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Articles

Characterization of the R-State Insulin Hexamer and Its Derivatives. The Hexamer Is Stabilized by Heterotropic Ligand Binding Interactions†

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ABSTRACT: ^{1}H NMR and UV-visible electronic absorption studies have been performed to investigate the effects of anions and cyclic organic molecules on the interconversion of the T- and R-conformational states (Kaarsholm et al., 1989) of hexameric M(II)-substituted insulin in solution (M = Zn or Co). Two ligand binding processes that stabilize the R-state conformation of the M(II)-substituted insulin hexamer [M(II)-R_6] have been distinguished: (i) The binding of neutral organic molecules to the six, crystallographically identified, protein pockets in the Zn(II)-R_6 insulin hexamer (Derewenda et al. 1989) generate homotropic site-site interactions that stabilize the R-state. Cyclohexanol, phenol, 4-nitrophenol, and 4-hydroxymethylbenzoate are shown to bind at these sites. (ii) The coordination of singly charged anions that are able to gain access to the two HisB10 coordinated metal ions of the M(II)-R_6 hexamer stabilizes the R-state. Adducts of the M(II)-R_6 hexamer are formed, thereby, in which the solvent-accessible fourth coordination position of the M(II) ion is replaced by a competing anion. Binding to these two classes of sites introduces strong heterotropic interactions that stabilize the R-state. UV-visible spectral data and apparent affinity constants for the adducts formed by the Co(II)-R_6 hexamer with a wide range of anionic ligands are presented. The Co(II)-R_6 adducts have a strong preference for the formation of pseudotetrahedral Co(II) centers. The HCO_3^- and pyridine-2-thiolate ions form Co(II)-R_6 adducts that are proposed to possess pentacoordinate Co(II) geometries. The relevance of the Co(II)-R_6 complexes to carbonic anhydrase catalysis and zinc enzyme model systems is discussed.

Insulin is synthesized in the β-cells of the pancreas where it is stored as crystalline hexameric aggregates containing high concentrations of Zn^{2+} and Ca^{2+} ions (Howell, 1974; Havu et al., 1977). The biologically active form of the hormone is a 51 amino acid residue monomer comprised of A and B chains of total molecular weight 5800. Little is known about the molecular recognition process and the biochemical signal-transducing events that take place when the insulin monomer binds to its receptor; however, it is probable that these processes require the accessibility of specific conformational states by the insulin molecule (Blundell, 1979; Chothia et al., 1983). Consequently, the self-aggregation properties and conformational behavior of monomeric insulin and higher aggregates are subjects of great importance to understanding the expression of its hormonal activity.

The R-state insulin hexamer (Scheme IB) is a new conformational variant of hexameric insulin that recently has been described by X-ray crystallography (Derewenda et al., 1989) and identified spectroscopically in solution (Wollmer et al., 1987; Thomas & Wollmer, 1989; Kaarsholm et al., 1989; Roy

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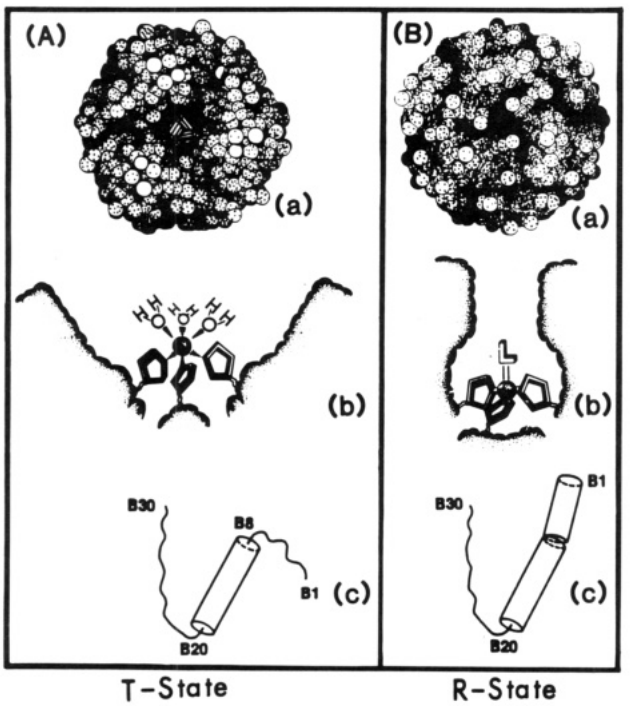
Scheme I: Comparison of Structural Differences between the Zn(II)–T_6 (A) and Zn(II)–R_6 (B) Forms of the Insulin Hexamer[a]



[a] (a) Space-filling models viewed down the 3-fold symmetry axes [redrawn from Smith et al. (1984)]. The three water molecules coordinated to Zn(II) in the T-state are shown as striped balls. The exchangeable ligand coordinated to Zn(II) in the R-state is shown as a cross-hatched ball at the center. (b) Cartoons depicting the metal cavities and coordination geometries. (c) Cartoons depicting the extended (T-state) and helical (R-state) conformations of the B-chain, residues 1–9.

et al., 1989; Brader & Dunn, 1990; Brader et al., 1990; Krüger et al., 1990). Depending upon the nature of the crystallization media, the insulin crystal structures reported to date show that the Zn(II)–insulin hexamer may adopt one of three distinct conformations (Blundell et al., 1972; Peking Insulin Structure Research Group, 1974; Bentley et al., 1976; Dodson et al., 1979; Sakabe et al., 1981; Smith et al., 1984; Baker et al., 1988; Derewenda et al., 1989). These structures have been designated as T_6, T_3R_3, and R_6 in accordance with allosteric nomenclature,[1] the subunit conformation within each hexamer, and the affinities exhibited by each hexamer for small molecule ligands (Kaarsholm et al., 1989). Each structure consists of three equivalent dimers associated about a 3-fold axis to form a hexamer. In the T-state insulin hexamer (Scheme IA), residues B1–B8 of all six subunits exist in an extended conformation (Baker et al., 1988). The two Zn(II) ions of this hexamer each reside in octahedral ligand fields comprising three B10 histidine residues and three water molecules. In the R-state hexamer (Scheme IB), residues B1–B8 of all six subunits take up a helical conformation to form a region of helix contiguous from B1 to B19 (Derewenda et al., 1989). This change in conformation causes each of the two Zn(II) ions to adopt a tetrahedral coordination geometry that comprises three B10 histidines and a single anion or small-molecule ligand. The crystal structure of the Zn(II)–R_6 hexamer shows that the hexamer subunits are each stabilized via homotropic interactions mediated by the binding of six phenol molecules to hydrophobic pockets on the protein surface. This binding involves a number of van der Waals contacts between the phenol ring and various side chain atoms of the insulin A and B chains as well as two hydrogen bonds between the phenol OH group and the amide NH and the carbonyl oxygen of CysA11 and CysA6, respectively. Zn(II)–T_3R_3 crystals are obtained by incorporating into the crystallization media large concentrations (typically 0.03–0.5 M) of lyotropic anions (de Graaf et al., 1981). In the Zn(II)–T_3R_3 structure, three of the hexameric subunits exist in the T-state conformation and the remaining three exist in the R-state conformation (Bentley et al., 1976; Cutfield et al., 1981; Smith et al., 1984). When the lyotropic anion is Cl, this arrangement causes one axial zinc ion to adopt a tetrahedral coordination sphere that is analogous to the zinc sites in the Zn(II)–R_6 hexamer. In addition, new, disordered off-axial zinc binding sites are also created. The coordination of the second axial zinc ion remains unchanged relative to that of the Zn(II)–T_6 hexamer. The octahedral zinc site is fully occupied, whereas the two types of tetrahedral zinc sites are not. A total content of 2.67 zinc ions/hexamer has been estimated from the site occupancy factors of the Zn(II)–T_3R_3 crystal structure. The existence of a T_3R_3 structure in solution has been predicted (Ramesh & Bradbury, 1980; Reinscheidt et al., 1984; Wollmer et al., 1987; Palmieri et al., 1988; Kaarsholm et al., 1989; Thomas & Wollmer, 1989; Krüger et al., 1990).

One aim of the experiments presented herein was to distinguish between the effects of anion binding to the M(II)–R_6 metal sites and the effects of phenol-like molecules binding to the protein pockets of this hexamer. A second aim was to make a qualitative characterization of heterotropic and homotropic effects involving ligand binding to these two classes of sites. Spectroscopic studies have shown that the Co(II)- and Cu(II)-substituted insulin hexamers also can undergo the T to R conformational transition (Roy et al., 1989; Brader & Dunn, 1990; Brader et al., 1990). Furthermore, these studies have shown that the R_6 conformation of the insulin hexamer can stabilize transition metal coordination that mimics characteristic features of blue copper proteins and of the zinc enzyme carbonic anhydrase (Brader & Dunn, 1990; Brader et al., 1990). We have performed studies to characterize the adducts formed with the Co(II)–R_6 hexamer and to further investigate the relevance of these species to Co-substituted metalloproteins.

Materials and Methods

Materials

The chemicals employed 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). Phenol-d_6, cyclohexanol-d_{12}, NaOD (40% solution), D_2SO_4 (98% solution), 2,2-dimethyl-2-silapentane-5-sulfonate-2,2,3,3-d_4 (DSS), and D_2O, were purchased from Aldrich.

Methods

Ligand Binding Titrations. Titration isotherms were measured by addition of the appropriate ligand to Co(II)–R_6. Hexamer solutions were prepared with 50 mM Tris–ClO_4 buffer, pH 8.0, in the presence of 100 mM phenol. Half-saturation values were obtained from the resulting titration curves. Metal-substituted insulin hexamers (M = Zn or Co)

[1] Abbreviations: M, Zn or Co; T, the conformation of an insulin subunit in the two-zinc hexamer; R, the conformation of an insulin subunit in the phenol-induced insulin hexamer; T_6, T_3R_3, and R_6, the three crystallographically identified allosteric forms of the insulin hexamer; ppm, parts per million; pH*, the pH meter reading in D_2O solutions; NMR, nuclear magnetic resonance, 2D-COSY, two-dimensional correlated spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate-2,2,3,3-d_4, ESR, electron spin resonance spectroscopy; CD, circular dichroism spectroscopy; MCD, magnetic circular dichroism spectroscopy; CA, carbonic anhydrase.

Table I: Electronic Absorption Data[a] for Various Co(II)–R_6 Adducts Formed with Ligands (L)

L	K_{app} (mM)	[L] (mM)	band maxima (nm), molar absorptivity (M^{-1} cm^{-1})
thiocyanate	0.31	20	310 (2600), 548[b] (500), 572 (750), 586[b] (730)
cyanate	0.31	20	538[b] (430), 562 (590), 602 (480)
chloride	19.8	200	554[b] (430), 580 (610), 594[b] (570)
bromide	22.4	200	562[b] (480), 596[b] (750)
iodide	25.2	200	566 (440), 598 (600), 618 (700)
acetate	45	200	520[b] (280), 536 (310), 594 (160)
nitrate	107	500	522[b] (290), 546 (370)
bicarbonate[f]		500	525[b] (270), 538 (300), 592 (210)
acetazolamide		50	512 (420), 564 (510), 584[b] (480)
imidazole		160	534[b] (420), 556 (540), 570[b] (490)
cyanide		10	548[b] (570), 574 (820)
nitrite		40	526[b] (290), 556 (360)
azide		20	314 (2000), 552[b] (420), 580 (670), 604 (660)
hydrosulfide		1	540 (390), 598 (550), 616 (550)
benzenethiolate		4	345 (2000), 402 (1600), 524 (500), 610[c] (700)
4-methylbenzenethiolate		4	350 (2600), 410 (2100), 522 (800), 610[c] (700)
pentafluorobenzenethiolate		4	333 (3400), 374 (1200), 398[b] (900), 528 (500), 596[c] (700)
pyridine-4-thiolate		4	532 (560), 604[c] (700)
pyridine-2-thiolate		8	532 (240), 582 (300)
phenol		100	359 (560), 508 (230), 544 (300), 622 (410), 640[b] (310)
4-hydroxymethylbenzoate[d,g]		40	520[b] (180), 550 (230), 616 (330), 636[b] (200)
4-nitrophenol[d,e,g]		100	524[b] (230), 550 (260), 612 (420), 632[b] (210)
pentafluorophenol[d,h]		40	525[b] (170), 544 (190), 604 (200), 628[b] (90)

[a] Spectra were recorded on 0.33 mM insulin solutions in the presence of 100 mM phenol at pH 8.0 except where indicated. [b] Shoulder. [c] Broad. [d] Recorded in the absence of phenol. [e] Difference spectrum. [f] Recorded in the presence of 10 mM phenol. [g] Recorded at pH 9.0. [h] Recorded in the presence of 100 mM cyclohexanol.

were prepared by addition of the appropriate metal ion to buffered solutions of metal-free insulin in the ratio of two metal ions per hexamer.

UV–Visible Spectra and Kinetic Studies. UV–visible spectra were recorded on a Hewlett-Packard 8450A spectrophotometer. The adducts of Table I were prepared in solutions of 50 mM Tris–ClO_4 buffer, pH 8.0, that incorporated saturating concentrations of the appropriate ligands as indicated.

^1H NMR Spectroscopy. Spectra were recorded at 299 K on a GN-500 spectrometer equipped with a Nicolet 1280 computer. A D_2O field frequency lock was used for these measurements. Chemical shifts are reported in parts per million (ppm) relative to the methyl resonance of DSS. The spectra of Figures 1 and 2 were recorded with use of presaturation pulse sequences to suppress the H_2O resonance. Samples (0.33 mM) for ^1H NMR were prepared by use of a Zn to hexamer ratio of 2:1. The spectra reported herein were largely reproducible, although we note that the appearance of the C2 His region of these spectra (specifically 7.5–8.0 ppm) is very sensitive to minor variations in sample preparation, e.g., the Zn to insulin ratio and pH. The acidity of solutions measured in D_2O are reported as pH*, the pH meter reading of the solution.

Results

Electronic Absorption Spectra of Co(II)–R_6 Adducts. Table I documents the electronic absorption spectra of a number of Co(II)–R_6 adducts, recorded over the region 300–800 nm. The ability of the Co(II)–R_6 hexamer to form pseudotetrahedral adducts in which the fourth ligand positions of the Co(II) ions are occupied by suitably small anions has been noted previously (Roy et al., 1989; Brader & Dunn, 1990; Brader et al., 1990). The spectra of Table I were recorded on 0.33 mM Co(II)–R_6 solutions, prepared in 50 mM Tris–ClO_4 buffer, pH 8.0, and except where indicated incorporating 100 mM phenol. The adducts were obtained by the addition of the respective anions until no further significant changes in the spectra occurred. The measured extinction coefficients (normalized to metal ion concentration) are apparent values only and correspond to minimum values. Even under the "saturating" conditions employed, it is likely that the species of interest is present together with small fractions of the Co(II)–T_6 hexamer and the Co(II)–R_6–phenolate adduct. To our knowledge, the only organic compounds that are able to induce the T to R conformational transition in the Co(II)-substituted insulin hexamer are phenol and certain ring-substituted phenol derivatives. The presence of these compounds necessarily results in the introduction of the respective phenolate anions, species that coordinate to the Co(II) ions of the Co(II)–R_6 hexamer. Consequently, we have thus far been unable to attain the anion-free conditions necessary to record spectra of the hydroxide or H_2O adducts of the Co(II)–R_6 hexamer.

Characterization of Zn(II)-Substituted R-state Species by ^1H FT NMR. Figure 1 shows the aromatic regions of the ^1H NMR spectra of hexameric Zn(II) insulin (0.33 mM) recorded at pH* 8.0 in D_2O under the following conditions: (a) in the absence of additives, (b) in the presence of 50 mM NCS^-, (c) in the presence of 100 mM cyclohexanol-d_{12}, (d) in the presence of 10 mM phenol-d_6, (e) in the presence of 100 mM cyclohexanol-d_{12} and 200 mM Cl^-, and (f) in the presence of 100 mM cyclohexanol-d_{12} and 50 mM NCS^-.

The spectrum of the Zn(II)–T_6 hexamer (Roy et al., 1989) is shown in Figure 1, spectrum a. Spectra b–f show that the presence of certain organic molecules and inorganic anions causes large perturbations in the ^1H NMR spectrum of Zn(II)–insulin. The resonances in the region 5.0–6.5 ppm in spectrum d are characteristic of the phenol-induced Zn(II)–R_6 hexamer (Roy et al., 1989). Spectrum c exhibits features in this region that are comparable to those of (d), notably a resonance at 6.27 ppm. This demonstrates that, like phenol, cyclohexanol stabilizes the Zn(II)–R_6 hexamer. Spectra c, e, and f show that, in the presence of 100 mM cyclohexanol-d_{12}, large spectral changes occur upon subsequent addition of NCS^- or Cl^- ion. In (e) and (f), the intensities of the resonances at 6.3 ppm are increased relative to (c), indicating that additions of these anions result in an increase in the fractions of the Zn(II)–R_6 hexamers present in these solutions. We infer from these results that the combined effects of anions and 100 mM cyclohexanol are to stabilize

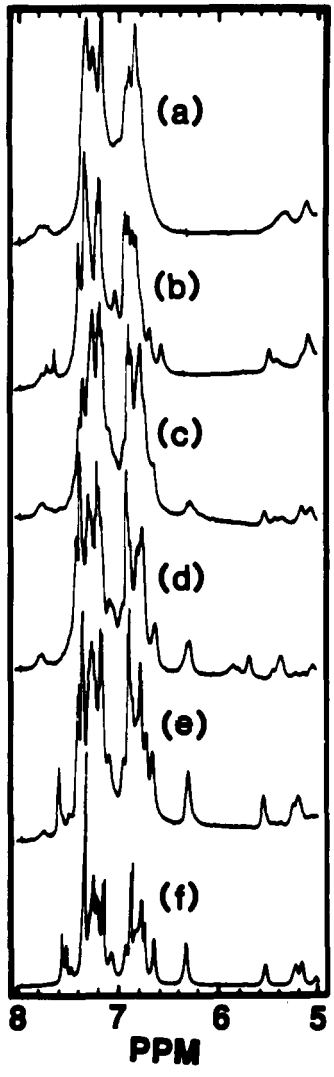


FIGURE 1: Aromatic regions of the 500-MHz ^{1}H NMR spectra of hexameric zinc insulin in D_2O (0.33 mM, pH* 8.0) recorded in the absence of additives (a) and in the presence of the following compounds: 50 mM NCS^- (b), 100 mM cyclohexanol-d_{12} (c), 10 mM phenol-d_6 (d), 100 mM cyclohexanol-d_{12} and 200 mM Cl^- (e), and 100 mM cyclohexanol-d_{12} and 50 mM NCS^- (f).

the Zn(II)–R_6 structure as a consequence of anion coordination to the Zn(II) ions (two per hexamer) and the binding of cyclohexanol molecules to the protein pockets (presumed to be six per hexamer). Spectrum b shows that the presence of 50 mM NCS^- produces a spectrum that is clearly different from (a) or (d) and that is similar to those reported by Williamson and Williams (1979) and Palmieri et al. (1988), which were recorded under comparable conditions. Spectrum b exhibits characteristic resonances in the 5.0–6.7-ppm region possessing chemical shifts of 6.65, 6.53, 5.48, 5.48, and 5.08 ppm. In view of its distinctive appearance, it is tempting to assign this spectrum to that of the Zn(II)–T_3R_3 hexamer. However, we note that the occurrence in this spectrum of resonances possessing unique chemical shifts does not preclude the possibility that this spectrum arises from an equilibrium mixture of those species that give rise to spectra a and d. The ^{1}H NMR spectrum of such a mixture would depend heavily upon the proportional composition of the equilibrium and upon the exchange rate for the interconversion of states relative to the NMR time scale. This spectrum may, therefore, be attributable either to the Zn(II)–T_3R_3 hexamer or to a mixture of the Zn(II)–T_6 and Zn(II)–R_6 hexamers. Resonances in the 7.5–8.0-ppm spectral region of hexameric human Zn(II)–

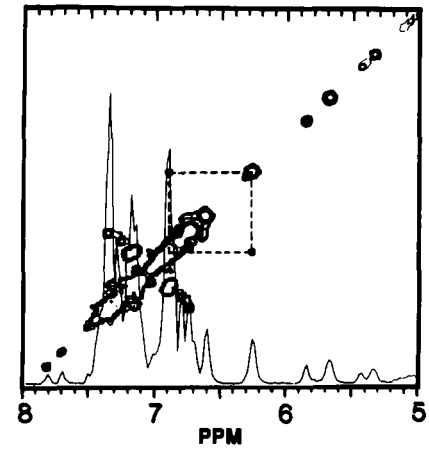


FIGURE 2: Aromatic region of the 500-MHz 2D-COSY ^{1}H NMR spectrum of hexameric zinc insulin in D_2O (0.33 mM, pH* 8.0) recorded in the presence of 10 mM phenol-d_6.

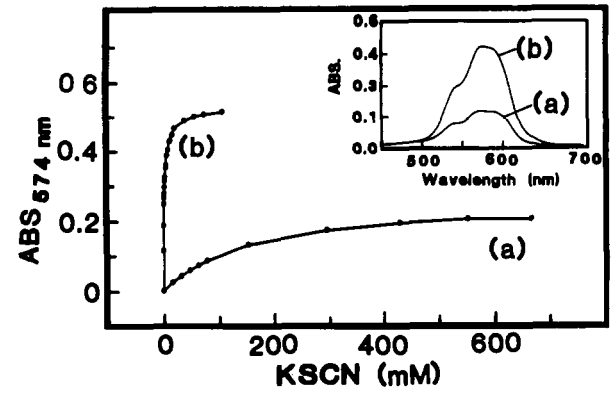


FIGURE 3: Binding isotherms for the titration of 0.33 mM hexameric cobalt(II)–insulin with NCS^- ion in the absence of additives (a) and in the presence of 100 mM cyclohexanol (b). The absorbance at 574 nm is plotted as a function of [NCS^-]. Curve fitting to a single hyperbolic expression gave apparent dissociation constants of (a) 154 mM in the absence of cyclohexanol and (b) 0.96 mM in the presence of cyclohexanol. (Inset) Electronic absorption spectra of hexameric cobalt(II)–insulin in the presence of 600 mM NCS^- (a) and 100 mM NCS^- and 100 mM cyclohexanol (b).

insulin have been assigned to the C2 protons of the histidine residues B5 and B10 (Bradbury et al., 1981; Palmieri et al., 1988). The complex multiplicity of resonances that occur in this region in some of the spectra of Figure 1 suggests that these spectra correspond to solutions comprising comparable fractions of more than one conformational state (Palmieri et al., 1988).

Figure 2 shows the 2D-COSY ^{1}H NMR spectrum of Zn(II)–insulin recorded in the presence of 10 mM phenol-d_6 at pH* 8.0. The 6.3-ppm resonance exhibits a cross-peak that correlates with the aromatic envelope at 6.88 ppm. This resonance, therefore, arises from an aromatic residue exhibiting chemical shift and coupling parameters consistent with the 3,5 ring protons of a tyrosine or phenylalanine group.

Evidence for Heterotropic Site–Site Interactions. Figure 3 shows the isotherms obtained from titrations of 0.33 mM Co(II)–T_6 with NCS^- ion (a) in the absence of additives and (b) in the presence of 100 mM cyclohexanol. Thiocyanate is a pseudohalide anion that coordinates strongly to transition metal ions. In contrast, cyclohexanol is a noncoordinating species. The data of Figure 3 show that the presence of 100 mM cyclohexanol enhances the apparent binding affinity of the Co(II)–insulin hexamer for NCS^- ions by 150-fold. These binding curves may each be approximated by single hyperbolae possessing apparent dissociation constants of (a) 154 mM and

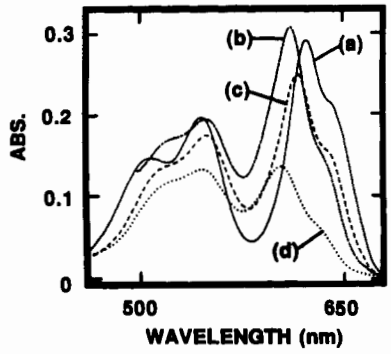


FIGURE 4: Electronic absorption spectra of hexameric cobalt(II)–insulin (0.33 mM) recorded in the presence of 100 mM phenol (a), 20 mM 4-nitrophenol (difference spectrum) (b), 40 mM 4-hydroxymethylbenzoate (c), and 20 mM pentafluorophenol and 100 mM cyclohexanol (d).

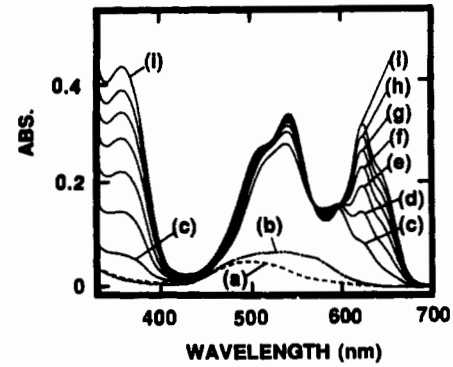


FIGURE 5: Electronic absorption spectra of hexameric cobalt(II)–insulin (0.66 mM, pH 8.0) recorded in the absence of additives (a) and in the presence of 500 mM HCO_3^- ion (b). Spectra c–i show the effects of subsequent incremental additions of phenol to (b). Phenol concentrations range from 10 mM (c) to 100 mM (i).

(b) 0.96 mM. The spectra shown in the inset to Figure 3 correspond to (a) Co(II)–insulin in the presence of 600 mM NCS^- ion and (b) Co(II)–insulin in the presence of 600 mM NCS^- ion and 100 mM cyclohexanol. The intensity, band shape, and positions of maxima of spectrum b correspond closely to those of the phenol-induced Co(II)–R_6 NCS^- adduct (see Table I). This finding indicates that the combination of 600 mM NCS^- ion and 100 mM cyclohexanol gives a heterotropic effect that results in near-complete conversion of the Co(II) ions to the tetrahedral coordination geometry of the Co(II)–R_6 hexamer. In contrast, the intensity of spectrum a is *less than* half that of spectrum b, indicating that *saturating* concentrations of NCS^- ion cause only partial conversion of the Co(II) centers to tetrahedral coordination.

Evidence That Phenolate–Metal Coordination Stabilizes the R-state. Figure 4 shows the visible electronic absorption spectra of 0.33 mM Co(II)–insulin recorded in the presence of (a) 100 mM phenol, (b) 100 mM 4-nitrophenol, (c) 40 mM 4-hydroxymethylbenzoate, and (d) the combination of 20 mM pentafluorophenol and 100 mM cyclohexanol. These spectra are documented in Table I. Spectra a and c were recorded in 100 mM TAPS buffer, pH 9.0. Spectra b and d were recorded in 50 mM Tris–ClO_4 buffer, pH 8.0. Intense absorption bands ($\epsilon > 300$ M^{-1} cm^{-1}) in the region 400–800 nm are diagnostic of the R-state tetrahedral Co(II) centers (Roy et al., 1989; Brader et al., 1990). Spectra a–c of Figure 3 show that phenol, 4-nitrophenol, and 4-hydroxymethylbenzoate, respectively, induce the T to R conformational transition in Co(II)–insulin and that these derivatives give rise to characteristic spectra. On the basis of this criterion, we have found that a large number of additional ring-substituted phenol derivatives are able to induce the T to R conformational transition.[2] It is very probable that these phenol derivatives induce the conformation change by binding to the six ligand sites on the Co(II)–R_6 hexamer in a manner similar to that adopted by phenol. The spectral variation evident in Figure 3 indicates that these different phenol derivatives cause perturbations in the electronic energy levels of the Co(II) ions in the Co(II)–R_6 hexamer. Such perturbations may, in principle, arise from alterations in the coordination geometry of the Co(II) ion and/or changes in the donor properties of one or more of the atoms coordinated to the Co(II) ion. In order to investigate these possibilities, it is useful to consider pentafluorophenol, which is of similar size to phenol but which possesses a much lower pK_a value due to the electron-withdrawing fluorine substituents [pK_a(phenol) = 10.0, pK_1(pentafluorophenol) = 5.5; Serjeant & Dempsey, 1979]. We have found that in 50 mM Tris–ClO_4 buffer at pH 8.0, a 20 mM concentration of pentafluorophenol causes negligible change in the Co(II)–T_6 spectrum. However, in the presence of 100 mM cyclohexanol, spectrum d of Figure 3 is obtained. At pH 8.0 pentafluorophenol exists largely as the pentafluorophenolate anion, which evidently is unable to bind to the phenol sites of the Co(II)–R_6 hexamer. Our unpublished work (Brader, Choi, Aguilar, and Dunn) indicates that only the neutral form of these phenolic compounds binds tightly to the protein pockets. The combination of cyclohexanol molecules binding to the protein pockets of the Co(II)–R_6 hexamer and the pentafluorophenolate ions coordinating to the Co(II) ions drives the conformation change and stabilizes the Co(II)–R_6 hexamer. This heterotropic effect gives rise to a spectrum corresponding to the pentafluorophenolate adduct of the Co(II)–R_6 hexamer (spectrum d). These observations suggest strongly that spectra a–d of Figure 3 each correspond to the respective phenolate adducts of the Co(II)–R_6 hexamer. Although the structural differences among these phenolic compounds could give slightly different Co(II) geometries, it is likely that the spectral variation of Figure 3 arises primarily as a result of the variation in donor properties of the coordinated phenolate oxygen atoms.

The Co(II)–R_6 Hexamer Forms a HCO_3^- Adduct. Figure 5 shows the effect of titrating phenol into a 0.33 mM Co(II)–R_6 solution in the presence of 500 mM HCO_3^- ion. The Co(II)–R_6 solution was prepared in 100 mM Tris–ClO_4 buffer, pH 8.0. Spectrum a corresponds to that of the Co(II)–T_6 hexamer in the absence of additives. Spectrum b shows that the subsequent addition of 500 mM HCO_3^- ion (final pH 8.0) causes an increase in the spectral intensity and a shift in the absorption maxima. This observation suggests that HCO_3^- (or CO_3^{2-}) ion coordinates to the octahedral Co(II) ions of the Co(II)–T_6 hexamer. As increasing concentrations of phenol are added, the observed spectra become progressively similar to that of the phenolate ion adduct (Figure 3a and Table I). In the presence of 10 mM phenol (spectrum c), the observed spectrum consists of components attributable to three species, the Co(II)–R_6–HCO_3^- adduct, the Co(II)–R_6–phenolate adduct, and Co(II)–T_6, with the Co(II)–R_6–HCO_3^- species predominating. From (a) it is apparent that the Co(II)–R_6–HCO_3^- spectrum exhibits bands at 525, 538, and 592 nm. The spectra of Figure 5 indicate that as the phenol concentration is increased, the very weakly coordinating HCO_3^- ion is replaced by the phenolate ion.

[2] Detailed ligand binding and kinetic studies on the interactions between a wide range of cyclic molecules and the Co(II)-substituted insulin hexamer will be presented elsewhere (Choi et al., manuscript in preparation).

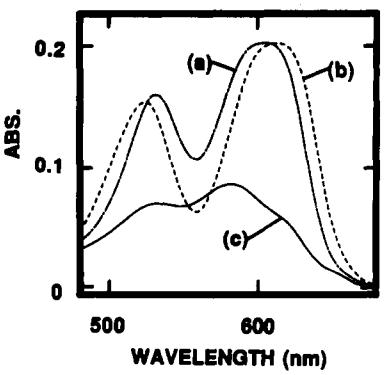


FIGURE 6: Electronic absorption spectra of the Co(II)–R_6 hexamer (0.33 mM; 100 mM phenol) adducts formed in the presence of 4 mM pyridine-4-thiolate (a), 4 mM benzenethiolate (b), and 8 mM pyridine-2-thiolate (c).

Evidence for an R-state Five-Coordinate Pyridine-2-thiolate Adduct. Figure 6 compares the Co(II)–R_6 adduct spectra obtained for the ligands pyridine-4-thiol (a), benzenethiol (b), and pyridine-2-thiol (c). Spectra a and b are very similar in band shape and intensity ($\epsilon > 300$ M^{-1} cm^{-1}), showing only small differences in absorption maxima (see Table I). We conclude that these two ligands form Co(II)–R_6 adducts via coordination of the thiolate sulfurs forming structurally analogous tetrahedral Co(II)N_3S centers. In contrast, spectrum c shows large differences in band shape and intensity (Table I). These comparisons indicate that the coordination of pyridine-2-thiol occurs in a different fashion to that of pyridine-4-thiol and thiophenol. We have been unable to detect formation of a coordination complex with pyridine. Although spectrum c does not display a spectral band in the region 700–900 nm, a feature sometimes present in the spectra of pentacoordinate Co(II) complexes (Bertini et al., 1978), the markedly reduced extinction coefficient of spectrum c relative to those of the tetrahedral Co(II) spectra a and b is consistent with pentacoordinate Co(II) coordination (Corwin et al., 1987; Banci et al., 1982). Therefore, we propose that pyridine-2-thiol coordinates in a bidentate fashion as the thiolate anion, thus forming pentacoordinate Co(II)N_4S centers.

Discussion

Conformational Equilibria. The recognition that the M(II)–T_6, M(II)–T_3R_3, and M(II)–R_6 hexamers constitute members of an allosteric series (Kaarsholm et al., 1989) has presented the challenge of defining the mechanism of the T to R transition. The two most widely applied schemes for describing the allosteric regulation of biological activity are the concerted model (Monod et al., 1965) and the sequential model (Koshland et al., 1966). These models each propose that allosteric binding processes may be described in terms of a particular series of protein–ligand intermediates. The mathematical nature of this binding will depend upon the number of ligand sites on the protein, the affinities of each of these sites for the ligands, and the equilibria in which the intermediates are involved. Therefore, in order to apply these schemes to a description of the mechanism of the T to R conformational transition, both qualitative and detailed quantitative knowledge of the interrelationships between the species in solution is required. Previous studies utilizing X-ray diffraction, ^1H NMR, ^{19}F NMR, ESR, CD, and electronic absorption spectroscopy have concluded that, in solution, the M(II)–T_6 and M(II)–R_6 (M = Cu, Co, or Zn) hexamers yield data consistent with species possessing structures that correspond to the crystal structures of the Zn(II)–T_6 and Zn(II)–R_6 hexamers, respectively (Brill & Venable, 1968; Reinscheidt et al., 1984, 1989; Roy et al., 1989; Brader & Dunn, 1990; Brader et al., 1990). In the crystalline state, interconversion of the Zn(II)–T_6 and Zn(II)–T_3R_3 structures has been shown to depend upon the concentration of lyotropic anions present in the crystallization media (de Graaf et al., 1981). With Cl^- ion, this transformation is reversible in the crystal whereby Cl^- ion concentrations greater than 1 M favor crystallization of the Zn(II)–T_3R_3 structure. The significance and enhanced stability of the T_3R_3 structure under these conditions is intriguing. An attractive explanation for the formation of M(II)–T_3R_3 is that this species represents a particularly stable intermediate in the T_6 to R_6 mechanistic pathway (Kaarsholm et al., 1989). Alternatively, it has been suggested that crystal packing forces may be predominantly responsible for stabilizing this conformation (Chothia et al., 1983; Smith et al., 1984). Studies using such techniques as ^1H NMR, rapid kinetics, and UV–circular dichroism spectroscopy have been invoked to study solutions from which Zn(II)–T_3R_3 crystals grow (Ramesh & Bradbury, 1986; Williamson & Williams, 1979; Wollmer et al., 1987; Palmieri et al., 1988; Kaarsholm et al., 1989; Thomas & Wollmer, 1989; Krüger et al., 1990). These studies have shown, qualitatively, that structural transformations induced by anions such as Cl^- and SCN^- do occur in solution. However, the difficulties inherent in achieving a spectroscopic distinction between a single T_3R_3 hexamer species and an equilibrium mixture consisting of the T_6 and R_6 hexamer species complicate further interpretation of such results. In our opinion, an unambiguous identification of the hexamer(s) present in solution under conditions appropriate for T_3R_3 crystallization has not been made because neither the symmetry states of species present nor a quantitative determination of the distribution of these species has been achieved.

Ligand Binding Studies on Zn(II)–Insulin by ^1H NMR. The aromatic region of the ^1H NMR spectrum of monomeric human insulin consists of resonances attributable to two histidines (B5 and B10), three phenylalanines (B1, B24, and B25), and four tyrosines (A14, A19, B16, and B26). The involvement of these aromatic residues in specific interactions between subunits of the insulin hexamer makes the 5.0–8.0-ppm region of the ^1H NMR spectrum a sensitive indicator of hexamer conformation. Figure 1 shows that the ^1H NMR spectrum of hexameric Zn(II)–insulin is altered extensively by the presence of NCS^- ion, phenol, or cyclohexanol. One effect these species have is to produce a noticeable general sharpening in the profile of the aromatic region relative to that of the Zn(II)–T_6 spectrum (a). Also evident is the appearance of new resonances between 5.0 and 6.6 ppm. In the phenol-induced hexamer, Roy et al. (1989) have attributed the appearance of these new resonances to altered anisotropic ring current effects arising both from the new R_6 subunit conformation and from the bound phenol molecules. We note that the contacts between aromatic side chains across the monomer–monomer interfaces in the Zn(II)–R_6 structure are almost certainly extensively altered from those present in the Zn(II)–T_6 structure. Therefore, it is very likely that the differences evident in spectra a and d reflect the changes that occur in this region of the hexamer. The clear similarities that exist between spectra c and d suggest that cyclohexanol produces a conformational change in the zinc–insulin hexamer that is analogous to that induced by phenol. In view of the similar steric and hydrogen-bonding properties of phenol and cyclohexanol, it is likely that in (c) this stabilization is achieved via the binding of six cyclohexanol molecules to the hydrophobic pockets identified in the Zn(II)–R_6 X-ray crystal

structure (Derewenda et al., 1989). This binding probably involves favorable van der Waals contacts and hydrogen-bonding interactions analogous to those that stabilize the phenol-induced structure. Cyclohexanol is a useful phenol analogue for NMR studies on insulin because it is devoid of an aromatic ring current. The distinctive resonance at approximately 6.3 ppm, which is present in both spectra c and d evidently is not influenced by the phenol ring currents. The dramatic change in chemical shift that occurs for this resonance upon the addition of phenol must, therefore, arise as a result of new ring current effects that originate from aromatic protein residues that reside in chemical environments characteristic of the R-state protein folding and hexamer association. The 2D-COSY spectrum of Figure 2 shows that the 6.3-ppm resonance correlates with the aromatic region of the spectrum at 6.88 ppm, thus indicating that the 6.3-ppm resonance may be assigned to a tyrosine residue. Inspection of the contacts that occur in the three Zn–insulin hexamer crystal structures[3] suggests that significant anisotropic ring current effects present in R_6 and T_3R_3 but not in T_6 could arise across the monomer–monomer interfaces in the R_6 structure as a result of the contacts B16Tyr–B26Tyr and B16Tyr–B5His. The T to R transition involves the spatial rearrangement of residues B1–B9 from an extended conformation to a helical one. This invariably causes large changes both in the chemical environments of residues B1–B9 and the aromatic residues B16Tyr and B26Tyr. On the basis of modeling studies,[3] we suggest that it is likely that the resonances at 6.3 ppm, evident in spectra c, d, e, and f, originate from the B16Tyr ring protons. These modeling studies indicate that B16Tyr forms contacts with the rings of B5His and B26Tyr that should cause the ring protons to experience upfield ring current shifts due to the close proximity of B5His and/or B26Tyr.

Ligand Binding Studies on Co(II)–Insulin. The results of our ^{1}H NMR experiments on Zn(II)–insulin appear largely to parallel the results obtained from UV–visible absorption studies on Co(II)–insulin. Hexameric insulin incorporating two metal ions per hexamer has been shown to form rhombohedral crystals with a range of divalent transition metal ions including Zn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, Pb^{2+}, Fe^{2+}, and Ni^{2+} (Brill & Venable, 1968; Schlichtkrull, 1956). We previously have shown that in solution the Zn(II)-, Co(II)-, and Cu(II)-substituted insulin hexamers can undergo the phenol-induced T to R conformational transition (Kaarsholm et al., 1989; Roy et al., 1989; Brader & Dunn, 1990; Brader et al., 1990). Cobalt(II)-substitution provides a convenient means for studying the kinetics and mechanism of the transition. The visible spectral bands of the Co(II) ions provide a sensitive spectroscopic signature that is particularly useful for the investigation of the interactions of anions and phenol or phenol analogues with Co(II)–insulin. The titration experiments of Figure 3 show that NCS^- ion causes conformational changes in hexameric Co(II)–insulin and that saturating concentrations of NCS^- ion result in partial conversion of the Co(II) centers to tetrahedral coordination. The amplitude ratio of approximately 1:2 for spectra a:b is consistent with the formation of the Co(II)–T_3R_3 species in (a) and the Co(II)–R_6 species in (b) (such species would contain one and two tetrahedral Co(II) ions, respectively). However, since the Co(II) ions of the Co(II)–T_6, Co(II)–T_3R_3, and Co(II)–R_6 hexamers all may coordinate NCS^- ion, albeit with different affinity constants, it is possible that a *saturating* concentration of NCS^- ion will effect an equilibrium comprising significant fractions of each of these hexamers. Therefore, for Co(II)–insulin in the presence of 600 mM NCS^- ion, the results of Figure 3 do not distinguish between an equilibrium mixture comprising comparable fractions of Co(II)–T_6 and Co(II)–R_6 hexamers and one consisting largely of Co(II)–T_3R_3. In the presence of cyclohexanol (Figure 3, spectrum b), the equilibrium is shifted strongly in favor of Co(II)–R_6, indicating that cyclohexanol molecules bind to the protein pockets of this hexamer and that strong heterotropic site–site interactions between the metal ion site and the protein pockets stabilize the R_6 species. These experiments corroborate our interpretation of the ^{1}H NMR experiments on Zn(II)–insulin reported herein and illustrate that the M(II)–R_6 hexamer is stabilized by heterotropic ligand-binding processes involving two loci: anion coordination to the Co(II) ions, and the binding of cyclic organic molecules such as cyclohexanol or phenol to the hydrophobic pockets on the protein. The isotherms of Figure 3 show no obvious sigmoidicity, indicating that there is little or no positive cooperativity *between metal sites*. More detailed information relating ligand binding and subunit interactions in the R_6 hexamer derived from studies on a variety of phenol analogues possessing widely variable insulin binding characteristics will be presented elsewhere (Choi, Brader, Aguilar, and Dunn, manuscript in preparation).

Cobalt(II) Coordination. The active sites in a variety of zinc enzymes consist of a tetrahedral or pentacoordinate zinc ion with a water molecule serving as one ligand, e.g., carbonic anhydrase, carboxypeptidase, thermolysin, alcohol dehydrogenase, and alkaline phosphatase (Bertini et al., 1986). The M(II) coordination spheres of the M(II)–R_6 hexamer comprise pseudotetrahedral arrays of three HisB10 nitrogen atoms and a fourth ligand site that can be occupied by a variety of small molecule ligands from solution. These features are comparable to those of the tetrahedral zinc centers at the actives sites of several zinc enzymes, notably, carbonic anhydrase (Brader et al., 1990).

The utility of Co(II) substitution as a spectroscopic probe of the active site cavity in metalloproteins is well recognized. However, the spectroscopic complexities of high-spin Co^{2+} dictate that the information gained from electronic absorption and ESR studies on cobalt enzymes be interpreted largely at a qualitative level. These constraints accentuate the importance of developing Co(II) model systems that duplicate specific characteristics of Co(II)-substituted metalloproteins. The high-spin Co^{2+} ion can often be ascribed to a particular coordination geometry on the basis of its visible electronic absorption spectral intensities. Octahedral complexes give rise to broad spectral envelopes of weak intensity such that $\epsilon < 100\ M^{-1}\ cm^{-1}$, whereas tetrahedral complexes possess intense, multibanded spectra where $\epsilon > 300\ M^{-1}\ cm^{-1}$. Pentacoordinate complexes display spectra with intermediate intensities where $100 < \epsilon < 300\ M^{-1}\ cm^{-1}$ and sometimes possess a band in the region 700–900 nm (Bertini et al., 1978).

Most of the adducts listed in Table I exhibit spectra possessing ϵ_{max} values $> 400\ M^{-1}\ cm^{-1}$ and are thus described as possessing pseudotetrahedral Co(II) geometries. The d–d absorption maxima are observed in the 500–650-nm region of these spectra and may be assigned to $4A_2$–$4T_1$ electronic transitions of the high-spin tetrahedral Co(II) ion. In the presence of phenol at alkaline pH values, the optical spectrum of Co(II)–R_6 is remarkably similar to that of the alkaline form of Co(II)–carbonic anhydrase (Brader et al., 1990). The data presented in Figures 4 and 5 establish that the species that gives rise to this spectrum is the Co(II)–R_6–phenolate complex.

[3] Deductions were made from the Zn(II)–T_6 crystal structure atomic coordinates (Brookhaven Protein Data Bank) and from examination of a CPK space-filling model of the Zn(II)–R_6 structure.

Detailed MCD, CD, and electronic absorption studies on Co(II)–CA and comparisons with small molecule Co(II) spectra indicate that this distinctive alkaline spectrum arises from a trigonally distorted tetrahedral Co(II) coordination geometry (Coleman & Coleman, 1972). We note that many of the Co(II)–R_6 adducts documented in Table I possess spectra that are *extremely* similar to the corresponding Co(II)–CA adducts (viz., imidazole, hydrosulfide, bicarbonate, and acetazolamide) (Bertini et al., 1978).

Inspection of space-filling models of the Zn(II)–R_6 X-ray structure indicate that the dimensions of the tunnel extending from solution along the 3-fold axis to the metal center are too small to accommodate the steric bulk of the phenolate ring.[3] Since the evidence for phenolate and aryl thiolate complexes is unambiguous (viz., Figures 4–6 and Table I), we conclude that the R-state structure is sufficiently flexible to allow adjustments in polypeptide folding that increase the tunnel dimensions to a size that can accommodate these bulky ligands.

The high degree of similarity between the Co(II)–R_6 phenolate spectrum and the alkaline Co(II)–CA spectrum shows that the respective Co(II) geometries in these two proteins are very similar. The Co(II) centers of the Co(II)–R_6 hexamer have high affinities for anionic ligands. This is evidenced by the observation that this hexamer forms adducts with very weakly coordinating anions such as NO_3^- and HCO_3^- that usually do not coordinate to the Co(II) ions of small-molecule Co(II) complexes in aqueous solution (Dennard & Williams, 1966). Table I shows that the various Co(II)–R_6 adducts possess spectra that display considerable variations in band structure. The Co(II)–R_6 adducts generally possess spectra with fewer well-resolved bands in comparison to the Co(II)–R_6 phenolate spectrum. Similarly, the large splitting between the visible bands of the alkaline spectrum of Co(II)–CA is not evident in the spectra of its adducts. This has led to the conclusion that, upon the formation of four-coordinate Co(II)–CA adducts, the replacement of the OH^- ion by another anion causes a displacement of the trigonally distorted tetrahedron to a more regular tetrahedral geometry. We conclude by analogy that the Co(II)–R_6 adducts formed with inorganic anions possess more regular tetrahedral Co(II) geometries than do the Co(II)–R_6 phenolate adducts. The maximum extinction coefficients of most of the Co(II)–R_6 adduct spectra fall within the range 500–700 M^{-1} cm^{-1}. In contrast, the series of Co(II)–CA adducts display a much greater variation in spectral intensities. This variation originates from a propensity of Co(II)–CA to form both four- and five-coordinate Co(II) centers. It is clear that the Co(II)–R_6 hexamer has a much greater preference for the formation of four-coordinate Co(II) centers than does Co(II)–CA. Phenol has been shown to be a competitive inhibitor of CO_2 hydration catalyzed by CA (Simonsson et al., 1982). However, the region of the CA active cavity with which the phenol molecule associates is not established. Our observation that the Co(II)–R_6 hexamer readily coordinates phenolate anions raises the likelihood that phenol inhibits CA catalysis by coordinating directly to the metal as the phenolate anion.

Evidence for a HCO_3^- Complex with Co(II)–R_6. The HCO_3^- ion is the natural substrate for carbonic anhydrase, hence the HCO_3^- adduct spectrum of Figure 5 deserves special comment. An important aspect of CA catalysis pertains to the details of how HCO_3^- associates with the active site. Yeagle et al. (1975) have carried out studies of the paramagnetic enhancement of ^{13}C relaxation rates and thereby have shown that HCO_3^- ion binds directly to the Co(II) ions of Co(II)–CA. The complex formed between Co(II)–CA and HCO_3^- ion has been ascribed to a pentacoordinate Co(II) geometry on the basis of the visible electronic absorption spectrum (Bertini et al., 1978) and XAFS studies (Yachandra et al., 1983). We note that the HCO_3^- adduct of Co(II)–R_6 (Figure 5c and Table I) possesses a visible electronic absorption spectrum that is similar to that of the Co(II)–CA–HCO_3^- complex reported by Bertini et al. (1978), the former being shifted to shorter wavelength by approximately 15 nm. The Co(II)–R_6 adducts formed with acetate and the HCO_3^- ion exhibit spectra that are extremely similar to each other and that possess substantially lower intensities than virtually all those listed in Table I. On the basis of these observations we consider it likely that both the acetate and the HCO_3^- ions associate with the Co(II)–R_6 hexamer to form structurally analogous pentacoordinate Co(II) centers that are closely comparable to the HCO_3^- complex formed with Co(II)–CA.

In view of the similarity between the Co(II)–CA and Co(II)–R_6–HCO_3^- complexes, we are surprised that we have been unable to detect any catalytic activity in the Co(II)–R_6–HCO_3^- system (Brader et al., 1990). Possible reasons for this lack of catalysis have been previously discussed (Brader et al., 1990).

Pentacoordinate Co(II)–R_6 Complexes. Crystallographic studies have shown that Zn(II)–CA, in the presence of SCN^- ion at alkaline pH, forms a five-coordinate Zn(II) center (Eriksson et al., 1988). The Zn(II) coordination sphere comprises three histidine residues from the protein, a water molecule, and a terminally coordinated thiocyanate ion. ^1H NMR and electronic absorption spectroscopic studies have shown that Co(II)–CA forms five-coordinate adducts with many different anions (Bertini et al., 1978). In these complexes, the anion and the water molecule are both incorporated into the Co(II) coordination sphere. In contrast, most of these anions displace the water molecule and form tetrahedral adducts with the Co(II)–R_6 hexamer. The strong tendency to form tetrahedral adducts probably is a consequence of the greater steric constraints associated with the fourth coordination position in the Co(II)–R_6 hexamer that restricts an increase in the coordination number of the Co(II) ion. There has been much speculation about the significance of pentacoordinate M(II)–OH_2 intermediates in the catalytic processes of zinc enzymes (Makinen et al., 1984). Therefore, much attention has focused on the spectroscopic changes that occur in Co(II)-substituted enzymes upon transition from the initial tetrahedral Co(II) centers to the intermediate states. Unfortunately, the information contained in Co(II)–enzyme spectra can be ambiguous because the delineation between tetrahedral spectra and pentacoordinate spectra is often unclear. For example, the question of coordination number remains uncertain for Co(II)–carboxypeptidase, for the acidic form of Co(II)–CA, and for some of the spectroscopic intermediates observed during Co(II)–liver alcohol dehydrogenase catalysis (Sartorius et al., 1987, 1988). This problem is augmented by the fact that there are few structurally well-characterized pentacoordinate Co(II) complexes that serve as suitable models for the active sites of Co(II)-substituted zinc enzymes.

The Co(II)–R_6 hexamer evidently forms pentacoordinate Co(II) centers with appropriate bidentate ligands. This is illustrated by the spectra of the adducts formed with benzenethiol, pyridine-2-thiol, and pyridine-4-thiol (Figure 6). The coordination chemistry of pyridine-2-thiol has been studied in detail (Kitagawa et al., 1990; Mura et al., 1985). This molecule may adopt a variety of coordination modes either as the neutral ligand or as the conjugate base, the pyridine-

2-thiolate anion. The anionic ligand may function as a monodentate N-, or preferably S-, bonded ligand or as an N,S-bonded chelate. The spectra of the Co(II)–R_6 adducts shown in Figure 6 suggest that the pyridine-2-thiolate adopts the latter coordination, whereas pyridine-4-thiolate and benzenethiolate each coordinate as monodentate ligands producing tetrahedral Co(II) centers. Spectrum c is comparable to those of complexes formed by Co(II)–thermolysin and Co(II)–carboxypeptidase that have been postulated to contain pentacoordinate Co(II) centers (Bertini, 1983).

Insulin Structural Flexibility and Function. The protein pockets of the R-state hexamer are formed by contributions from neighboring subunits and, therefore, are characteristics of the R_6 hexameric state. The variety of organic molecules that are able to bind to these pockets and thereby induce the T_6 to R_6 conformational change attest to the steric versatility of these sites. The Zn(II)–T_3R_3 structure possesses three protein pockets analogous to those in the Zn(II)–R_6 hexamer that should also be capable of binding phenol analogues. Our inspection of the Zn(II)–T_6 crystal structure has not revealed any features recognizable as specific protein sites capable of binding phenol analogues. Consequently, we conclude that the principal stabilization derived from ligand binding interactions within the M(II)–T_6 hexamer likely involve the solvent-accessible coordination positions of the octahedral metal ions. Our data show that the Zn(II)-substituted insulin hexamer undergoes the conformational change more readily than does the Co(II)-substituted derivative, presumably a consequence of the different ligand field stabilization energies conferred upon these two metal ions as a result of the change from octahedral to tetrahedral coordination geometry.

The relationship between insulin subunit conformation in the hexameric and monomeric states is only partially understood. The subunit conformations in the T_6 and R_6 structures are stabilized by subunit interactions, metal ion effects, and, for the latter, homotropic and heterotropic ligand–ligand interactions. It has been proposed that crystal packing forces in the crystalline Zn(II)–T_6, Zn(II)–T_3R_3, and Zn(II)–R_6 structures also influence conformation. Collectively, these coercive effects may cause large discrepancies between the conformations of the hexamer subunits in the crystals and the solution-state monomer conformation(s) recognized by the membrane receptor. Roy et al. (1990a) have presented ^{1}H NMR data that demonstrates that, in solution, a conformational change accompanies monomer–dimer aggregation. However, since the assignments for many of the resonances in the ^{1}H NMR spectrum of monomeric insulin correspond to predictions based on the subunit conformation in the Zn(II)–T_6 crystal structure at neutral pH (Roy et al., 1990a,b) and at acidic pH (Kline & Justice, 1990; Boelens et al., 1990; Hua & Weiss, 1990), it appears that in solution the insulin monomer possesses a structure that, although not identical with the Zn(II)–T_6 subunit conformation, is at least very similar. The insulin conformations(s) adopted in the insulin–receptor complex must become complementary to the receptor binding surface. The conformation(s) of insulin in this complex clearly will depend upon the nature of the receptor–insulin association and the way in which the interaction of insulin with the receptor initiates the sequence of events that constitute the biological response of the receptor. The conformations involved in this process could be T-like or R-like or some new conformation not yet identified.

Conclusions

The thermodynamic stability of the M(II)–R_6 hexamer is influenced to different extents by two ligand binding processes: (i) The binding of phenol, cyclohexanol, or cyclic analogues to the six protein pockets identified in the Zn(II)–R_6 crystal structure. These compounds are bound as electrostatically neutral molecules and confer stability upon the R_6 hexamer via the formation of hydrogen-bonding interactions and favorable van der Waals contacts. (ii) Anions stabilize the M(II)–R_6 hexamer by coordinating to the fourth ligand position of the tetrahedral metal ions; our results indicate that ligands that are capable of forming singly charged anions and that are small enough to gain access to the metal ion via the narrow channel may coordinate to it. Cyclohexanol binds to the six protein sites on the R_6 hexamer in a manner analogous to that of phenol.

It is clear that the NCS^- ion also induces conformational changes in the Zn- and Co-substituted insulin hexamers; however, on the basis of the present data an unambiguous description of the resultant conformational state(s) has not been made. The ^{1}H NMR and visible absorption spectroscopic results presented herein are consistent with the existence of an M(II)–T_3R_3 species in solution. Further ^{1}H NMR studies on Zn–insulin are currently underway to provide an improved understanding of the effects of NCS^- ion on the conformational states of hexameric insulin.

The Co(II) ions of the Co(II)–R_6 hexamer have a strong preference for distorted tetrahedral geometries. Our results show that pentacoordinate Co(II) is attainable with the pyridine-2-thiolate anion and, hence, that the Co(II) coordination number may be influenced by the design of the ligand that occupies the fourth coordination site on the 3-fold axis of the protein. The Co(II) centers of the Co(II)–R_6 hexamer have very high affinities for anionic ligands, and the adducts thus formed may be compared with the inhibitor complexes formed by carbonic anhydrase. Our observation of the Co(II)–R_6–HCO_3^- adduct and the similarity of its spectrum to that reported for the Co(II)–CA–HCO_3^- adduct supports suggestions that the HCO_3^- ion becomes incorporated into an inner-sphere metal ion coordination complex during CA catalysis.

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