Spectroscopic Signatures of the T to R Conformational Transition in the Insulin Hexamer*

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The cobalt(II)-substituted human insulin hexamer has been shown to undergo the phenol-induced T₆ to R₆ structural transition in solution. The accompanying octahedral to tetrahedral change in ligand field geometry of the cobalt ions results in dramatic changes in the visible region of the electronic spectrum and thus represents a useful spectroscopic method for studying the T to R transition. Changes in the Co²⁺ spectral envelope show that the aqua ligand associated with each tetrahedral Co²⁺ center can be replaced by SCN⁻, CN⁻, OCN⁻, N₅⁻, Cl⁻, and NO₃⁻. ¹⁹F NMR experiments show that the binding of m-trifluorocresol stabilizes the R₄ state of zinc insulin. The chemical shift and line broadening of the CF₃ singlet, which occur due to binding, provide a useful probe of the T₆ to R₆ transition. Due to the appearance of new resonances in the aromatic region, the 500 MHz ¹H NMR spectrum of the phenol-induced R₆ hexamer is readily distinguishable from that of the T₆ form. ¹H NMR studies show that phenol induces the T₆ to R₆ transition, both in the (GlnB₁₃)₆(Zn²⁺)₆ hexamer and in the metal-free GlnB₁₃ species; we conclude that metal binding is not a prerequisite for formation of the R state in this mutant.

Current understanding of insulin structure-function interrelationships is derived primarily from insulin crystal structures and from correlations of biological potency with chemically modified and/or naturally occurring insulin mutants. Except for the structure of des-pentapeptide-(B₂₆–3₀) insulin (1, 2), all of the published insulin structures are aggregated species, i.e. the hagfish dimer (3, 4), the "2Zn," (4–8), the "4Zn" (4, 9–12), and the "phenol-induced" (13) insulin hexamers. The structure of monomeric insulin in solution is not known, nor is it obvious which insulin conformation(s) is (are) recognized by insulin receptors (14–16). Crystal structures (8, 12, 13) have established that hexameric zinc insulin can assume three quite different conformations, the 2Zn conformation (designated T₆), the 4Zn conformation (designated T₆R₆), and the phenol-induced conformation (designated R₆). The T₆, T₆R₆, and R₆ designations (17) appear to be gaining wide acceptance (13). Interconversion of the T and R conformations (the T to R transition) involves an extended chain to helix transition of residues B₁–B₈ (Fig. 1) in which some residues move by as much as 20–25 Å (12, 13). It is unclear what the relevance of either structure is to the biologically active form of insulin (the insulin monomer).

When subjected to lyotropic anions such as SCN⁻, I⁻, Br⁻, or Cl⁻ (11), three of the six subunits of the crystalline zinc insulin hexamer undergo the T to R conformational change to GlnB₁₃. Crystallization in the presence of phenol gives an R₆ hexamer in which all six subunits have undergone the T to R conformational change (13). This conformational transition creates six essentially identical, well defined pockets within the hexamer that bind phenol via hydrophobic and H-bonding interactions. The available spectroscopic evidence from ¹H NMR (18, 19) and circular dichroism (20, 21) and the rapid kinetic studies of Kaarsholm et al. (17) indicate that the conformational states found in the crystal can be induced in solution by the binding of lyotropic anions or by phenol. The X-ray crystallographic studies have shown that, in the crystal, the T₆ zinc hexamer (Fig. 1A) incorporates two Zn²⁺, each coordinated in an octahedral arrangement by three histidyl B₁₀ nitrogens and three water molecules. In the R₆ form (Fig. 1B), there are two identical zinc binding sites in which each Zn²⁺ is coordinated in a tetrahedral arrangement by three histidyl B₁₀ nitrogens and a water molecule. If the conformational behavior of the cobalt(II)-substituted insulin hexamer (In₀)(Co²⁺)₆, parallels that of the zinc hexamer, (In₀)(Zn²⁺)₆, then the different ligand fields experienced by the cobalt(II) ion in the T₆ and R₆ conformations should be manifested as distinct electronic spectral signatures of the two forms (cf. the behavior of carbonic anhydrase, carboxypeptidase, and alcohol dehydrogenase (22)).

In this communication, we establish that the T₆ to R₆ conformational transition occurs in the (In₀)(Co²⁺)₆ hexamer and induces a change in the ligand field about Co²⁺ from octahedral to tetrahedral. Via ¹H and ¹⁹F NMR, UV-visible, CD spectroscopy, and rapid kinetics, we have undertaken studies to investigate the nature of the T₆ to R₆ conformational transition and the phenol binding process. We report here our preliminary findings from these studies and show that these spectroscopic tools can be used to quantitate the kinetics and thermodynamics of the T₆ to R₆ transition in

* The abbreviations used are: human insulin hexamers are designated as (In₀)(M²⁺)₆, where M²⁺ is either Zn²⁺ or Co²⁺. The GlnB₁₃→Gln mutant hexamer is designated as (GlnB₁₃)₆, the metal-free hexamer; and as (GlnB₁₃)(Zn²⁺)₆, the zinc-substituted hexamer. The crystalline hexamers are designated as follows: T₆, 2Zn insulin; T₆R₆, 4Zn insulin; R₆, phenol-induced hexamer; ppm, parts per million.

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solution and that metal coordination is not a prerequisite for the conformation change.

EXPERIMENTAL PROCEDURES

Materials

The chemicals employed in these studies were reagent grade or better. Metal-free human insulin, GluB13 human mutant insulin, and phenol were supplied by Novo Research Institute (Denmark); m-trifluororesol and d-phenol were obtained from Aldrich. NaOD (40% solution), DCl (20% solution) and 2,2-dimethyl-2-silapentane-5-sulfonate-2,2,3,3-d4 were obtained from Sigma; D2O (99.8%) was purchased from Aldrich.

Methods

Metal-free insulin stock solutions containing <0.002 mol of Zn2+ per insulin monomer were prepared as described previously (6, 17, 19, 23). The metal ions of choice were added as required just prior to use.

UV-visible Spectra, CD Spectra, and Kinetic Studies—UV-visible spectra were collected on a Hewlett-Packard 8450A UV-visible spectrophotometer. CD spectra were recorded on a Jobin-Yvon Dichrograph Mark V. Single wavelength, rapid kinetic measurements and the kinetic analyses were made as described previously (23, 24). All experiments were carried out in 50 mM Tris-HCl, pH 8.0.

NMR Spectra—Samples for NMR spectroscopy were typically 1–2 mM insulin. 1H NMR spectra were recorded at 25 °C on a GN-500 spectrometer equipped with a Nicolet 1280 computer. Chemical shifts are reported in parts/million relative to the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonate-2,2,3,3-d4. 3P NMR spectra were recorded on a Nicolet NT300 NMR spectrometer. A capillary tube containing 60 mM m-trifluororesol as reference standard was inserted into the sample tubes. The temperature of the sample probe was thermostated at 26 °C. A D2O field frequency lock was used for both 3P and 1H NMR measurements.

RESULTS

The visible electronic absorption spectrum of (In)6(Co3+)2 is shown in Fig. 2A, a). The broad and weakly intense absorption band centered on 495 nm is typical of d → d transitions observed in octahedral cobalt(II) complexes (25). Fig. 2A, b–e, shows the effects of increasing concentrations of phenol on the 400–700 nm region of the absorption spectrum in the presence of Cl−. The appearance of intense bands (λmax = 580 nm, ε1 = 1000 M−1 cm−1, and λmax = 558 nm, ε2 = 700 M−1 cm−1 (shoulder)) are consistent with the d → d transitions expected for a tetrahedral cobalt(II)-Cl− complex (22, 25) and graphically illustrate that phenol promotes the conversion of the cobalt metal centers from octahedral to tetrahedral coordination geometry. Furthermore, marked changes occur in the CD spectrum of (In)6(Co3+)2 upon the addition of phenol. Fig. 2B, a, shows that the CD spectrum of (In)6(Co3+)2 exhibits a CD band centered on 500 nm. Fig. 2B, b, shows that, in the presence of 30 mM phenol and Cl−, intense CD spectral bands appear with negative components at 553 and 574 nm and a positive component at 633 nm. Collectively, these observations for (In)6(Co3+)2 are consistent with the spectral changes.
Fig. 3. A, effects of 10 mM d$_6$-phenol on the 500 MHz $^1$H NMR spectrum of the native zinc-insulin hexamer, (ln)$_6$(Zn$^{2+}$)$_2$, at pH* 8.0 D$_2$O. a, 2 mM (ln)$_6$(Zn$^{2+}$)$_2$; b, 2 mM (ln)$_6$(Zn$^{2+}$)$_2$ plus 10 mM d$_6$-phenol. Note the large changes in the spectrum, and in particular the new resonances at 0.2, 5.0, 5.2, 5.35, 5.7, and 6.3 ppm in b, 2,2-dimethyl-3-silapentane 5-sulfonate-2,2,3,3-td, (DSS), external standard. Inset to A, dependence of the area under the 6.3 ppm resonance (viz., spectrum b in A) on the concentration of phenol. B, comparison of the effects of phenol on the 500 MHz spectra of the metal-free GlnB13 mutant and on the (GlnB13)$_6$(Zn$^{2+}$)$_2$ hexamer in D$_2$O at pH* 8.0. a, 0.33 mM (GlnB13)$_6$(Zn$^{2+}$)$_2$; b, 0.33 mM (GlnB13)$_6$(Zn$^{2+}$)$_2$ plus 10 mM d$_6$-phenol; c, 2 mM metal-free mutant GluB13 plus 10 mM d$_6$-phenol. Internal standard (DSS).

predicted from consideration of the geometries of the zinc sites in the crystal structures of T$_6$ and R$_6$ zinc insulin hexamers. These findings establish that (ln)$_6$(Co$^{2+}$)$_2$ undergoes the phenol-induced T$_6$ to R$_6$ conformation change in an analogous manner to (ln)$_6$(Zn$^{2+}$)$_2$. The addition of CN$^-$, OCN$^-$, SCN$^-$, Cl$^-$, or NO$_2$ ion to phenol-saturated (ln)$_6$(Co$^{2+}$)$_2$ results in distinct changes in the spectrum. Fig. 2A, f, shows the effect of adding CN$^-$ to (ln)$_6$(Co$^{2+}$)$_2$ in the presence of a saturating concentration of phenol on the 400–700 nm region. The spectral changes are attributed to the substitution of CN$^-$ at each tetrahedral Co$^{2+}$ of the R$_6$ hexamer. The resulting modification of the Co$^{2+}$ ligand field caused by the CN$^-$ coordination accounts for the shift in wavelength and intensity of the d $→$ d absorption bands ($\lambda_{\text{max}}$ = 574 nm, $\epsilon_1$ = 1250 M$^{-1}$ cm$^{-1}$, $\lambda_{\text{max}}$ = 550 nm, $\epsilon_2$ = 900 M$^{-1}$ cm$^{-1}$ (shoulder)). Binding curves for the phenol-(ln)$_6$(Co$^{2+}$)$_2$ system at pH 8.0 were determined by measuring the absorbance at 580 nm relative to the concentration of added phenol (data not shown). Attempts to fit these curves indicate that an expression with greater complexity than that of a single rectangular hyperbola is needed to describe the phenol concentration dependence. The kinetic time course for the phenol-induced T$_6$ to R$_6$ transition (data not shown) is biphasic and consists of an initial fast phase (1/\tau_1 = 0.42 s$^{-1}$), followed by a slow phase (1/\tau_2 = 0.062 s$^{-1}$) that is complete after approximately 20 s.

Fig. 3A shows the changes in the aromatic region of the $^1$H NMR spectrum that occur in the phenol-induced conversion of (ln)$_6$(Zn$^{2+}$)$_2$ from the T$_6$ state (a) to the R$_6$ state (b). The aromatic region and the region between 5.0 and 6.5 ppm show remarkable changes; there are new peaks in the R$_6$ state spectrum at 5.00, 5.25, 5.40, 5.72, 5.80, 6.37, and 6.65 ppm, many of the resonances in the aromatic envelope have undergone significant changes in chemical shift, and the HisB5 and B10 C-2 proton resonances have disappeared. These signals allow quantitation of the fraction of insulin subunits that have undergone the T to R conformation change. Measurement of the area under the 6.27 ppm resonance as a function of the phenol concentration (Fig. 3A, inset) shows the occurrence of a saturation phenomenon with $K_{\text{eq}}$ $\sim$ 2 mM.

The spectra in Fig. 3B show that phenol induces the T$_6$ to R$_6$ transition both in the mutant (GlnB13)$_6$(Zn$^{2+}$)$_2$ hexamer (compare spectra a and b and in the metal-free GlnB13 species spectrum c). Comparison of spectra b and c strongly suggests that the metal-free species is a hexamer with the R$_6$ conformation.

To further investigate the binding process, we have utilized $^1$H NMR spectroscopy to qualitatively study the interaction of m-trifluorocresol with zinc-insulin by observing the chemical shift of the CF$_3$ singlet. The $^1$F NMR spectra (Fig. 4),
measured with varying concentrations of m-trifluorocresol show that in the presence of \((\text{In})_6(\text{Zn}^{2+})_2\), the CF₃ singlet is broadened and shifts downfield. The observed single indicates that m-trifluorocresol is in rapid to intermediate exchange (relative to the NMR time scale) between the insulin-bound environment and that of the solution. The line broadening, which usually occurs when a small molecule binds to a macromolecule, is interpreted as indicative of intimate contact between the fluorine atoms of m-trifluorocresol and the phenol binding site of the zinc-insulin hexamer. Spectra a–c show that in the presence of \((\text{In})_n(\text{Zn}^{2+})_2\) the chemical shift of the CF₃ singlet is dependent upon the m-trifluorocresol concentration. The CF₃ resonance is shifted upfield as the m-trifluorocresol concentration is increased from 0.5 to 5.0 mM, reflecting the influence of the increasing proportion of unbound m-trifluorocresol on the averaged chemical shift. Spectrum d shows the effect of incorporating 17 mM \(d_4\)-phenol into a sample of 2 mM zinc-insulin with 2.5 mM m-trifluorocresol. Comparison of spectra b and d indicates that phenol displaces m-trifluorocresol from the binding sites of the insulin hexamer and that the position of the CF₃ resonance shifts toward that of the unbound ligand with an accompanying narrowing of the linewidth. Control experiments performed in the absence of insulin show that the chemical shift of the m-trifluorocresol CF₃ resonance is essentially invariant over the concentration range 0.5 to 5.0 mM and also invariant to the presence of phenol.

**DISCUSSION**

In the hemoglobins of higher organisms, the binding of dioxygen forces a change in the coordination geometry of the heme iron from 5- to 6-coordinate. This reduces the Fe–N bond distances by ~0.2 Å, allowing the iron to move ~0.6 Å into the heme plane. The 0.6-Å motion triggers a conformation change that alters subunit interfaces, moves hinge groups on the \(\beta\) subunits closer together by as much as 6 Å and increases the affinity for dioxygen by 2 orders of magnitude (26–28). The single-crystal x-ray diffraction studies of Derewenda et al. (13) show that the phenol-mediated \(T_6\) to \(R_e\) transition of \((\text{In})_n(\text{Zn}^{2+})_2\) involves a gross molecular re-arrangement of all six insulin B chains in which B1 moves 20–25 Å. This allosteric transition changes the topography of the hexamer surface, creates six binding sites for phenol and dictates a new metal coordination geometry at the HisB10 sites (Fig. 1).

Our spectroscopic and kinetic studies establish that phenol or m-trifluorocresol mediates large changes in the conformations of the (GlnB13)₉(\(\text{Zn}^{2+}\))₉, (In)₉(\(\text{Zn}^{2+}\))₉, (In)₉(\(\text{Co}^{2+}\))₉, and metal-free (GlnB13)₉ hexamers in solution. Since the absorbance and CD electronic spectral changes induced by phenol (Fig. 2) are only reasonably explained as arising from the transformation of the Co²⁺ ligand field from octahedral to tetrahedral, we conclude that the phenol-mediated spectroscopic signatures identified in these studies have their origins in a conformational change that corresponds to an allosteric transition between the crystallographically identified \(T_6\) and \(R_e\) states. Using the same spectroscopic criteria, it appears that SCN⁻ and OCN⁻ also induce \(T\) to \(R\) transitions in the \(\text{Zn}^{2+}\) and \(\text{Co}^{2+}\)-substituted hexamers (data not shown).

The GlnB13 carboxylates of \((\text{In})_n(\text{Zn}^{2+})_2\) have been shown to form a cage that binds Ca²⁺ (19, 29–32). In the absence of Ca²⁺ or other divalent metal ions, Coulombic charge repulsions between the six B13 Glu carboxylates destabilize the hexamer (19, 30, 33). The GlnB13 mutant (34) lacks this Ca²⁺ site and was designed to enhance the stability of the hexamer and thus slow the release of insulin following subcutaneous injection in diabetes therapy.² Osmotic pressure molecular weight studies (data not shown) show that the metal-free GlnB13 mutant is predominately a molecular weight 36,000 species at pH 7.5 and 1–10 mg/mL, while in the same concentration range, the molecular weight of metal-free human insulin is variable and less than 36,000. The data in Fig. 3 indicate that phenol induces a conformational change in the metal-free GlnB13, that is highly similar to that induced in \((\text{In})_n(\text{Zn}^{2+})_2\) and in (GlnB13)(\(\text{Zn}^{2+}\))₂; hence, metal binding is not essential for the allosteric transition. Circular dichroism studies on the GlnB13 mutant (35) have led to similar conclusions. Under the same conditions of pH and insulin concentration, we find no evidence that phenol induces an \(R_e\) state in metal-free human insulin. The rate of the \(T\) to \(R\) transition is 6–8 orders of magnitude slower than that expected for a simple coil-to-helix peptide transition in solution (36).

In the \(T\) state, the HisB10 zinc sites are positioned at the bottom of shallow clefts at opposite ends of the cylindrically shaped hexamer, Fig. 1B. The three water molecules coordinated to each octahedral \(\text{Zn}^{2+}\) ion extend out into the cleft and can be readily replaced by tridentate chelators (17, 23, 30, 31). In the \(R_e\) state, the HisB10 sites are buried, and the water molecules access the tetrahedrally coordinated \(\text{Zn}^{2+}\) ion via an 8-Å-long tunnel that is too narrow to accommodate large tridentate chelators (12, 17). Our studies (Fig. 2A) show that in the \(R_e\) form of \((\text{In})_n(\text{Co}^{2+})_2\), this site is accessible to small anions such as \(\text{CN}^-, \text{OCN}^-, \text{N}^-, \text{SCN}^-, \text{NO}_2^-, \text{Cl}^-\), and that these anions can replace the coordinated \(\text{H}_2\text{O}\) with an accompanying change in the UV-visible electronic spectrum. Phenol binding stabilizes all six of the insulin subunits in the \(R\) conformation, giving an \(R_e\) structure, whereas in the crystalline state, the transition induced by lyotropic anions converts only three of the six insulin subunits to the \(R\) state, giving a \(T_6R_e\) structure. The anion-induced \(T_6R_e\) crystalline hexamer may be constrained to the \(T_6R_e\) state by crystal lattice forces (10, 12). It is not known whether or not the \(T_6R_e\) state is a stable species in solution. Four positions and three types of zinc sites are identified in the \(T_6R_e\) crystal structure. Some of these sites involve both HisB5 and HisB10. However, the total \(\text{Zn}^{2+}\) occupancy was calculated to be 2.67 (12). In the phenol-induced \(R_e\) structure, there are only two identical, high affinity tetrahedral \(\text{Zn}^{2+}\) sites (Fig. 1B), each one obvious top of three HisB10 B chains in one monomer molecule. Phenol is bound via a large number of van der Waals contacts between the ring and various side chain atoms of the A and B chains and via two hydrogen bonds between the phenolic hydroxyl and the A chain backbone, one to the carbonyl oxygen of CysA6, the other to the amide N-H of CysA11 (13). The side chains of both HisB5 and HisB10 (from adjacent subunits) come close to the same phenol ring. The disappearance of the HisB5 and B10 C-2 proton NMR signals (Fig. 3) probably is due to anisotropic ring current effects from the phenol molecules which shift these signals upfield. Either the C-2 proton signals are located under the aromatic envelope (6.5 to 7.5 ppm) or they appear as new signals between 5.0 and 6.5 ppm. The resonances located between 5.0 and 6.5 ppm almost certainly are due to ring current effects either from phenol or from new interactions involving the aromatic side chains of insulin.

The spectropolarimetric titration of \((\text{In})_n(\text{Co}^{2+})_2\) with phenol gives rise to an isotherm which may be approximated by two hyperbolic functions. This suggests that the binding of phenol is a negative cooperative process which would presumably arise from allosteric interactions in the \(T\) state to \(R\) transition.

² J. Brange, manuscript in preparation.
The high resolution x-ray structures of the T₆, T₃R₃, and R₆ states, show that the phenol- and anion-mediated conformation changes of the insulin hexamer are interesting examples of an allostERIC transition. Because there are only a few examples of allosteric systems where the details of the changes in three-dimensional structure are well defined, this allostERIC transition seems worthy of further investigation. The spectroscopic probes described in this study make it possible to carry out detailed thermodynamic and kinetic investigations to determine the mechanism of the transition.

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