroacetate       | 546 (530)          | (+, +)     | 578 (−) | 578 (−)  | 578 (−)                        | 2,000 | 18,100 | −0.52 |
| Cyanoate               | 562 (590)          | (+, +)     | 556 (−) | 556 (−)  | 556 (−)                        | 2,000 | 18,200 | −0.59 |
| 1-Methyl imidazole     | 565 (500)          | (+, +)     | 556 (−) | 556 (−)  | 556 (−)                        | 1,900 | 19,000 | −0.29 |
| Nitrate                | 546 (590)          | (+, +)     | 556 (−) | 556 (−)  | 556 (−)                        | 1,800 | 18,500 | −0.59 |
| Imidazole              | 556 (500)          | (+, +)     | 572 (−) | 595 (−)  | 595 (−)                        | 1,800 | 17,700 | −0.67 |
| Azide                  | 581 (670)          | (+, +)     | 572 (−) | 572 (−)  | 572 (−)                        | 1,800 | 17,200 | −1.18 |
| Iodide                 | 617 (700)          | (+, +)     | 572 (−) | 572 (−)  | 572 (−)                        | 1,700 | 17,500 | −1.03 |
| Bromide                | 595 (600)          | (+, +)     | 572 (−) | 595 (−)  | 595 (−)                        | 1,700 | 17,800 | −0.71 |
| Chloride               | 581 (610)          | (+, +)     | 572 (−) | 595 (−)  | 595 (−)                        | 1,500 | 18,100 | −0.95 |
| Cyanide                | 575 (820)          | (+, +)     | 575 (−) | 575 (−)  | 575 (−)                        | 1,500 | 18,100 | −0.95 |

*Sample showed a slight decrease in intensity during the data collection; the molar absorptivities and MCD magnitudes are therefore considered approximate values. sh, denotes shoulder.*

Complexes with distorted tetrahedral Co(II) centers that exhibit somewhat comparable electronic spectra have been reported (31, 32).

**Comparison of Co(II)-R6 with Co(II)-Carbonic Anhydrase—** The His-B10 metal chelate site in the R-state insulin hexamer bears some structural similarity to the active sites in several zinc enzymes. We have reported previously that the absorption spectra for many of the Co(II)-R6 complexes are remarkably similar to those of the corresponding adducts formed with Co(II)-carbonic anhydrase (33). The metal chelate site in CA consists of three histidine residues and is completed by one or two small molecule ligands (5). The spectroscopic and crystallographic data for Zn(II) and Co(II)-CA indicate that the metal center may accommodate expanded coordination numbers; thus pseudotetrahedral and pentacoordinate geometries are possible for Co(II)-CA (26, 34). In the absence of added anions at alkaline pH values, the absorption and MCD spectra of Co(II)-CA exhibit maxima at 518 nm (+), 556 nm (+), and 641 nm (−). Although the interpretation of Co(II)-CA spectra has been complicated by uncertainty regarding equilibria involving multiple forms, the exogenous coordinated anion in this species has been postulated to be a hydroxide ion. The distinctive appearance of the Co(II)-CA spectrum has fueled ongoing speculation that it signifies the mechanistically relevant structural motif of a key intermediate in the HCO₃⁻ dehydration pathway for Co(II)-CA (23, 35). The large splitting between the bands of the alkaline Co(II)-CA spectra has been interpreted as indicative of a trigonally distorted tetrahedral Co(II) geometry (23). This interpretation is supported by comparison of the MCD spectra of a series of five-coordinate Co(II)-CA adducts with that of alkaline Co(II)-CA (36). The highly similar electronic and MCD spectra observed for the Co(II)(NH₃)₅O(phenolate), Co(II)(NH₃)₅O(hydroxide), and Co(II)(NH₃)₅S(Cys) centers of the Co(II)-R₆ hexamer, alkaline Co(II)-CA, and Co(II)-β-lactamase, respectively, suggest that the distinctive nature of these spectra originate primarily from the effect of a specific Co(II) geometry. For the Co(II)-R₆-phenolate complex, the effect of increasing the electronegativity of the coordinated phenolate oxygen atom is demonstrated by examining the spectra of the Co(II)-R₆-pentfluoro phenolate adduct (Fig. 2, C and D). The more electron withdrawing pentfluoro phenolate oxygen results in electronic and MCD spectra that possess profiles substantially analogous to those of the Co(II)-R₆-phenolate spectra. The consequence of this large perturbation in the donor properties of the phenolate oxygen atom is manifested as relatively minimal changes in the splitting of the MCD spectral components and the transition energy (Table I). The observation that the Co(II)-CA-like electronic and MCD spectral profiles are preserved, in spite of this perturbation, confirms that these distinctive spectral features are related intimately to a specific type of Co(II) coordination geometry. The two low energy bands have been assigned to transitions to the doublet states $^2T_2(G)$ and $^2E(G)$ which can become enhanced by spin orbit coupling only. Via spin-orbit coupling, a trigonal distortion of the Co(II) geometry is expected to give rise to an increased mixing of these doublet states into the quartet states. It is noteworthy that the symmetry of the M(II) chelate site in the M(II)-R₆ hexamer is predisposed toward a trigonally distorted tetrahedral geometry because the cylindrical channel to the metal is expected to constrain a bulky fourth ligand, such as the phenolate group, to an orientation along the 3-fold symmetry axis of the hexamer. Such an arrangement is observed for the Zn(II)(His₅)⁻-phenolate center in the monoclinic Zn₃(II)-R₆ crystal structure (21).

The interaction of phenol with Zn(II)-CA and Co(II)-CA has been studied in detail because phenol is known to be a competitive inhibitor of the CO₂ hydration reaction catalyzed by CA. Phenol molecules have been shown to bind to CA; however, the phenolate anion does not displace the metal-bound hydroxide ligand of either Co(II)-CA or Zn(II)-CA, even at high pH values (37, 38). The phenolate-binding affinities of the metal centers are clearly very different for the M(II)-CA and M(II)-R₆ species even though our results show that the structures of the respective Co(II) centers must be extremely similar for at least some of the adducts, notably CN⁻. This observation suggests that the residues forming the outer coordination sphere must play a critical role in defining the coordinative selectivity of the active site metal ion in CA.

**Comparison of Co(II)-R₆ with Co(II)-β-Lactamase—** In β-lactamase, three histidines and a cysteine have been identified as ligands to the active site Zn²⁺ (8). The Co(II) derivative of β-lactamase exhibits absorption and MCD spectra that are remarkably similar to those of alkaline Co(II)-CA. However, there is some conflict regarding the interpretation of these spectral features as indicative of either a pentacoordinate Co(II)(His₅)(S-Cys)(H₂O) arrangement (11) or a distorted tetrahedral Co(II)(His₅)(S-Cys) arrangement (39). The putative coordinated water of the former arrangement has been postulated...
to play a key role in the mechanistic pathway of β-lactamase catalysis (11). In view of the strong spectral congruency between Co(II)-R₆, Co(II)-CA and β-lactamase, it is reasonable to infer that the spectra observed for Co(II)-β-lactamase are consistent with a distorted tetrahedral (Cₛₑₐₚₑ₄) Co(II)His₃(S-Cys) donor set. Although an activated water molecule may well play a role in catalysis, this spectral interpretation suggests that in the resting enzyme the putative water molecule does not interact with the Co(II) strongly enough to significantly influence the electronic structure of the Co(II) ion.

Thiolate Complexes Give MCD Spectra Similar to the Spectra of Co(II)-substituted Blue Copper Proteins—Co(II)-substituted blue copper proteins possess visible absorption spectra indicative of highly distorted tetrahedral Co(II) sites (40, 41). These spectra are in accord with the x-ray crystal structures of blue copper proteins which all identify low symmetry Cu(II) geometries comprising two histidines, a methionine, and a cysteine as ligands. The native blue copper proteins are characterized by small ESF hyperfine coupling constants and intense visible S(Cys) → Cu(II) charge transfer bands, features uncommon for Cu(II) chromophores (42). The Cu(II)-R₆ hexamer exhibits similar spectral features when a thiolate ligand occupies the fourth coordination site (43, 44). Furthermore, the visible absorption spectra of Co(II)-R₆ thiolate complexes are qualitatively very similar to those of Co(II)-substituted blue copper proteins. Because the spectral properties of blue copper proteins and the corresponding Co(II)-substituted derivatives have long been considered highly distinctive signatures of the active site stereochemistries and electronic structures, the spectroscopic analogy with the M(II)-insulin hexamer is indeed remarkable. The MCD spectrum of the Co(II)-R₆-benzenethiolate complex (Fig. 3H) extends this analogy. The MCD spectrum of the Co(II)-R₆-benzenethiolate complex appears to be very similar to those of Co(II)-substituted azurin, -plastocyanin, and -stel-lacyanin. The two transitions occurring in the UV region of the Co(II)-substituted blue copper proteins have been ascribed to LMCT transitions from the 3pπ and 3pσ orbitals of the cysteine thiolate sulfur to the Cu(II) d vacancy (40, 41). The seemingly analogous transitions in the MCD spectrum of the Co(II)-R₆-benzenethiolate complex are resolved at 427 and 377 nm, a separation of 3100 cm⁻¹. The splitting of the π and σ orbitals will depend on the symmetry of the ligand field and the donor properties of the ligating atoms. The value of 3100 cm⁻¹ may be compared with the values of 3600–4900 cm⁻¹ reported for the aforementioned Co(II)-substituted blue copper proteins (41).

Insulin Hexamer Conformations—The Co(II)-substitution experiment for insulin (45, 46) has become widely utilized as a convenient method for spectroscopically characterizing the conformational behavior and metal-binding characteristics of insulin and its derivatives in solution. Co(II)-insulin crystals are isomorphous to Zn(II)-insulin crystals, and consequently, the Co(II)-hexamer appears to represent a good chromophoric facsimile of its biologically relevant Zn(II) counterpart. Several crystal structures have shown that, in Zn(II) hexamers, R₆₃ trimers impose tetrahedral coordination upon the Zn(II) atom, whereas T₃₄ trimers are observed to coordinate Zn(II) in an octahedral arrangement (4, 19–21). These structures show that the coordination geometry of the metal ion appears to correlate with the conformation of the subunits within each trimer. The identity and oxidation state of the metal ion at the His-B₁₀ site have been shown to influence the conformational properties of the hexamer (15, 44, 46). These effects can be attributed to the unique coordinative preferences of the different metal ions. The d¹⁵ electronic configuration of the Zn(II) ion means that no ligand field stabilization energy is derived upon Zn(II) complexation. Consequently, the resulting Zn(II) stereochemistry is dictated only by the bonding and steric requirements of the ligating environment. A recent Zn(II)-insulin crystal structure (29) presents evidence for dual coordination about one Zn(II) atom in a Zn(II)-T₃R₄ type structure. In this structure, the Zn(II) ion of the T₃ trimer exhibits both tetrahedral and octahedral coordination as a result of a 2-fold disorder. On the basis of this observation it was suggested that a similar situation could exist for Co(II) hexamers, thus calling into question the interpretation of spectroscopic results. However, it must be noted that, in contrast to Zn(II), the ligand field stabilization effect is expected to be a significant factor in determining the stereochemistry of the high spin Co(II) ion. Our MCD studies show that the orbital splitting of the Co(II) center in the Co(II)-R₆₃ trimer is, indeed, significantly affected by the nature of the fourth ligand. The ligand field stabilization effect is expected to result in a degree of thermodynamic selection for Co(II) stereochemistry within the Co(II)-hexamer, and this selection will be modulated by the nature of the fourth exogenous ligand. The stabilization of the Co(II)-R₆₃ hexamer via heterotropic ligand binding interactions involving the Co(II) ion has been reported (3, 16–18). We consider it unlikely, therefore, that a given insulin trimer conformation would simultaneously stabilize octahedral and tetrahedral Co(II) geometries in solution.

In conclusion, the His-B₁₀ site in the M(II)-R₆ hexamer provides a fortuitous example of a biological chelate that stabilizes pseudotetrahedral transition metal coordination. This system has enabled us to examine a series of pseudotetrahedral Co(II)(His)₃L centers in which the variation of the structural and electronic features of the Co(II) ion originates from the variation in the nature of one exogenous ligand. Several factors are expected to influence the geometry of the Co(II) chromophore in the Co(II)-R₆₃ hexamer, the steric bulk of the exogenous fourth ligand, the electronic structure of this ligand, and its disposition of donor atoms. The data of Table I show that near-tetrahedral geometries are obtained with the halides, pseudohalides, trichloroacetate, nitrate, imidazole, and 1-methylimidazole. Considerably distorted geometries are obtained with the larger aromatic ligands. In order to accommodate the additional steric bulk of these ligands, an alternative arrangement of the B₁-B₁₀ residues is required which evidently results in a distorted Co(II) geometry. For the near-tetrahedral adducts, the transition energies are influenced by the different donor properties of the ligands in conformity with the spectrochemical series. Although the electronic absorption spectra of these adducts show considerable diversity, the qualitative appearance of the MCD spectral profiles fall into distinct categories. These ligand perturbation experiments demonstrate that the MCD dispersion is highly sensitive to the Co(II) coordination geometry and appears to be less affected by the actual chemical nature of the exogenous ligand donor atoms in the Co(II)(His)₃L unit. The present study provides evidence for a structural analogy between the Co(II) sites of Co(II)-R₆₃-phenolate, alkaline Co(II)-CA, and Co(II)-β-lactamase.

Although structural conclusions based on spectral methods alone are necessarily tentative, the spectra reported herein fall into distinct spectral classes that almost certainly correspond to discrete Co(II) coordination geometries. The intriguing spectral parallels with blue copper proteins (3, 43, 44), carbonic anhydrase (33), and Co(II)-β-lactamase attest to the utility of an artificial metalloprotein system to model the unique spectrochemical and structural features of spectroscopically unusual metalloproteins.

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