Structural signatures of the complex formed between 3-nitro-4-hydroxybenzoate and the Zn(II)-substituted R₆ insulin hexamer

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Abstract

3-Nitro-4-hydroxybenzoate (3N4H) is a probe of the structure and dynamics of the metal-centered His B10 assembly sites of the insulin hexamer. Each His B10 site consists of a ~12 Å-long cavity situated on the threefold symmetry axis. These sites play an important role in the storage and release of insulin in vivo. The allosteric behavior of the insulin hexamer is modulated by ligand binding to the His B10 zinc sites and to the phenolic pockets. Binding to these sites drives transitions among three allosteric states, designated T₆, T₃R₃, and R₆. Although a wide variety of mono anions bind to the His B10 zinc sites of R₃, X-ray structures of ligands complexed to this site exist only for H₂O, Cl⁻, and SCN⁻. This work combines one- and two-dimensional ¹H NMR and UV-Vis absorbance studies of the structure and dynamics of the 3N4H complex, which establish the following: (1) relative to the NMR time scale, 3N4H exchange between free and bound states is slow, while flipping among three equivalent orientations about the site threefold axis is fast; (2) binding of 3N4H perturbs resonances within the His B10 zinc site and generates NOEs between ligand resonances and the insulin C-α and side chain resonances of ValB2, AsnB3, LeuB6, and CysB7; and (3) 3N4H exchange for other ligands is limited by a protein conformational transition. These results are consistent with coordination of the 3N4H carboxylate to the His B10 zinc ion and van der Waals interactions with Val B2, Asn B3, Leu B6, and Cys A7.

Keywords: Insulin allostery; positive and negative cooperativity; His B10 sites; NMR spectroscopy

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The insulin hexamer is an allosteric protein that exhibits both positive and negative cooperativity and half-of-the-

sites reactivity in ligand binding (Kaarsholm and Dunn 1987; Kaarsholm et al. 1989; Roy et al. 1989; Brader and Dunn 1991; Brader et al. 1991; Choi et al. 1993, 1996; Brzovic et al. 1994; Bloom et al. 1995, 1997a,b, 1998). This allosteric behavior consists of two interrelated allosteric transitions designated L^A₀ and L^B₀, three interconverting allosteric conformation states (Fig. 1A), designated T₆, T₃R₃, and R₆ (Kaarsholm et al. 1989; Bloom et al. 1995, 1997a,b) and two classes of allosteric ligand binding sites designated as the phenolic pockets and the His B10 anion sites (Fig. 1A,B; Derewenda et al. 1989; Kaarsholm et al. 1989; Huang et al. 1997). These allosteric sites are associ-

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Abbreviations: 3N4H, 3-nitro-4-hydroxybenzoate; T₆, T₃R₃, and R₆, the three allosteric conformation states of the insulin hexamer; NOE, nuclear overhauser effect; NOESY, nuclear overhauser effect spectroscopy; TOCSY, total correlated spectroscopy; FID, free induction decay.

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Figure 1. (A) Cartoon depicting the allosteric model for the interconversion of the T₆, T₃R₃, and R₆ conformations of the insulin hexamer. The hexamers are viewed along the threefold symmetry axis with subunits represented by circles (T-state, light and dark blue) and ellipses (R-state, red and yellow), respectively. The zinc ion bound to the His B10 sites is shown as a brown circle located on the threefold symmetry axis. The phenolic pockets are represented as silver circles (three in T₃R₃, and six in R₆). (B) Molecular model of the R₃ unit of the insulin hexamer viewed down the threefold symmetry axis, presenting the B1–B15 part of each helix segment. The Zn-site pocket is shown in surface representation and the Zn ion as a black ball. (C) View perpendicular to the threefold symmetry axis in same representation as (B). Structures are drawn from PDB file, 1EVV (Smith et al. 2000).

ated only with insulin subunits in the R conformation (Derewenda et al. 1989, 1991; Kaarsholm et al. 1989; Roy et al. 1989; Brader and Dunn 1991; Brader et al. 1991; Smith and Dodson 1992a,b; Ciszak and Smith 1994; Bloom et al. 1995, 1997a,b; Whittingham et al. 1995; Birnbaum et al. 1996; Huang et al. 1997; Smith et al. 2000). While the *in vivo* function of this allosteric behavior is not known, these properties of the insulin hexamer provide an interesting and relatively well-characterized paradigm for the allosteric phenomena designated as negative cooperativity and half-of-the-sites reactivity in ligand binding (Brzovic et al. 1994; Bloom et al. 1995, 1997a,b).

The T-to-R transition of the insulin hexamer involves transformation of the first nine residues of the B chain from an extended conformation in the T-state to a α -helical conformation in the R-state (Derewenda et al. 1989). This coil-to-helix transition causes the N-terminal residue, Phe B1, to undergo a ~ 30 Å change in position. This change in conformation creates hydrophobic pockets (the phenolic pockets) at the subunit interfaces (three in T₃R₃, and six in R₆), and the new B-chain helices form 3-helix bundles (one in T₃R₃ and two in R₆) with the bundle axis aligned along the

hexamer threefold symmetry axis. The His B10 Zn(II) in each R₃ unit (Fig. 1) is forced to change coordination geometry from octahedral to either tetrahedral (monodentate ligands) or pentahedral (bidentate ligands). Formation of the helix bundle creates a narrow hydrophobic tunnel in each R₃ unit that extends ~ 12 Å from the surface down to the His B10 metal ion (Fig. 1). This tunnel and the His B10 Zn(II) ion form the anion binding site (Brader and Dunn 1991; Brader et al. 1991, 1997; Choi et al. 1993, 1996; Brzovic et al. 1994; Bloom et al. 1995, 1997a,b, 1998; Huang et al. 1997). Two detailed studies of the structure of the Zn²⁺–R₆ hexamer in solution via NMR have been reported (Jacoby et al. 1996; Chang et al. 1997). These studies showed a high degree of correspondence between the solution structures and the crystal structures.

Most ligand binding sites in proteins are highly asymmetric. Because the HisB10 Zn(II) anion sites reside on the threefold symmetry axis (Fig. 1), these sites possess a symmetry that is unusual, but not unique. Several other proteins have highly symmetric ligand binding sites. For example, the HIV-1 protease has two identical subunits and a single catalytic site that resides on the subunit interface. Owing to the D2 symmetry of this dimeric protein, the substrate binding site cavity also has twofold symmetry (Navia et al. 1989). The hemoglobin allosteric site for phosphorylated ligands (the 2,3-diphosphoglycerate site) is located in the tunnel that passes through the Hb tetramer. This site has a true twofold symmetry and a pseudo-tetrahedral symmetry (Perutz 1989). The catalytic chamber of the *Thermoplasma acidophilum* proteasome multienzyme complex has D7 symmetry (Lowe et al. 1995).

As shown in Figure 1B, the HisB10 anion site consists of a tunnel or cavity with an approximately triangular-shaped cross-section. The dimensions of this cross-section vary along its length and depend on the crystal form. The coordinates of a number of different R-state human insulin hexamers have been deposited with the PDB. These structures involve different ligands residing in the phenol pockets and the Zn²⁺ anionic sites, respectively. However, the protein portions of these structures are either R₆ or T₃R₃. In the R₆ structures the B-chain helix extends from B19 through B1, while in T₃R₃, the R-state B-chain helices appear “frayed,” that is, the helix stops at B3. The degree of definition of the B1–B2 residues is highly variable among the deposited structures; however, the difference between the extended (of R₆) and the “frayed” (of T₃R₃) R-helices give rise to two different conformations of AsnB3. The walls of the tunnel leading to Zn²⁺ are made up of the side chains of the amino acid residues along one face of each α -helix. Because the helix side chains that make up the lining of the tunnel are Phe B1, Asn B3, and Leu B6, the relatively poor definition of B1 and the two conformations of B3 lead to variations at the opening of the tunnel as judged by crystal structures. Except for the zinc ion, which

is coordinated to three HisB10 residues and is positioned at the bottom of the tunnel, the site is principally hydrophobic. Depending on the structure of the ligand, it may be possible for substituents on the ligand to make H-bonding interactions with the amido group of Asn B3.

Huang et al. (1997) reported that the chromophoric ligand, 3-nitro-4-hydroxybenzoate (3N4H), binds to the His B10 sites of R-state hexamers with a relatively high affinity. Bloom et al. (1998) developed 3N4H for use as an indicator of the allosteric transitions of the insulin hexamer. Ferrari et al. (2001) investigated the vibrational spectrum of the 3N4H complex via Raman spectroscopy. Therefore, 3N4H has emerged as a sensitive probe of the structure and dynamics of the metal-centered protein assembly site of the insulin hexamer, the His B10 site, a site that plays an important role in the storage and timely release of insulin in vivo (Rahuel-Clermont et al. 1997).

The work presented herein, is undertaken with the objective of determining the set of weak bonding interactions between 3N4H and the HisB10 anion site that account for the electronic and vibrational spectroscopic properties of bound 3N4H. To this end, we present optical spectroscopy and one- and two-dimensional ^1H NMR studies to further characterize the structure of the complex formed between the Zn(II) R_6 hexamer and 3N4H.

Results

Spectroscopic and kinetic characterization of 3N4H binding

The data presented in Figure 2 summarize some of the spectroscopic properties and kinetic behavior that characterize the binding of 3N4H to the His B10 sites of the R_6 human insulin hexamer. At pH values where 3N4H exists as the dianion, the free ligand exhibits a long wavelength, π - π^* absorption band (Fig. 2A, spectrum 1) with $\lambda_{\text{max}} = 410$ nm ($\epsilon_{410} = 4.3 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$). When bound to the His B10 site of an Zn(II)- R_3 trimeric unit of either R_6 or T_3R_3 , this band is red-shifted to 426 nm and the extinction coefficient is slightly increased ($\epsilon_{426} = 5.2 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$; Fig. 2A, spectrum 2). The difference spectrum (bound-free ligand) has a maximum at 440 nm and a minimum at 380 nm (data not shown; Huang et al. 1997; Bloom et al. 1998). The 3N4H spectrum is similarly red-shifted when bound to the Co(II)-substituted R_6 hexamer (Fig. 2A, spectrum 3; Brader et al. 1990, 1997; Huang et al. 1997; Bloom et al. 1998). The circular dichroism (CD) spectra of the 3N4H complex formed with the Zn^{2+} and Co^{2+} - R_6 hexamers also provide useful signatures of the 3N4H interaction with the His B10 anion site (Huang et al. 1997; Bloom et al. 1998). These signatures can be used, for example, to determine the apparent dissociation constant for the binding of 3N4H (Huang et al. 1997; Bloom et al. 1998), to observe the

Figure 2. Summary of UV/Vis absorption spectroscopic properties and kinetic behavior for the binding of 3N4H to the HisB10 sites of the Zn(II) and Co(II) R_6 human insulin hexamers. (A) UV/Vis spectra showing the π - π^* absorption bands of 3N4H free (1) and 3N4H bound to the Zn(II) (2) hexamer measured under conditions where the hexamer is present in large excess over 3N4H (Huang et al. 1997). When 3N4H binds to the Co(II)-substituted R_6 hexamer, again the spectrum of 3N4H (3) is red-shifted. Spectrum (4) is the spectrum of the phenol stabilized R_6 Co(II) hexamer. The d,d transitions in spectra (3) and (4) are dominated by the large excess of the R_6 Co(II) hexamer with phenolate bound to the His B10 sites. (Fig. 2A is redrawn from Huang et al. 1997.) (B) Typical stopped-flow rapid mixing time course for the displacement of 3N4H by Cl^- , measured by following the disappearance of the red-shifted absorption spectrum of the Zn^{2+} - R_6 -3N4H complex following mixing of the complex with Cl^- . The dependence of the relaxation rate on the concentration of Cl^- is shown in (C), and the dependence of the rate on the concentration of 3N4H is shown in (D).

kinetic time course for 3N4H binding and displacement, and to investigate the allosteric transitions of the insulin hexamer (Bloom et al. 1998).

Figure 2B presents a typical stopped-flow rapid mixing time course for the displacement of 3N4H by Cl^- , measured by following the disappearance of the red-shifted absorption spectrum of the 3N4H complex with Zn^{2+} - R_6 following mixing of the complex with Cl^- . This time course consists

of a single relaxation, $A_t = -Ae^{-t/\tau} + A_{\text{inf}}$, where A_t is the absorbance at time t , A is the amplitude of the relaxation, and A_{inf} is the absorbance at time infinity. The dependence of this relaxation rate on the concentration of Cl^- is shown in Figure 2C. This dependence shows that the relaxation exchange rate decreases and then approaches a constant value as the concentration of Cl^- is increased. The rate of the relaxation slightly increases as the concentration of 3N4H is increased (Fig. 2D). Dependencies on the concentration of the displacing anion similar to that shown in Figure 2C were obtained with a variety of displacing ligands, including SCN^- , and p -aminobenzoate. This dependence appears hyperbolic in form, and the data are well described when fit to an expression of the form

$$1/\tau = 1/\tau_{\infty} + \{(1/\tau_0)(K)\}/\{K + [Y]\} \quad (1)$$

In Equation 1, K is the hyperbolic constant, $1/\tau_{\infty}$ is the relaxation rate at infinite concentration of the displacing ligand, Y , and $1/\tau_0$ is the relaxation rate extrapolated to $[Y] = 0$. This analysis gives values of $1/\tau$ that range from $1/\tau_0 = 4.53 \pm 0.5 \text{ sec}^{-1}$ to $1/\tau_{\infty} = 1.51 \pm 0.2 \text{ sec}^{-1}$.

The dynamics of 3N4H binding were examined by analysis of the line widths of the ^1H NMR signals for the free and bound species. When 3N4H is present in solutions of the Zn^{2+} - R_6 insulin hexamer, two sets of ligand resonances appear in the ^1H NMR spectrum (Choi et al. 1996). The resonances of the first set equal those known from the ligand spectrum, whereas the other set represents the ligand bound to the insulin hexamer. The presence of two distinct sets of resonances demonstrates that the exchange between ligand in its unbound and its bound states is slow on the NMR time scale.

Treating the binding of 3N4H to the His B10 anion sites as a second order exchange process and assuming that the affinity of ligand to the two binding sites are equivalent and noncooperative, the equilibrium (Equation 2) reads,

with the equilibrium constant defined by Equation 3

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[\text{site}][\text{ligand}]}{[\text{complex}]} \quad (3)$$

The lifetime of the ligand in the unbound state is given by Equation 4

$$\tau_{3\text{N4H}} = \frac{1}{k_{\text{on}}[\text{site}]} = \frac{[\text{ligand}]}{k_{\text{off}}[\text{complex}]} \quad (4)$$

Therefore, it can be inferred that the linewidths of the resonances of the free ligand will be additive according to Equation 5 (Lian and Roberts 1996)

$$\frac{1}{T_{23\text{N4Hobs}}} = \frac{1}{T_{23\text{N4H}}} + \frac{1}{\tau_{3\text{N4H}}} = \frac{1}{T_{23\text{N4H}}} + k_{\text{off}} \frac{[\text{complex}]}{[\text{ligand}]} \quad (5)$$

Equation 5 states that the width of the lines corresponding to resonances of the free ligand will be broadened when the ligand is present in the insulin solution. This linewidth approaches the inherent linewidth of the ligand when $[\text{ligand}] \gg [\text{binding site}]$. It follows from this equation that measurements of the linewidth of the free ligand resonance in a series of spectra with increasing concentration of ligand will allow a determination of the value of k_{off} . Provided that the exchange rate is slow on the NMR time scale, the intensity ratio of two resonance lines of the same proton in insulin with/without 3N4H bound corresponds to the ratio $[\text{complex}]/[\text{site}]$, and from the knowledge of the total concentration of insulin and 3N4H the concentrations of all species involved, are readily calculated. At the same time the value of K_D can be determined by rewriting Equation 4 to Equation 6

$$\text{fraction}_{\text{sites bound}} = \frac{[\text{complex}]}{[\text{site}] + [\text{complex}]} = \frac{K_D^{-1} [\text{ligand}]}{1 + K_D^{-1} [\text{ligand}]} \quad (6)$$

and by fitting the fraction of bound sites to the concentration of free ligand for the complete series of spectra.

Figure 3A shows the dependence of the R_6 fingerprint region of the insulin spectrum on the concentration of 3N4H. Figure 3B shows the results obtained when spectra calculated from the parameters (linewidth, frequency, phase, and intensity) of part of the resonances in Figure 3B are fitted to the experimental spectra. The value of the $[\text{complex}]/[\text{site}]$ ratio was calculated from each of the insulin lines of A6 α , A7 α , or B5 δ (the free form summed from two lines) through the entire concentrations series, and the value of K_D was determined from Equation 6, giving an averaged value of $131.0 \pm 9.0 \mu\text{M}$ (estimated uncertainty given as the standard deviation of the average calculation). The data points and the fitted lines are shown in Figure 3C.

In Figure 4A another excerpt from the same set of spectra shows the ligand resonances from the 3N4H aromatic ring protons at C-2 in the bound and free forms (8.57 ppm and 8.43 ppm, respectively) and from the protons at C-5 in the bound and free forms (7.85 ppm and 7.78 ppm, respectively). The value of k_{off} was determined from a fit of Equation 6 to the linewidths of the 8.43 ppm resonance versus the ratio $[\text{complex}]/[\text{ligand}]$, giving the average value $k_{\text{off}} = 4.13 \pm 0.45 \text{ sec}^{-1}$. Again, the estimated uncertainty repre-

Figure 3. (A) Excerpts from the 1D ¹H NMR spectra of the Zn²⁺-R₆ hexamer with increasing concentration of 3N4H. At the first addition of 3N4H, part of the proton signals from the protein are seen to populate two distinct chemical shift positions in slow exchange on the NMR time scale corresponding to the single chemical shift seen without ligand and the single chemical shift seen at large excess of ligand. Note that binding of 3N4H in the His B10 anion site narrows the width of the lines in the insulin spectrum. Experimental conditions: 2.1 mM amide deuterated human insulin; 0.7 mM Zn²⁺; 40 mM deuterated phenol; 10 mM deuterated Tris in D₂O; and pH = 8.0 (direct meter reading) at 27°C. (B) The simulated spectra resulting from a least squares fit of the spectral parameters of the resonances selected in the 5.2 ppm to 6.0 ppm region of each spectrum in (A). In (C) the ratio [complex]/([complex] + [site]), calculated from the A7α signals of the fitting results versus the concentration of free ligand, are shown as triangles together with the results obtained by fitting the value of K_D using Equation 6. The result of the fits using the A7α, A6α, and B5δ signals averages to $K_D = 131.0 \pm 9.0 \mu\text{M}$, the estimated uncertainty of K_D is the standard deviation of the average calculation.

sents the standard deviation of the average calculation. From the K_D and k_{off} values, the value of k_{on} was estimated to be $\sim 3.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$.

¹H NMR studies—chemical shift perturbations induced by 3N4H binding

The 4.9–6.5 ppm region of the 1D ¹H NMR spectra of the Zn²⁺-R₆ hexamer as a function of increasing concentrations of 3N4H is shown in Figure 3. Upon addition of ligand, the

hexamer species with 3N4H bound appears as a new set of insulin resonances. As the titration progresses, the intensities of these resonances increase at the expense of resonances corresponding to the same protons in the hexamer without Zn-site ligand. Note that resonances sharpen as a result of complex formation. Also, the resonances that change chemical shift as a result of ligand addition are confined to the His B10 site and its immediate vicinity. In Figure 3, the R₆ fingerprint region of the spectrum show perturbations of the B5δ, A6α, and A7α, resonances that represent part of the lining between the phenol pockets and the His B10 anion site of the R₆ hexamers. In contrast, the resonances from the α protons of A20, B24, and B25 and δ-proton of B16 are essentially unaffected by the presence of the ligand. The His B10 binding sites possess threefold symmetry (vide supra), and the fact that only a single set of new resonances appear upon binding of the ligand implies that this symmetry is preserved or that any asymmetry imposed by the accommodation of the nonsymmetric and planar ligand is part of a set of substates in fast exchange on the NMR time scale. The high degree of similarity between the spectra of the Zn²⁺-R₆ hexamer with and without 3N4H bound establishes that any potential change in the conformation of the protein upon binding of 3N4H in the two binding sites is of limited scope.

¹H NMR studies—assignments for the complex of 3N4H with Zn²⁺-R₆

Due to the stability of the Zn²⁺-R₆ hexamer and inasmuch as the three-dimensional conformation of each of the six monomers of the aggregate is the same, the NMR spectrum of this 36-kD species is relatively simple. From a series of 2D spectra consisting of TOCSY and NOESY, the majority of resonances of the insulin R₆ spectrum are readily assigned. When compared to previously published assignments (Jacoby et al. 1996; Chang et al. 1997), only minor differences are seen that can be ascribed to the divergences in experimental conditions (temperature and concentration of phenol). Based on the high degree of similarity between resonance assignments, the three-dimensional structure of the R₆ hexamer examined in this work is by every probability similar to the one previously determined (Chang et al. 1997). As a consequence, the assignment of the NOESY spectrum in this investigation is limited to the sequential assignment stage and analysis of internal NOEs in secondary structural elements, and will not be described in more detail. Because only a small subset of insulin resonances is perturbed upon binding of 3N4H, the assignment of the insulin spectrum of the Zn²⁺-R₆ hexamer with ligand bound in the His B10 site is easily carried out. It is noteworthy that NOEs from the side chain of Val B2 to the amide proton of His B5 are present in both

Figure 4. (A) Excerpts from the 1D ¹H NMR spectra of the Zn²⁺-R₆ hexamer with increasing concentration of 3N4H. When 3N4H binds in the His B10 anion site of the hexamer, two resonance lines in slow exchange on the NMR time scale of the C-2 and C-5 aromatic protons of 3N4H are present; these correspond to bound ligand (broad lines at 8.57 ppm and 7.85 ppm, respectively), and free ligand (sharp lines at 8.43 ppm and 7.78 ppm, respectively). (B) The triangles depict the fitted values of 1/T_{2,obs} of the 8.43 ppm line versus the ratio of bound ligand to free ligand (calculated from the A7α signals). The line represents the best fit of k_{off} and the inherent value of 1/τ₂, according to Equation 8. The result of the fits using the A7α, A6α, and B5δ signals averages to $k_{\text{off}} = 4.13 \pm 0.45 \text{ sec}^{-1}$, the estimated uncertainty of k_{off} is the standard deviation of the average calculation. Experimental conditions: 2.1 mM amide deuterated human insulin; 0.7 mM Zn²⁺; 40 mM deuterated phenol; 10 mM deuterated Tris in D₂O; pH = 8.0 (direct meter reading) at 27°C and concentration ratios of [3N4H] to [binding sites] as marked in the figure.

sets of spectra, indicating that the helical conformation in this part of the B-chain is preserved upon addition of 3N4H. The affected amino acid residues, the chemical shift assignments, and the chemical shift perturbations arising from the binding of 3N4H are given in the supplementary material presented in the electronic edition of this work. The insulin resonances that undergo the largest change in chemical shifts belong to residues Asn B3, Leu B6, and His B10. The chemical shifts of residues not involved in forming the His B10 site were found to be unchanged (i.e., within ±0.02 ppm) from those of the hexamer measured in the absence of 3N4H.

Figure 5, right panel, presents a superimposition of the aliphatic–aliphatic region of three 2D TOCSY spectra measured for ratios of the concentration of binding sites to 3N4H of 0:1, 1:1, and 10:1. The left panels in the same figure are excerpts from a NOESY spectrum at the chemical shifts of the C2 and C5 protons of bound 3N4H measured at a large excess of 3N4H. These spectra show the collection of NOEs between the two 3N4H protons and the insulin protons; the C6 proton of 3N4H overlaps the aromatic signals of insulin, making NOEs from this proton hard to distinguish from insulin NOEs. The NOEs from 3N4H to insulin are limited to the α- and side chain protons of Val B2, Asn B3, Leu B6, and Cys B7. These side chains form the surface of the His B10 anion site.

Discussion

Insulin allostery

The allosteric properties of the insulin hexamer have become an interesting paradigm for mixed forms of allostery consisting of positive and negative cooperativity, half-of-the-sites reactivity in ligand binding, and both homotropic and heterotropic ligand binding interactions (Kaarsholm et al. 1989; Roy et al. 1989; Brader and Dunn 1991; Choi et al. 1993, 1996; Brzovic et al. 1994; Bloom et al. 1995, 1997a,b, 1998; Rahuel-Clermont et al. 1997). Current knowledge of the relationship between the structure of the insulin hexamer and its allosteric properties is derived from a wide variety of experimental approaches including structure studies via X-ray crystallography (Derewenda et al. 1989; Bentley et al. 1992; Smith and Dodson 1992a,b; Ciszak and Smith 1994; Whittingham et al. 1995) and NMR spectroscopy (Roy et al. 1989; Brzovic et al. 1994; Jacoby et al. 1996; Chang et al. 1997), and via biophysical studies in solution (Kaarsholm et al. 1989; Brader and Dunn 1991; Bloom et al. 1995, 1997a,b, 1998; Birnbaum et al. 1996; Choi et al. 1996). These works have established that the mechanism of the allosteric transition involves the interconversion among three allosteric states, T₆, T₃R₃, and R₆, and is well-described by the Seydoux, Malhotra, and Bernhard (SMB)

Figure 5. 2D ^1H NMR spectra of the Zn^{2+} - R_6 hexamer measured at different concentrations of 3N4H. (A, B) Excerpts from a NOESY spectrum (mixing time 150 msec) showing the complete collection of NOEs from the 3N4H signals at 8.57 ppm and 7.86 ppm (respectively, the protons at positions C-2 and C-5 of the aromatic ring) to insulin resonance lines. The NOEs are confined primarily to residues in the lining of the His B10 anion binding site, B2, B3, B6, and B7. Experimental conditions of the NOESY spectrum: 2.1 mM amide deuterated human insulin, 0.7 mM Zn^{2+} ; 40 mM deuterated phenol; 10 mM deuterated Tris in D_2O , pH = 8.0 (direct meter reading) at 27°C. In (C), three TOCSY spectra (mixing time 65 msec) are superimposed; red, without 3N4H; blue, at a 1:1 stoichiometry of 3N4H to binding sites; and black (on top), at a [binding site]:[ligand] ratio of 10:1. Resonance lines that are perturbed upon binding of 3N4H are annotated by their residue position (for further details see the supplementary material in the electronic edition of this paper). Experimental conditions: 6.0 mM human insulin; 2.0 mM Zn^{2+} ; 80 mM deuterated phenol; pH = 8.0, at 27°C. The deviations in experimental conditions between the NOESY and TOCSY series had no effect on the chemical shifts of either the insulin or the ligand lines.

model for allostery (Bloom et al. 1995, 1997a,b, 1998; Fig. 1A). According to this model, the T_6 , T_3R_3 , and R_6 states preexist in the absence of allosteric ligands, a prediction in agreement with experimental observations (Bloom et al. 1995, 1997a,b, 1998). Because the binding sites for allosteric ligands are present only for the R-state trimeric units (R_3), ligand binding to T_3R_3 and to R_6 stabilizes these states, causing a redistribution of conformations in favor of the R-state species. Bloom et al. (1995, 1997a,b) have established that at pH 8, both L^{A}_0 and L^{B}_0 (Fig. 1A) are $\gg 1.0$. This happenstance gives rise to positive cooperativity in ligand binding. However, because $L^{\text{B}}_0 \gg L^{\text{A}}_0$, ligand binding isotherms tend to saturate upon formation of T_3R_3 , giving rise to half-of-the-sites reactivity in ligand binding and negative cooperativity. The preference for T_3R_3 formation

is also dependent on ligand structure. This structural dependence has its origins in differences in the shapes of the phenolic pockets of R-state subunits in T_3R_3 versus R_6 (Bloom et al. 1995, 1997a,b). Nevertheless, the structural origins of the subunit interactions that give rise to $L^{\text{B}}_0 \gg L^{\text{A}}_0$ are not completely understood.

Properties of the His B10 anion site—symmetry, polarity, flexibility, and proximity of the phenolic sites

Through investigations of the spectroscopic properties of the Co(II)-, Cu(II)-, and Zn(II)-substituted R-state insulin hexamers, the His B10 anion site has been shown to bind a wide variety of monovalent inorganic and organic anions

(Roy et al. 1989; Brader et al. 1990, 1991, 1997; Brader and Dunn 1990; Brzovic et al. 1994; Bloom et al. 1995, 1997a,b; Choi et al. 1996; Huang et al. 1997). Inorganic anions that are known to bind to this site include the halides (Cl^- , Br^- , I^-), the pseudo-halides (SCN^- , OCN^- , CN^- , N_3^-), nitrate ion, and bicarbonate ion. The organic anions include organic carboxylates, phenolates, thiophenolates, alkylthiolates, and sulfonamide anions. The imidazoles are the only class of neutral ligands demonstrated to bind to this site (Brader et al. 1990). As determined from the UV/Vis absorption, CD, and MCD spectral signatures of the d,d transitions of the Co(II)-substituted R-state hexamers, the binding of each of the anionic ligands listed above occurs via innersphere coordination to the metal center. Where spectroscopic data is available for the Cu(II) complexes, again the spectroscopic properties of the Cu(II) center establish that complex formation involves coordination to the metal center. Most of these complexes are classified as either near tetrahedral or distorted tetrahedral complexes based on the Co(II) spectra, and to a lesser extent the Cu(II) spectra, of the corresponding Co(II)- and Cu(II)-substituted R-state complexes. The CD and MCD spectra of these complexes are consistent with trigonally distorted tetrahedral Co(II) geometry (C_{3v} ; Brader et al. 1997).

The published X-ray crystallographic studies of R-state complexes have been restricted to structure determinations of Zn(II) hexamers. Most of these structures involve coordination of Cl^- to the HisB10 anion sites. All of the Cl^- structures show a pseudo-tetrahedral Zn(II)(His) $_3$ - Cl^- complex where Cl^- is positioned on the threefold symmetry axis of the hexamer. In one instance, phenolate ion is reported to be coordinated to a HisB10 anion site, giving a pseudo-tetrahedral Zn(II)(His) $_3$ -phenolate complex (Smith and Dodson 1992a,b). However, no corresponding coordinates have been deposited with the PDB. In 1998, Turkenburg et al. deposited the structure 1znj in which the outer portion of one of the His B10 cavities of the Cl^- ligated Zn $^{2+}$ -R $_6$ hexamers is occupied by a phenol molecule with the ring CH groups in van der Waals contact with Cl^- and with the walls of the tunnel (Phe B1, Asn B3, and Leu B6). Smith et al. (2000) have reported structures of three Zn $^{2+}$ -R $_6$ hexamers (1evr, 1ev3, and 1ev6) in which the Zn $^{2+}$ ligands are Cl^- with pseudo-tetrahedral geometry. In one R $_3$ unit of the 1evr structure, the outer portion of the His B10 cavity is occupied by resorcinol oriented such that the phenolic hydroxyls point out toward the solvent and one also is H bonded to the amide of Asn B3. Except for the presence of the phenolic ligand lodged in one of the His B10 tunnels the structures of the R $_3$ units of these complexes are very similar and not different from those of the 1znj structure.

Ligands with the potential of forming bidentate complexes such as nitrate ion, bicarbonate ion, and certain organic bidentate ligands (provided the organic substituent is not too bulky) such as carboxylates and 2-pyridinethiolate

ion, give complexes with the Co(II)-R $_6$ hexamer that exhibit absorption and CD signatures consistent with the formation of five-coordinate complexes (Brader et al. 1997).

The X-ray diffraction structures of R-state Cl^- complexes show a nearly tetrahedral geometry for the Zn(II)(His) $_3$ - Cl^- site with Cl^- located on the threefold symmetry axis and forming van der Waals contacts with the Leu B6 methyl groups. The B1-B8 peptide segments that define the His B10 anion sites of the R $_6$ complexes with Cl^- typically are fully formed α -helices. In the crystal structures of many T $_3$ R $_3$ complexes with Cl^- , the first three residues of the B chain take up a nonhelical conformation (Ciszak and Smith 1994) that has been designated as the "frayed" conformation, R' (Ciszak et al. 1995). Indeed, the frayed conformation prevails for the R-state portion of all deposited T $_3$ R $_3$ human insulin complexes including also the structure (Whittingham et al. 1995) of the T $_3$ R $_3$ complex with SCN^- in which the R $_3$ unit looks like the Cl^- structures with SCN^- coordinated to the nearly tetrahedral Zn(II) via nitrogen and oriented along the threefold symmetry axis. Ciszak et al. (1995) attributed the frayed structure to steric clashes between hexamer molecules in the crystal lattice. The solution structural investigations by NMR of Jacoby et al. (1996), report evidence of helix extending from B2 to B19, while Chang et al. (1997) report that the region B3-B19 is helical. Our results indicate that the helix starts at B2 and extends to B19. Consequently, the predominating solution structure of the R $_6$ hexamer does not appear to be frayed.

These high-resolution crystal structures and the solution studies of Huang et al. (1997) show that the His B10 anion site is relatively flexible and can adapt to accommodate a variety of small ligand structures as long as the ligand is not too large, and provides complementarity with respect to the charge/polarity requirements of the His B10 Zn(II) ion and the tunnel walls.

Ligand exchange at the His B10 site

The kinetic constants for ligand exchange measured by stopped-flow rapid-mixing UV/Vis spectroscopy and by ^1H NMR linewidth measurements are in good agreement (Figs. 2-4). We conclude that the most reasonable explanation for the hyperbolic dependence of $1/\tau$ on the concentration of the displacing ligand (Fig. 2C; Equation 2) involves a mechanism where the ligand binding and dissociation steps are fast relative to the rate of a conformational transition in the insulin hexamer. If the binding steps are sufficiently fast, then a steady-state approximation can be made that assumes the two protein conformations are in equilibrium with the ligands at any given time. The equations below define such a model for an R $_3$ unit of the insulin hexamer:

Provided that [Y] is buffered, the time course for the replacement of 3N4H by Y will be exponential. The dependence of $1/\tau$ on [Y] will be hyperbolic (with values of $1/\tau$ that decrease with increasing [Y]). Simulations of this mechanism for the conditions described in Figure 2C were found to generate reaction time courses and concentration dependencies in good agreement with the observed kinetic behavior. Consequently, as [Y] approaches zero, $1/\tau$ approaches $(k_1 + k_{-1}) = 4.53 \pm 0.5 \text{ sec}^{-1}$, and as [Y] approaches infinity, $1/\tau$ approaches $k_1 = 1.51 \pm 0.2 \text{ sec}^{-1}$. Therefore, $k_{-1} = 3.02 \pm 0.35 \text{ sec}^{-1}$. We speculate that the conformation change that limits ligand exchange is the conversion of R-state trimeric units between “capped” and “open” conformations of the His B10 site. The detection of NOE’s between the ligand and the ring protons of Phe B1 are not inconsistent with the formation of a “cap.”

Alternative explanations that were considered consist of: (a) an S_N1 -like mechanism where dissociation of 3N4H to give a trigonal intermediate is rate-determining for ligand exchange, (b) an S_N2 -like mechanism wherein exchange occurs via expansion of the coordination sphere to a five-coordinate intermediate involving both the entering and the departing ligands, and (c) a mechanism that involves a rate-determining conformational transition in the protein that converts the R_6 conformation back to the T_3R_3 conformation followed by rapid exchange of the ligand at the His B10 T_3 unit of T_3R_3 via its octahedral coordination sphere. The S_N1 -like mechanism will also explain the hyperbolic dependence of $1/\tau$ on [Y]. However, the bimolecular rate constant for the recombination of 3N4H with the trigonal intermediate predicted by this fit ($\sim 1.9 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$) seems too slow to be plausible unless strong steric constraints on the recombination process are invoked. The S_N2 -like mechanism was rejected for the reason that this mechanism fails to predict the concentration dependence on Y. The R-to-T state conformational change mechanism was rejected for the reason that this mechanism predicts strong rate dependencies on both the affinity of the phenolic ligand and the concentration of the phenolic ligand that are not observed (Leuenberger-Fisher 2000).

Structural implications of the 3N4H-insulin NOEs

The chemical shift perturbations arising from the binding of 3N4H to the Zn^{2+} - R_6 hexamer (see the supplemental material given in the electronic edition of this article), together with the TOSCY and NOESY data (Fig. 5; Table 1), further confirm that 3N4H binds to the His B10 anion binding site.

The NOEs found between the C-2 proton of 3N4H and insulin undoubtedly arise from the close approach of this edge of the ring to Leu B6 and Cys B7, and the NOEs found between the C-5 hydrogen of 3N4H and Leu B6 also place this edge of the ring in contact with Leu B6. The threefold symmetry of the site predicts that ligands such as 3N4H will bind to the site with three equivalent, but overlapping orientations in each R_3 unit. In any one of these orientations, each of the three Leu B6 residues will have a different distance from the C-2 proton of 3N4H. The same argument can be made for the distance relationships between the Cys B7 protons and the C-2 proton of 3N4H and for the distance relationships between the Leu B6 protons and the C-6 proton of 3N4H. One possibility is that 3N4H switches among these three equivalent positions on a time scale that is rapid relative to the NMR time scale. If this were the case, then each of the three sets of B-chain residues that make up the site will experience identical, time-averaged interactions with the ligand, and therefore, only a single set of chemically shifted resonances and a single set of NOEs would be generated in the NMR spectrum of the complex. If the switching of 3N4H between equivalent positions were slow with respect to the NMR time scale, then there should be more than one set of perturbed resonances for the 3N4H complex, and there likely would be more than one set of NOEs between the ligand protons and the protons of residues that make close contact with the ligand. Because only a single set of resonances and NOEs were actually observed, it seems reasonable to conclude that 3N4H rapidly switches among the three equivalent positions dictated by the site symmetry.

The side chains of Phe B1 residues show several strong NOEs to the Thr A8 side-chain protons and two very weak

Table 1. List of insulin protons with NOEs to C-2 and/or C-5 proton of 3N4H

Residue	Proton	Ch.sh. (ppm)
B2 Val	γ	0.82
B3 Asn	α	3.97
	β	2.71/2.57
	γ	1.47
B6 Leu	α	3.58
	β	1.65/1.23
	δ	0.74/0.37
B7 Cys	α	4.08
	β	3.95/3.22

NOEs to the ligand protons. This finding indicates that these side chains switch between different positions on a rapid time scale.

A structural hypothesis for the 3N4H complex

Previous studies of the 3N4H complex with $\text{Zn}^{2+}\text{-R}_6$ have established that this ligand binds as the dianion with the carboxylate bound to the Zn(II)- or Co(II)-substituted HisB10 sites giving five-coordinate complexes (Huang et al. 1997; Bloom et al. 1998; Ferrari et al. 2001). Binding occurs with a relatively high affinity compared to most other ligands for the His B10 site, and with a stoichiometry of one 3N4H per HisB10 anion site (Huang et al. 1997). The spectrum of the 3N4H dianion is red-shifted in these complexes (Fig. 2A), the envelope of d,d-transitions of the Co(II)-complex corresponds to a five-coordinate geometry (Huang et al. 1997), and the Raman difference spectrum of bound 3N4H (Ferrari et al. 2001) indicates that the carboxylate is coordinated with one O—Co bond much shorter than the other. Ligand exchange occurs on a relatively slow time scale. The slow rate of ligand exchange (Fig. 2) is consistent with the ^1H -NMR spectra (Figs. 3–5), establishing that exchange occurs on a millisecond time scale.

The changes in chemical shift that accompany the binding of 3N4H are restricted to a small subset of the protein resonances. The amino acid residues that give rise to these shifted resonances are either constituents of the HisB10 anion site, or are located near this site. This clustering of the perturbed resonances implies that the binding of 3N4H causes perturbations in the hexamer structure that are localized to the vicinity of the His B10 anion site, and that more distant portions of the structure are essentially unaffected.

Consequently, the above-described spectroscopic and kinetic studies establish that 3N4H is coordinated through its

carboxylate to the His B10 Zn(II) metal ion in a five-coordinate complex with nonequivalent O—Zn bond lengths. This interaction causes localized perturbations in the structure of the R-state His B10 anion sites that generate small chemical shift differences in a subset of resonances assigned to amino acid residues in and around the sites. The set of assigned NOE's determined for the $\text{Zn}^{2+}\text{-R}_6$ complex with 3N4H (Fig. 5; Table 1) further defines the structure of the complex. These NOEs provide distance constraints between the ligand ring C—H and the protein side chain C—H and backbone N—H atoms of less than 5.0 Å, but greater than or equal to the van der Waals H-H contact distance, and provide a plausible basis for the prediction of a moderately high resolution structural model for the 3N4H complex with $\text{Zn}^{2+}\text{-R}_6$.

The complex is assumed to retain the overall folding motif of the R-state with an His B10 anion site consisting of a tunnel-like cavity within a three-helix bundle created by the helical segments of residues B2–B8 of each R_3 unit of the hexamer extending from the surface down to the His B10 Zn(II) ion with dimensions similar to the crystallographically identified His B10 anion sites (Smith et al. 2000).

A structural model incorporating these features is presented in Figure 6. With offset in the structure of the $\text{Zn}^{2+}\text{-R}_6$ hexamer (PDB-code 1evr, Smith et al. 2000), 3N4H has been docked into the site originally occupied by Cl^- and resorcinol by adjusting the distances from the two oxygens of the carboxylate group to the zinc ion to 2.1 and 2.3 Å, respectively (Alberts et al. 1998), and by maximizing the overlap with resorcinol. Two of the Asn B3 sidechains have been adjusted slightly so that the oxygens from the nitro group and the hydroxy group of the ligand are in hydrogen bond distance to these two side chains. Given the fact that the Phe B1 side chains apparently take up different positions

Figure 6. Model of the insulin hexamer with 3N4H bound in the His B10 anion site, the 3N4H ligand has been docked into the 1evr (Smith et al. 2000) structure as described in the discussion. The structure is viewed down the threefold symmetry axis in (A) and perpendicular to the threefold axis in (B). The residues to which NOEs from the ligand are assigned have been color coded, whereas residues that experience perturbation of chemical shift values upon ligand binding are shown without color codes.

on a rapid timescale, that is, one close to the Thr A8 side chain and the other close to the ligand, no modeling has been applied to these residues. The two Phe B1 side chains shown in the figure are the ones seen in the original structure.

As established by Rahuel-Clermont et al. (1997) and by Bloom et al. (1995, 1997a,b), ligand binding to the His B10 site plays an important role in the dynamics of hexamer assembly and in the stabilization of the R-state conformation of the hexamer. The structure and dynamics of the hexamer are believed to play critically important roles in the storage and timely release of insulin *in vivo*, and these properties of the hexamer certainly play an important role in the stabilization of insulin formulations for the treatment of diabetes.

Materials and methods

Materials

Native human insulin was provided by Novo Nordisk A/S. All other chemicals used were purchased from Sigma-Aldrich Fine Chemicals (reagent grade or better) and used without additional purification. The insulin solutions used in these studies were prepared as previously described (Porter 1953; Brzovic et al. 1994; Bloom et al. 1997a,b, 1998).

Methods

UV-visible spectroscopy and rapid kinetics

Static UV-Vis spectroscopy was performed with a Hewlett-Packard 8452A diode array spectrophotometer. Rapid kinetic studies of 3N4H binding to the Zn(III) R₆ hexamer were carried out using an Applied Photophysics SFMV17 stopped-flow rapid mixing unit with customized optical, data acquisition, and data analysis systems as previously described (Weber-Ban et al. 2001). The optical spectroscopy experiments were all performed in 50 mM Tris-perchlorate buffer at pH 8.0 and 25°C. Kinetic simulations were carried out using the program KINETIC version 3.11 (Loren Milesescu).

NMR spectroscopy

Proton 2D NMR spectra of the R₆ hexamer with/without 3N4H ligand were analyzed with a focus on assignment of spin systems. The protons affected by 3N4H binding (i.e., those with altered chemical shifts) were mapped and NOEs between the ligand and insulin were identified. NOESY (Jeener et al. 1979; Kumar et al. 1981), TOCSY (Braunschweiler and Ernst 1983; Bax and Davis 1985) and 1D presaturation sequences with WATERGATE (Piotto et al. 1992) water suppression were recorded at 600 MHz Varian Unity Inova at 300 K. Mixing times for TOCSY spectra were 35 msec and 65 msec and for NOESY spectra 100 msec, 150 msec, and 200 msec. Processing of the NMR data was performed using XWINNMR (Bruker) on SGI equipment. Line broadening using squared sinebell-shifted $\pi/2.6$ or exponential algorithms were applied prior to Fourier Transformation. The software PRONTO (PRONTO Software Development and Distribution) was used for spectral assignments.

Two sets of conditions were used. The first set was 6.0 mM human insulin, 2.0 mM Zn²⁺, 80 mM phenol-d₆, 10 mM Tris-d₁₁, 90% H₂O/10% D₂O, pH = 8.0 and three levels of ligand 0.0–2.1–20.0 mM 3N4H, that is, binding [site]:[ligand] ratios 1:0, 1:1.0, and 1:10.0. Conditions for the second set were 2.0 mM HI-d (see below), 0.7 mM Zn²⁺, 40 mM phenol-d₆, 10 mM Tris-d₁₁, in D₂O pH = 8.0 (direct pH-meter reading) and levels of ligand 0.0–0.35–0.52–0.68–1.01–1.34–3.31–4.91 mM 3N4H, that is, [binding site]:[ligand] ratios 1:0, 1:0.5, 1:0.74, 1:1.0, 1:1.44, 1:4.72, and 1:7.01. For HI-d human insulin was dissolved in D₂O, left at room temperature for approximately 6 h, freeze dried overnight, redissolved in D₂O, left at room temperature for another 6 h, and subjected to a second freeze drying overnight. This procedure ensured that all amide and labile side chain protons were substituted by deuterons. The differences in human insulin and phenol concentrations between the two series did not cause observable changes in chemical shifts of insulin or 3N4H.

For the purpose of investigating the dynamics of ligand binding, estimation of linewidths and intensities of a limited number of ligand and insulin resonance lines in a series of 1D spectra was performed. The processed data was transferred from XWINNMR to Microsoft Excel, Office2000. An FID was simulated from spectral parameters, frequency, linewidth, intensity, phase, and spin-spin coupling constants of the number of lines suitable for the relevant parts of the spectra, and the Fourier transformed data set was fitted to excerpts of the spectra by adjustments of the spectral parameters in a least squares fit. A standard minimization algorithm from the Excel Solver add-in was used.

Electronic supplemental material

Supplementary material is provided in the electronic edition of this paper in the form of tables summarizing the proton chemical shifts of the human insulin R₆ hexamer, the complex of the hexamer with the 3N4H ligand, and the summary of the differences in proton chemical shifts (6.0 mM human insulin, 2.0 mM Zn²⁺, 80 mM phenol, in the presence and absence of 3N4H, 10 mM Tris-d₁₁, pH = 8, temp = 27°C).

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