Zinc Coordination, Asymmetry, and Allostery of the Human Insulin Hexamer

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Zinc coordination plays a functional role in the assembly, storage, and secretion of insulin. The physiological storage form of this hormone is believed to be the zinc hexamer,[1,2] and it is the zinc hexamer that is the primary component of all insulin pharmaceutical preparations used for the treatment of diabetes mellitus. The innovation of therapeutically improved insulin preparations requires a detailed understanding of hexamer assembly and structural selectivity in solution. Furthermore, the zinc insulin hexamer is an important model system for investigating fundamental mechanisms of intramolecular communication in multisubunit assemblies where cooperativity is mediated through protein subunit−subunit contacts. This report describes a novel chiroptical approach to probing the Zn(II) sites, revealing new intricacies of the Zn(II) coordination in the Zn-(II)-T_6 hexamer.[3]

The first X-ray crystal structure determinations[4] of the zinc insulin hexamer established that the hexamer exists as an oblate spheroidal assembly comprising three asymmetric dimers arrayed about a 3-fold crystallographic axis of symmetry. This arrangement generates two slightly different Zn(II) trimers, in which the Zn(II) ions exist in distorted octahedral Zn(II)-$(His^{B10})_3(H_2O)_3$ coordination geometries. This structure (subsequently designated Zn(II)-T_6)[3] has provided an elegant classical basis for understanding the oligozinc coordination fundamental to the physiological state and to many of the solution physicochemical and biological properties of insulin.[2,4] More recently, many additional crystal structures[5,6] have established that the conformation of each trimer within the hexamer is highly dependent upon the crystallization conditions, principally upon the identity and concentration of certain phenolic ligands, halide and pseudohalide anions.[7] Current understanding of this conformational and ligand-binding behavior is based on a three-state allosteric system in which the Zn(II)-T_3R_3 species has been proposed as an intermediate in the T_6-to-R_6 transition.[8,9] It has been generally assumed that octahedral coordination is representative of T_3 trimers, and pseudotetrahedral coordination is representative of R_3 trimers in solution.[8,9]

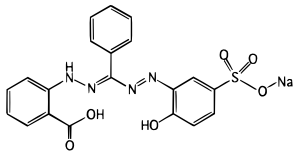

Figure 1. Zincon, monosodium salt.

The zinc insulin hexamer forms a colored complex[10] with the achiral, chelatometric zinc indicator 2-(1-(2-hydroxy-5-sulfophenyl)-3-phenyl-5-formazano)benzoic acid (zincon),[11] possessing band maxima at 630, 540, and 479 nm (Figure 2A-(a)). Complexation of zincon to the hexamer is confirmed by the induced circular dichroism that resolves the absorption spectrum into components with extrema at 422, 540, and 645 nm (Figure 2B). This induced CD is attributed to excitonic coupling of the zincon transition dipole moments dissymmetrically oriented within the hexamer, thus indicating intimate chromophoric contact between the zincon ligand and the insulin subunits. A spectropolarimetric titration of the 660 nm CD indicates a stoichiometry of 1 mol of zincon coordinated per mol of hexamer (Figure 2, inset). This result is surprising, because the crystal structure[4] of the Zn(II)-T_6 hexamer shows that the two Zn(II) ions reside in very similar distorted octahedral Zn(II)$(His^{B10})_3(H_2O)_3$ environments. It is proposed that the zincon ligand coordinates selectively to insulin-bound Zn(II) sites that have multiple open coordination positions.[12] Thus it is inferred that the Zn(II)-T_6-zincon complex exists with two coordinatively dissimilar Zn(II) centers consistent with an asymmetric Zn(II)-T_6 species in which one Zn(II) center is octahedral and the other pseudotetrahedral. The data of Figure 2 (inset) suggest that the coordination of one zincon ligand to the preformed hexamer induces conformational effects that significantly decrease the affinity of zincon for the second Zn-(II) site. The zincon ligand could potentially exert such effects by distorting the *tris*-His^{B10} geometry or by displacing one of the coordinated His^{B10} groups via a meridionyl coordination mode. These results show that in solution the Zn(II) ions of the Zn(II)-T_6 hexamer are not constrained to an arrangement with three open coordination positions.[4] In view of the crystallographic identification of both octahedral and pseudotetrahedral Zn(II) geometries in T_3 trimers of Zn(II)-T_3R_3

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(1) Steiner, D. F. *Diabetes* **1977**, *26*, 322−340.

(2) Blundell, T.; Dodson, G.; Hodgkin, D.; Mercola, D. *Adv. Prot. Chem.* **1972**, *26*, 279−402.

(3) Crystal structures of zinc−hexamer complexes have identified two general monomer subunit conformations designated T and R. The T-state conformation corresponds to that identified in the first hexamer crystal structure.[4] In the R-state, residues B1-B8 exist in partial (B4-B8) or fully α-helical conformations. The Zn(II)-R_6 hexamer possesses six hydrophobic phenolic-binding pockets. The Zn(II)-T_3 Zn(II) ion is accommodated in a shallow open cleft, whereas in the Zn(II)-R_3 trimer it is a considerably less accessible, trigonally distorted tetrahedral Zn(II)$(His^{B10})_3$(L) arrangement where L is a small molecule ligand.

(4) (a) Adams, M. J.; Baker, E. N.; Blundell, T. L.; Harding, M. M.; Dodson, E. J.; Hodgkin, D. C.; Dodson, G. G.; Rimmer, B.; Vijayan, M.; Sheat, S. *Nature* **1969**, *224*, 491−495. (b) Peking Insulin Structure Group. *Peking Rev.* **1971**, *40*, 11−16. (c) Baker, E. N.; Blundell, T. L.; Cutfield, J. F.; Cutfield, S. M.; Dodson., E. J.; Dodson, G. G.; Hodgkin, D. M. C.; Hubbard, R. E.; Isaacs, N. W.; Reynolds, C. D.; Sakabe, K.; Sakabe, N.; Vijayan, N. M. *Philos. Trans. R. Soc. Lond.* **1988**, *B319*, 369−456.

(5) (a) Smith, G. D.; Swenson, D. C.; Dodson, E. J.; Dodson, G. G.; Reynolds, C. D. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 7093−7097. (b) Derewenda, U.; Derewenda, Z.; Dodson, E. J.; Dodson, G. G.; Reynolds, C. D.; Smith, G. D.; Sparks, C.; Swenson, D. *Nature* **1989**, *338*, 594−596. (c) Smith, G. D.; Dodson, G. G. *Biopolymers* **1992**, *32*, 441−445. (d) Smith, G. D.; Dodson, G. G. *Proteins: Struct. Funct. Genet.* **1992**, *14*, 401−408. (e) Whittingham, J. L.; Chaudhuri, S.; Dodson, E. J.; Moody, P. C. E.; Dodson, G. G. *Biochemistry* **1995**, *34*, 15553−15563. (f) Smith, G. D.; Ciszak, E.; Pangborn, W. *Prot. Sci.* **1996**, *5*, 1502−1511.

(6) (a) Ciszak, E.; Smith, G. D. *Biochemistry* **1994**, *33*, 1512−1517. (b) Smith, G. D.; Ciszak, E. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8851−8855.

(7) De Graaff, R. A. G.; Lewit-Bentley, A.; Tolley, S. P. *Structural Studies on Molecules of Biological Interest*; Dodson, G., Glusker, J. P., Sayre, D., Eds.; Clarendon Press: Oxford, 1981; pp 547−556.

(8) (a) Brzovic, P. S.; Choi, W. E.; Borchardt, D.; Kaarsholm, N. C.; Dunn, M. F. *Biochemistry* **1994**, 33, 13057−13069. (b) Bloom, C. R.; Choi, W. E.; Brzovic, P. S.; Ha, J. J.; Huang, S. T.; Kaarsholm, N. C.; Dunn, M. F. *J. Mol. Biol.* **1995**, *245*, 324−330. (c) Birnbaum, D. T.; Dodd, S. W.; Saxberg, B. E. H.; Varshavsky, A. D.; Beals, J. M. *Biochemistry* **1996**, *35*, 5366−5378. (d) Choi, W. E.; Borchardt, D.; Kaarsholm, N. C.; Brzovic, P. S.; Dunn, M. F. *Proteins: Struct. Funct. Genet.* **1996**, *26*, 377−390.

(9) Jacoby, E.; Kruger, P.; Karatas, Y.; Wollmer, A. *Biol. Chem. Hoppe-Seyler* **1993**, *374*, 877−885.

(10) Highly purified metal-free biosynthetic human insulin, analytical grade zincon, and Zn(II) solutions prepared from atomic absorption standards were used. CD measurements utilized an Aviv 62A DS circular dichroism spectrometer equipped with a computer-controlled Hamilton Microlab titrator and thermoelectric cuvette holder.

(11) Rush, R. M.; Yoe, J. H. *Anal. Chem.* **1954**, *26*, 1345−1347.

(12) Addition of phenol to the Zn(II)-T_6-zincon complex causes the spectrum of Figure 2A (a) to revert to that of uncoordinated zincon, indicating that zincon does not coordinate to the sterically encumbered tetrahedral sites of the Zn(II)-R_6 hexamer.

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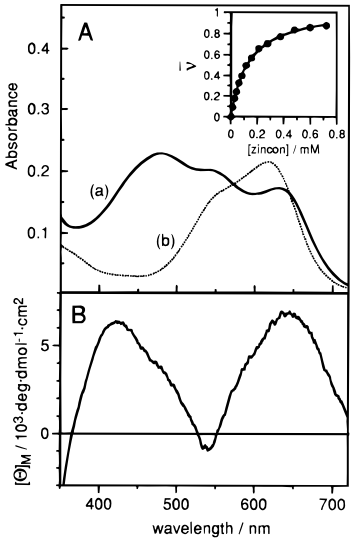

Figure 2. Absorbance spectrum (panel A spectrum (a)) and CD spectrum (panel B) of the Zn(II)-T_6-zincon complex formed with 0.21 mM zinc insulin hexamer in the presence of 0.016 mM zincon. Spectrum (b) of panel A shows the absorbance spectrum of 0.008 mM zincon in the presence of 0.42 mM Zn(II). Spectra were recorded in 50 mM Tris-$HClO_4$, pH = 7.50, at 22 °C. Absorbance and CD spectra utilized 1 and 5 cm pathlength cuvettes, respectively. CD is expressed as molar elipticity, $[\Theta]_M$, calculated with respect to zincon concentration. Inset: Plot of zincon binding to the Zn(II)-T_6 hexamer. The values of $\bar{\nu}$ were determined from a 660 nm spectropolarimetric titration. The experiment was performed in the presence of 0.20 mM hexamer in 50 mM Tris-$HClO_4$, pH = 7.50 at 22 °C. The solid curve has been generated from the binding equation, $\bar{\nu} = nK[\text{zincon}]/(1 + K[\text{zincon}])$, with the number of binding sites $n = 1.05$ and association constant $K = 7.47 \times 10^3\ M^{-1}$.

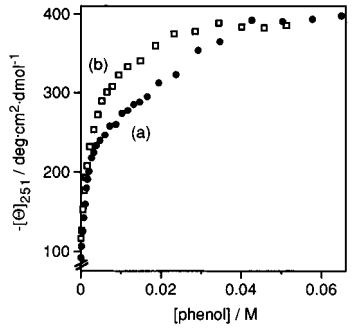

Figure 3. Spectropolarimetric titration of the Zn(II)-insulin hexamer (0.31 mM) with phenol performed in the presence (a) and absence (b) of 0.8 mM zincon. The titrations were performed in 50 mM Tris-$HClO_4$, pH = 7.50 at 22 °C. CD is expressed as mean residue ellipticity calculated using a mean residue weight of 113.9.

complexes,[6] it is deduced that in the absence of zincon, each T_3 trimer of the Zn(II)-T_6 hexamer may accommodate its Zn(II) ion in either an octahedral $Zn(II)(His^{B10})_3(H_2O)_3$ or pseudotetrahedral $Zn(II)(His^{B10})_3(H_2O)$ geometry in solution.

Binding isotherms corresponding to the titration of the zinc hexamer with phenol are shown in Figures 3 and 4. These isotherms correspond to two phenol-binding processes attributed to binding to the first trimer followed by a lower affinity binding to the second trimer in the T_6-to-R_6 transition.[8,9] The 251 nm CD of the Zn(II)-insulin hexamer has been monitored as a function of phenol concentration in the presence and absence of zincon (Figure 3). The 251 nm CD arises primarily from the disulfide chromophores and has been shown to provide a useful signature of insulin subunit conformation in the T_6-to-R_6 transition.[9] Figure 3 shows that the coordination of zincon

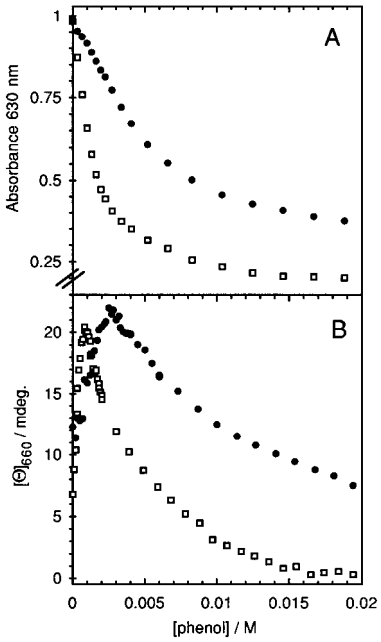

Figure 4. Spectrophotometric (A) and spectropolarimetric (B) titrations of the zinc insulin hexamer with phenol. The solution conditions were 0.32 mM zinc insulin hexamer with 0.27 mM zincon in 10 mM Tris-$HClO_4$, pH 7.50 at 22 °C. Open and solid symbols correspond, respectively, to titrations performed in the presence and absence of 25 mM Cl^-. The cell pathlengths were 0.5 and 1 cm, respectively, for the absorption and CD measurements.

accentuates the biphasicity of the phenol-binding transition, probably due to a stabilizing effect of the chelating zincon ligand on the T_6 and T_3R_3 states relative to the R_6 state. Notably, Figure 4B shows that titration of the first trimer causes a significant increase in the magnitude of induced CD at 660 nm, despite a major decrease in the absorbance of this chromophore (Figure 4A). This result suggests that the induced circular dichroism of the zincon ligand coordinated to the Zn(II)-T_3 trimer has increased significantly as a result of phenol binding to the opposing Zn(II)-R_3 trimer of the Zn(II)-T_3R_3 species. This intensification is consistent with the formation of a Zn(II)R_3-Zn(II)T_3(zincon) species in which the extrachromophoric environment of the zincon ligand is considerably more conformationally rigid[13] than it is in the Zn(II)-T_6(zincon) complex. This effect demonstrates structural tensioning and thus provides insight into the origins of the negative cooperativity between trimers[8,9] by suggesting that the conformational fluctuations of the T_3 trimer necessary to initiate formation of the phenol-binding pockets[14] become constrained upon formation of the T_3R_3 species. The data of Figure 4 also show that both the T_6-to-T_3R_3 and T_3R_3-to-R_6 transitions are modulated strongly by chloride, in accord with the observation that Zn(II)-T_3R_3 hexamers crystallize from solutions containing high concentrations of lyotropic anions.[7] Collectively, these results show that the sensitivity of the protein-bound Zn(II)-zincon chromophore to the chiral environment of the protein makes it a useful spectral reporter of allosteric conformational effects within the insulin hexamer.

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(13) Yoshida, N.; Yamaguchi, H.; Higashi, M. *J. Chem. Soc., Perkin Trans. 2* **1994**, 2507−2513.
(14) Jacoby, E.; Hua, Q. X.; Stern, A. S.; Frank, B. H.; Weiss, M. A. *J. Mol. Biol.* **1996**, *258*, 136−157.