Studies of the Association and Conformational Properties of Metal-free Insulin in Alkaline Sodium Chloride Solutions by One- and Two-dimensional \(^1\)H NMR*

(Received for publication, January 13, 1992)

Webe Kadima, Melinda Roy, Robert W.-K. Lee†, Niels C. Kaarsholm§, and Michael F. Dunn¶

From the Department of Biochemistry and the †Department of Chemistry, University of California, Riverside, California 92521-0129 and the §Novo Research Institute, DK 2880 Bagsvaerd, Denmark

One- and two-dimensional \(^1\)H NMR spectroscopy have been employed to probe the association and subsequent conformational changes of metal-free insulin in sodium chloride solution at pH 9 and 9.4. These studies establish that the proton resonances of His(B5) and His(B10) are useful signatures of aggregation and conformation. Changes in chemical shifts and areas of resonances due to the C2 protons of His(B10) and His(B5) and transfer of magnetization experiments served to identify the association as the assembly of tetramer from dimers under our experimental conditions (pH 9.4, [insulin] > 1 mM, [NaCl] = 0.1 M). Sodium chloride also alters the equilibrium distribution of species in favor of a tetrameric species. The association equilibrium constant was estimated from area measurements to be \(5 \times 10^4 \text{M}^{-1}\) at pH 9.4, 26 ± 0.1 °C, and 0.1 M sodium chloride. Under conditions of 0.1 M sodium chloride concentration, nuclear Overhauser effect experiments in the one- and two-dimensional modes revealed an operative nuclear Overhauser effect between the His(B5) C2 protons and the 2,6 ring protons of a Tyr residue provisionally assigned as Tyr(B16). We conclude that this interaction is a diagnostic signature of a conformational transition whereupon an extended chain from residues B1 to B9 (T-state) is transformed into an \(\alpha\)-helix (R-state) thus bringing the rings of His(B5) and Tyr(B16) from adjacent subunits across the monomer-monomer interface into van der Waals contact. This conformational flexibility is an added consideration to the discussion of the relevant structure of insulin for receptor binding.

It is well established that monomeric insulin is the receptor binding species in insulin action. Insulin must, therefore, undergo several transformations from its storage state in the pancreas, as a crystalline array of zinc-insulin hexamers, to its receptor binding, monomeric state. During exocytosis from the \(\beta\)-cells of the pancreas and transport to the target cells, the zinc-insulin complex must dissociate, leading to different association and possibly structural and conformational states of the insulin molecule. Self-association of insulin in the absence of zinc has long been known. Understanding the self-association of insulin is important for elucidating the mechanism of assembly of the zinc-insulin hexamer: a process both of pharmacological significance (Brange and Havelund, 1983) and of \textit{in vivo} significance to insulin biosynthesis (Steiner et al., 1975; Permutt, 1981). In view of its functional role, self-association of insulin has been a subject of much interest. Furthermore, the self-association pattern can be used as a model for the understanding of the interaction of insulin with the receptor. It has been suggested, for example, that the conformational changes induced in the monomer by dimerization are likely to occur upon receptor binding (Baker et al., 1988). Recent studies of the binding between insulin and site-mutated insulin receptors have suggested a domain of the insulin receptor compatible with the monomer interface in the insulin dimer (DeMeys et al., 1990).

Self-association of insulin has been investigated by sedimentation methods (Fredericq, 1954, 1956; Jeffrey and Coates, 1966a, 1966b; Jeffrey et al., 1976; Milthorpe et al., 1977; Pekar and Frank, 1972; Mark et al., 1987), concentration difference methods (Lord et al., 1973; Strazza et al., 1985), light scattering (Doty et al., 1952; Steiner et al., 1951; Martin et al., 1982; Bohidar and Geissler, 1984), rapid kinetics (Coffman and Dunn, 1988), and circular dichroism (Goldman and Carpenter, 1974; Pocker and Biawas, 1981). All these techniques are generally nonspecific for determining the exact nature, the number, and the proportions of the equilibrating species. Assumed association models must be employed to interpret the bulk response to aggregation. Because the structures of possible aggregates have been elicted by x-ray crystallography (Baker et al., 1988), a limited number of "realistic" models can be used to fit data. Understandably, insulin association studies have resulted in the proposal of a number of different modes of association.

Total agreement is encountered only on the first step in aggregation of insulin: the dimerization. The equilibrium constants reported in the literature are also in reasonable agreement. The nature of insulin oligomers formed beyond the dimer is still open to question, as various authors continue to disagree (Mark et al., 1987). In his earliest investigations, Jeffrey (Jeffrey and Coates, 1966a, 1966b) proposed a definite model of association consisting of monomers, dimers, tetramers, and hexamers. This model was accepted by other authors who examined this association at higher pH (Goldman and Carpenter, 1974; Holladay et al., 1977; Coffman and Dunn, 1988). Pekar and Frank (1972) proposed instead a mixture of monomers, dimers, hexamers and higher aggregates of the hexamers, as their data clearly indicated the presence of aggregates larger than the hexamer. Coffman and Dunn (1988) presented rapid kinetic evidence indicating that

---

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom all correspondence should be addressed: Dept. of Biochemistry, University of California, Riverside, CA 92521-0129. Tel.: 714-787-4226; Fax: 714-787-3590.
at alkaline pH the insulin tetramer predominates in concentrated solutions of metal-free insulin. To account for aggregates larger than hexamer, yet another model was proposed which favored instead iasodesmic self-association of the dimer (Jeffrey et al., 1976). The latter model implies the existence of only one type of dimer formed by self-interaction of one available reaction domain. This model was rejected based on the bifunctional nature of the monomer (Wollmer et al., 1980). Redefinition of the latter model taking into account the bifunctional nature of the monomer generated a model whereby two types of dimer are formed and the successive addition of monomers to each creates both odd and even numbered polymers (Nichol et al., 1984; Mark et al., 1987). Pocker and Biswas (1981), using UV difference circular dichroism data, reached a different conclusion. A stepwise assembly of the hexamer is proposed where the dimers associate to form the tetramer, and the tetramer and the dimer associate into the hexamer. According to estimated equilibrium constants, the dimer and the tetramer are the predominant species at concentrations higher than 400 μM. This conclusion has been reinforced by the findings of Coffman and Dunn (1988).

The insulin hexamer is known to undergo an allosteric transition now coined T to R (Kaarsholm et al., 1989; Roy et al., 1989) in the presence of phenol, phenol analogs, and lyotropic anions. X-ray crystallography studies (Smith et al., 1984; Derewenda et al., 1989) have shown that the T- to R-conformational transition involves the conversion of residues 1-9 of the B chain from an extended conformation (T-state) into a helix (R-state). When all six subunits of the hexamer undergo this transformation, six hydrophobic pockets capable of binding phenol and its analogs are formed. In metal ion-substituted hexamers, the coordination geometry of the metal ions in the His(B10) zinc sites changes from octahedral in the T-state to tetrahedral in the R-state (Roy et al., 1989; Brader et al., 1990, 1991; Brader and Dunn, 1990). Anions which bind to the fourth, solvent-accessible, ligand position of the tetrahedral metal ion also are allosteric effectors, and this effector site and the phenol binding pockets exhibit positive heterotropic allosteric interactions that stabilize the R-state (Brader et al., 1991). Salt induced aggregation in this work produces similar conformational changes in the tetramer.

The present paper reports a proton nuclear magnetic resonance (1H NMR) investigation of the association of metal-free insulin. It will be shown that the 1H NMR spectrum of the insulin His(B5) and His(B10) residues can be successfully used to characterize, both qualitatively and quantitatively, the aggregation of insulin, albeit in a limited range of pH and insulin concentration dictated by limits in sensitivity, overlapping of resonances, and precipitation. The NMR spectrum also contains signatures of local three-dimensional structure which are used herein to characterize some folding motifs for the aggregated species.

This study exploits the sensitivity to aggregation of the C2 proton resonances of the His(B10) and His(B5) residues. Attempts to assign these resonances in metal-free insulin have been made previously (Bradbury and Brown, 1977; William and Williams, 1979; Bradbury et al., 1980; Bradbury and Ramesh, 1985; Palmieri et al., 1988). Disagreements over assignments were resolved by the work of Palmieri et al. (1988). The detailed analysis of the 1H NMR spectra of divalent metal ion-substituted insulin hexamers (Palmieri et al., 1988) and the assignment of the aromatic region of native human monomeric insulin (Roy et al., 1990a) provide assignments which are used as a starting point.

The insulin concentration dependence of some chemical shifts, selective irradiation experiments, COSY experiments, NOESY experiments, and integration of relative areas of resonances are used to assign resonances and determine the nature of associated species. Interpretation of C2 His(B10) resonances were also used to determine equilibrium concentrations and to estimate the equilibrium constants.

**EXPERIMENTAL PROCEDURES**

**Materials**—Zinc-free porcine and human insulins and the Asp(B9)-Glu(B27) insulin mutant were gifts from Novo Nordisk (Bagsvaerd, Denmark). Sodium chloride (NaCl), deuterated water (D2O), deuterated sodium hydroxide (NaOD), deuterated hydrochloric acid (DCl), and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were purchased from Sigma. All chemicals were used as received without further purification.

**Preparation of Samples**—Metal-free insulin powder was dissolved in the solvent (D2O) containing the desired concentration of NaCl. The solvent was usually brought to pH 9-10 initially to facilitate dissolution of insulin. The pH was then adjusted by addition of small aliquots of NaOD or DCl (2 mM stock solutions). A Radiometer pH26 pH meter equipped with a combination electrode was used for pH measurements. The meter was calibrated between pH 7 and 10 with commercial buffers in H2O. Throughout this paper, pH* refers to the measuring uncompensated hydrogen ion concentration.

The unperturbed FID was obtained by subtracting the resonances from 5700 Hz, then applied to the hexamer. The pH was determined from absorbance measurements at 280 nm using e = 5700 M\(^{-1}\) cm\(^{-1}\) (Porter, 1953). For the latter purpose, a small (10-20 μl) aliquot of the sample was withdrawn and diluted with a volume of phosphate buffer sufficient to give absorbance measurements between 0.3 and 0.6.

**1H NMR Measurements**—1H NMR spectra were recorded either at 500 MHz on a G.N. spectrometer or at 400 MHz on a Bruker spectrometer at a probe temperature of 26 ± 1°C. The spectral width was usually ±3000 Hz for one-dimensional experiments and approximately 2250 Hz for two-dimensional experiments. Quadrature detection was employed in the first dimension. Spectra destined for quantitative analysis were obtained by the application of a single pulse followed by a waiting time of 17 s to insure return to equilibrium of all magnetization between successive pulses. Either 200 or 400 transients were coadded during these experiments. The FIDs were collected with 32,000-bit resolution to achieve a better definition of the resonance.

The longitudinal relaxation times (T\(_1\)) were roughy determined from the crossover relaxation delay during an inversion-recovery experiment using the relationship T\(_1\) = t(crossover)/ln2. The longest T\(_1\) measured for the C2 histidine resonances is approximately 2.7 s. Therefore, the waiting time was set to be at least 5 times T\(_1\). (Kadmon, 1986).

The relative integration of the C2 histidines resonances was obtained by weighing the cut-out plotted resonances. This procedure was employed, instead of integration measurements by the provided software, to avoid biases introduced by base-line correction needed prior to integration.

Selective irradiation of the resonances was performed according to a typical NOE difference experiment (Mersh and Saunders, 1982). Selective saturation was employed and the difference method applied to obtain spectra. The NOE or exchange effects were generated using gated decoupling to facilitate comparison of perturbed and unperturbed spectra. All NOEs were generated in a single experiment by cycling the irradiating frequency at all frequencies of interest every 8 scans. The longest T\(_1\) measured for the C2 histidine resonances is approximately 2.7 s. Therefore, the waiting time was set to be at least 5 times T\(_1\) (Kadmon, 1986).

The relative integration of the C2 histidines resonances was obtained by weighing the cut-out plotted resonances. This procedure was employed, instead of integration measurements by the provided software, to avoid biases introduced by base-line correction needed prior to integration.

**SELECTIVE IRRADIATION OF THE RESONANCES**

The COSY and NOESY pulse sequences were modified to include a presaturation pulse at the water signal. COSY spectra were recorded with increments in the f, dimension (t\(_1\)) equal to the dwell time, typically ±222 μs. For each increment, 64 FIDs of 1000 data points size were coadded. The total number of experiments (increments) was either 256 or 512. For NOESY experiments, t\(_1\) was set equal to 1 ps.

The abbreviations used are: NOE, nuclear Overhauser effect; FID, free induction decay; COSY, homonuclear two-dimensional correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.
the dwell time as well (±222 μs), and the mixing time was 100 ms. The total number of experiments was 256 (with 192 FIDs coadded per experiment).

All two-dimensional spectra were submitted to magnitude calculation after Fourier transformation. The FIDs were multiplied by a sine-bell window function without phase shift to improve resolution of the magnitude spectra (Bax, 1983).

RESULTS

Assignment of Resonances Due to C2 Protons of His(B10) and His(B5) in Two Aggregated States—The aromatic portion of the spectrum of the monomeric insulin Glu B9 → Asp Thr B27 → Glu species, obtained through site-directed mutation (Brange et al., 1988), is shown in Fig. 1A, along with the corresponding spectrum of native insulin at 36 μM concentration (Fig. 1B) (Roy et al., 1990b). This region of the spectrum comprises resonances due to aromatic residues (Tyr(A14), Tyr(A19), Phe(B1), Tyr(B16), Phe(B24), Phe(B25), Tyr(B26)) and the His(B5) and His(B10) C2 and C4 protons as assigned in Fig. 1A (Roy et al., 1990a). The His C2 protons give resonances located between 7.8 and 7.4 ppm, well removed from the main aromatic envelope (6.5–7.4 ppm) where there occurs a strong overlapping of resonances. At low concentration (spectra A–C), two resonances are observed in the His C2 region of the spectrum, one at 7.75 ppm and the other at 7.54 ppm, designated 1 and 2, respectively (see Fig. 1). These resonances were previously assigned to the C2 protons of His(B10) (1) and His(B5) (2) (Palmieri et al., 1988; Roy et al., 1990a, 1990b).

It has already been reported (Palmieri et al., 1988) that the chemical shifts and linewidths of the His C2 resonances are affected by changes in the concentration of insulin in a fashion that reflects the association of insulin. Both the number of resonances and the relative areas of resonances vary with insulin concentration (Fig. 1, spectra A–F).

The spectrum of the monomeric mutant (A) and the spectrum of 36 μM native insulin (B) shown in Fig. 1 are strikingly similar, indicating that at this concentration native insulin is also monomeric and that the two proteins have essentially the same solution structures (Roy et al., 1990a, 1990b). A slight increase in concentration (to 0.23 mM, spectrum C) causes broadening of resonances associated with aggregation to the dimer (Roy et al., 1990b). The chemical shifts of resonances 1 and 2 are unaffected by variation in the concentration of insulin between 36 μM and 5 mM.

Four new resolved resonances, designated 1’, 2’, 3, and 4, are detected when the concentration of insulin is increased above 1 mM (Fig. 1, spectra E and F); 1’ is located upfield of 1 at 7.43 ppm, 2’ (~7.66 ppm) overlaps with resonance 2, while 3 is located upfield of the main aromatic envelope at 6.70 ppm, and 4 is also located upfield at 6.55 ppm. As will be shown in a subsequent portion of “Results,” the two new upfield resonances, 1’ and 2’, are due to the His C2 protons of His(B10) and His(B5), respectively, in a higher aggregate species (viz. Figs. 2 and 5). The upfield peaks (3 and 4) are derived from other aromatic signals that are shifted upfield due to aggregation.

At pH* values >10.2 (Fig. 3), resonance 1 resolves into two
resonances (1a and 1b), and at all pH values >10.2, the intensities of 1b and 1' are similar and decrease as the pH increases. We assign resonance 1a to the (identical) C2 protons of His(B10) in the dimer, while resonances 1b and 1' are assigned to His(B10) C2 protons in the higher aggregate. This finding indicates that 1b is due to the C2 proton of a His(B10) that is not shielded in the higher aggregate.

**Determination of Equilibrium Ratios of Species Present at pH** 9.0 and 9.4 — Extensive area measurements were made at pH 9.0 and 9.4 in the presence of 0.1 M NaCl in order to provide information regarding the equilibrium concentrations of the dimer and the higher aggregate. Table I presents a summary of the results obtained.

**Table I**

<table>
<thead>
<tr>
<th>pH</th>
<th>[Insulin]_total</th>
<th>A1 - A1'</th>
<th>A1 + A1'</th>
<th>[Dimer]</th>
<th>[Tetramer]</th>
<th>K21</th>
<th>M⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>4.80</td>
<td>0.89</td>
<td>0.48</td>
<td>0.96</td>
<td>4.310⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>4.00</td>
<td>0.87</td>
<td>0.35</td>
<td>0.83</td>
<td>6.710⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>3.20</td>
<td>1.0</td>
<td>0.40</td>
<td>0.60</td>
<td>3.910⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>2.37</td>
<td>1.0</td>
<td>0.29</td>
<td>0.45</td>
<td>5.310⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>1.58</td>
<td>1.0</td>
<td>0.27</td>
<td>0.26</td>
<td>3.610⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>4.36</td>
<td>0.82</td>
<td>0.31</td>
<td>0.94</td>
<td>9.910⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>3.64</td>
<td>0.91</td>
<td>0.25</td>
<td>0.69</td>
<td>1.110⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>3.14</td>
<td>1.0</td>
<td>0.33</td>
<td>0.62</td>
<td>5.910⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>2.21</td>
<td>0.89</td>
<td>0.23</td>
<td>0.44</td>
<td>8.510⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>1.44</td>
<td>1.0</td>
<td>0.25</td>
<td>0.23</td>
<td>3.710⁵</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The total insulin concentration is expressed as subunits of 5,800 daltons.

* [Dimer] = \( \frac{A1 - A1'}{A1 + A1'} \times \frac{[\text{Insulin}]_{\text{total}}}{2} \)

* [Tetramer] = \( \frac{2A1'}{A1 + A1'} \times \frac{[\text{Insulin}]_{\text{total}}}{4} \)

* \( K_{21} = \frac{[\text{Tetramer}]}{[\text{dimer}]^2} \)

* The average values are \((4.7 \times 10^3 \pm 0.7 \times 10^3) \text{ M}^{-1} \) at pH 9.4 and \((7.8 \times 10^3 \pm 1.6 \times 10^3) \text{ M}^{-1} \) at pH 9.0.*

**Fig. 4. Sodium chloride effects on aggregation.** The aromatic region of the 500-MHz 1H NMR spectrum of metal-free human insulin is shown at pH 9.4: A, 2.5 mM insulin; B, 2.5 mM insulin plus 0.1 M NaCl. Inset, NaCl concentrations: a, 0; b, 0.02 M; c, 0.04 M; d, 0.06 M; e, 0.08 M; f, 0.1 M. 2,2-Dimethyl-2-silapentane-S-sulfonate (DSS) was used as the internal standard. Sample was not spun.

**Table II**

<table>
<thead>
<tr>
<th>pH*</th>
<th>[NaCl]</th>
<th>[Dimer]</th>
<th>[Tetramer]</th>
<th>( K_{21} )</th>
<th>M⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>0</td>
<td>0.15</td>
<td>0.19 \times 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>0.02</td>
<td>0.29</td>
<td>0.76 \times 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>0.04</td>
<td>0.33</td>
<td>1.2 \times 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>0.06</td>
<td>0.41</td>
<td>3.2 \times 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>0.08</td>
<td>0.44</td>
<td>8.3 \times 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>0.10</td>
<td>0.41</td>
<td>3.3 \times 10³</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effect of NaCl is, therefore, a shift of the equilibrium toward formation of a higher aggregate to the detriment of the dimer. This effect is seen to saturate above 40 mM NaCl (Fig. 4, spectra a-f).

**High NaCl Concentrations Induce a Conformational Change** — Portions of COSY and NOESY spectra of a concentrated insulin sample measured in the presence of 100 mM NaCl are shown in Fig. 5 (A and B, respectively). The COSY spectra with and without NaCl are similar; there are no detectable cross-peaks associated with the resonances previously identified as the C2 protons of the His(B5) and His(B10) residues (region between 7.8 and 7.4 ppm). Therefore, these resonances (1, 1', 2, 2') give no detectable i-correlation to each other or to resonances in the aromatic regions.

The NOESY spectrum (Fig. 5B) recorded in the presence of NaCl reveals two cross-peaks, designated a and b in the His C2 proton region. Cross-peak a connects resonances 1 and 1'. This signal is due to transfer of magnetization through...
**Discussion**

*The Higher Aggregate Is a Tetramer*—The organization of the His(B10) residues in the metal-free hexamer is depicted in Fig. 2. The high resolution x-ray structure of this species (Hill *et al.*, 1991), shows the 6 His(B10) residues on equivalent chelating sites designed to bind zinc ions. Each site is made up of 3 equivalent His residues, one from each asymmetric dimer. Formation of the hexamer in this fashion brings the C2 protons of each His(B10) residue under the imidazole ring of the equivalent residue in the next dimer. In this conformation, the C2 proton of each residue is positioned in the local field generated by the ring current from the adjacent imidazole ring where it opposes the applied field. We postulate that this screening shifts the His(B10) C2 resonance to higher field. Since resonance 1' experiences a net upfield shift of 0.25 ppm compared with resonance 1 (Figs. 1, 3, and 4), there is some change in the micromagnetic environment of the His(B10) C2 proton (as compared with B5) upon aggregation of dimers into the higher aggregate. If the sub-units of this oligomer are organized like those of the metal-free insulin hexamer (Hill *et al.*, 1991), the screening effect on the His(B10) C2 protons can be justified as resulting from anisotropic ring current effects from the adjacent His(B10) residues in the tetramer or hexamer (but not the dimer).

The rationale for the assignment of 1' and 1b as due to the tetramer is as follows. The nature and extent of the shift from position 1 to 1' undergone by the His(B10) C2 protons strongly suggest that, under conditions of low ionic strength, the (His(B10)) side chains of the higher aggregate species are arranged in a constellation essentially the same as that found in the T-state metal-free hexamer (Hill *et al.*, 1991). Since in a T-state hexamer, each C2 His(B10) proton senses ring currents of the imidazolyl ring of the equivalent histidine residue in the neighboring dimer (see Fig. 2A), all six of the C2 His(B10) protons are expected to resonate at the same frequency. However, in a T-state tetramer, only one of the C2 His(B10) protons (proton x in Fig. 2b) in each dimer senses the ring currents; the other remains in a micromagnetic environment practically equivalent to that in a free dimer (compare proton y in Fig. 2, b and c). The C2 His(B10) protons resulting from the tetrameric species are expected, therefore, to give a spectrum with two resonances of equal intensity, one at the chemical shift close or equal to that of the C2 His(B10) protons in the dimer, presumably 1b, and the other at a lower chemical shift; presumably that of resonance 1' (see Fig. 3).

Furthermore, the equality of the intensities of resonances 1b and 1' also argues against the existence of the hexamer. If the hexamer and the tetramer coexisted in solution, then the area under 1' would represent the sum of half the concentration of the C2 His(B10) protons in the tetramer and the concentration of all the C2 His(B10) protons in the hexamer, thus the intensity of 1' would be greater than the intensity of 1b. Consequently, we assign resonances 1' and 2' to the His(B10) and His(B5) C2 protons, respectively, of a T-like insulin tetramer.

A similar screening effect was observed in the spectrum of the two-zinc hexamer (Palmieri *et al.*, 1988). At pH* between 9 and 10, a single His C2 proton resonance occurs at 7.58 ppm. This resonance was assigned to the superimposed signals from the C2 protons of the Zn2+-coordinated His(B10) ring and the C2 protons of (metal-free) His(B5). The chemical shift of the His(B10) resonance was rationalized as due to the shielding ring current effect from adjacent B10 imidazole rings and the deshielding effect from Zn2+ coordination. Accordingly, in the absence of zinc, this resonance should be shifted to a lower chemical shift than 7.58 ppm, and it is found to
occurs at 7.43 ppm (resonance 1, Fig. 1). In contrast, the C2 His(B5) protons are minimally affected by further association, indicating that interaction between these C2 protons and other residues upon aggregation of the dimer to a T-state tetramer causes negligible chemical shift effects. Note that in the dimer, the C2 His(B10) protons resonate at a chemical shift position only 0.04 ppm higher than in the monomer, consistent with the absence of any ring current effects (viz. Fig. 1).

**On the Association Constants for a Dimer = Tetramer Model**—Given that only two of the four C2 His(B10) protons in the tetramer are expected to sense ring currents and the ratio \( (A1 - A1')/(A1 + A1') \) is the fractional concentration of C2 His(B10) protons in the dimer, then according to the assignments in the previous section, the ratio \( A1'/A1 + A1' \) shown in Table I should be equal to one-half the fractional concentration of C2 His(B10) protons in the tetramer. Using the concentration of insulin and the fractional concentrations, the concentrations of the dimer and tetramer were estimated according to the equations defined in the footnote to Table I. When fit to the dimer/tetramer model, the data yield calculated equilibrium constants (\( K_{eq} \) values, Table I) which vary between \( 3.6 \times 10^2 \) and \( 6.7 \times 10^4 \) at pH 9.4 with an average value of \( (4.7 \times 10^6 \pm 0.6 \times 10^6) \). Considering all possible errors in the procedure used to estimate concentrations, a standard deviation around 14% (as observed) is acceptable. The result at pH 9, \( K_{eq} = (7.8 \times 10^3 \pm 1.6 \times 10^3) \), carries a higher standard deviation of 20%.

The literature reports a value of \( 5 \times 10^4 \) M\(^{-1} \) for \( K_{eq} \) at pH 7 and low ionic strength (50 mM KHPO\(_4\)) (Pocker and Biswas, 1981). In the absence of NaCl at pH 9.4, the value of \( K_{eq} \) deduced from the present study is \( 1.9 \times 10^6 \) M\(^{-1} \), and at 0.1 M NaCl concentration is \( 4.7 \times 10^5 \) M\(^{-1} \). These results indicate that, at low ionic strength, the tetramer is approximately 25 times more stable at pH 7 than at pH 9.0 or 9.4. At high pH, a zinc free tetramer, assembled with a protein structure corresponding to the T-state of the two zehmers of hexamer (T-state), will experience strong electrostatic repulsions from interaction of the Glu(B13) carboxylates (Cowman and Dunn, 1988; Kaarsholm et al., 1990). This unfavorable interaction may explain the lower stability. In the crystalline T-state at pH 6.8, the central cavity of the hexamer is defined by the six carboxyl groups of the Glu(B13) residues which are arranged in hydrogen-bonded pairs that span the monomer-monomer interfaces (Baker et al., 1988; Hill et al., 1991). In the presence of divalent metal ions such as Ca\(^{2+}\) or Cd\(^{2+}\), these side chains reorganize to form binding sites for divalent ions which span the dimer-dimer interfaces (Eudesmeier et al., 1981; Storm and Dunn, 1986; Dunn et al., 1987; Baker et al., 1988; Hill et al., 1991). In the absence of metal ions coordinated to this site and at high pH, electrostatic repulsions between the B13 carboxylates would lower the stability of the tetramer and decrease the tendency to further assembly into the hexamer (Cowman and Dunn, 1988). Further evidence for the predominance of the tetramer at this pH has been obtained from dynamic light scattering measurements.7

**On the Sodium Chloride Effects**—Fig. 5C shows a NOE between His(B5) and a Tyr residue. The only known structures of insulin in which His(B5) is close enough to a tyrosine to give an NOE are the T3R3 and Rs structures. Our modeling of a T-R-R structure shows that the rings of B5 and B16 come within 4 Å and that no other aromatic residue comes in close contact with the His(B5) ring in any of the known structures. The latter fact is consistent with the absence of NOES between His(B5) proton resonances and any tyrosine resonances (Weiss, 1989; Kline and Justice, 1990; Hus and Weiss, 1990, 1991; Weiss et al., 1991). These two facts argue strongly in favor of the assignment of the spin system shown in Fig. 3B to the ring protons of Tyr(B16). According to this assignment, the Tyr(B16)2.6 and 3.5 ring protons resonate at 7.33 and 6.82 ppm, respectively. Incidentally, in the monomeric insulin mutants Ser(B9)→Asp, Thr(B27)→Glu at pH 9.3, the Tyr(B16) ring protons 2.6 and 3.5 give resonances at 7.31 and 6.85 ppm, respectively. The interpretation of the above cited NOE as an R-state signature is further reinforced by observed spectral changes in the aliphatic region, particularly the methyl region. The overall structure of the envelope of resonances due to methyl protons is altered in the presence of NaCl in a fashion similar to that observed in spectra of the phenol-induced R-state hexamers. A broad envelope of resonances becomes resolved into sharper individual resonances between 1.4 and 0.0 ppm (data not shown).

The structures of the phenol-induced Zn(II)-Rs species (Derewenda et al., 1989) and the Cl−-induced Zn(II)-Rs species (Smith et al., 1984) show residues Gly(B8) and Ser(B9) make close contacts with Tyr(B16); thus, cross-peaks connecting the resonances of these residues to those of Tyr(B16) should be expected. (Model building studies indicate the α carbons of Gly(B8) and Ser(B9) are located within 5 Å, respectively, of the furthest meta carbon of Tyr(B16).) The NOESY spectrum shows cross-peaks between the Tyr resonances assigned to B16 and resonances in the aliphatic region at 4.4, 4.1, and ~3 ppm (Fig. 6). In the absence of unusual anisotropic effects, the chemical shifts of resonances due to Gly α protons, the Ser α proton, and the Ser β protons on peptides occur in the vicinities of ~4.0, ~4.5, and ~3.9 ppm, respectively (Wuthrich, 1986). Consequently, two of the three experimental chemical shifts (4.4 and 4.1 ppm) are comparable with those expected for the α protons of Ser and Gly residues. The resonance at 3 ppm could be due to the β protons.

---

7 In some instances, the NOE between the C2 and C4 of a His residue can be detected. Consequently, an alternative interpretation for cross-peak b is that this signal is such an NOE. However, in the assignments of monomeric insulin (Roy et al., 1990a) the C4 proton of His(B5) gives a 6.78 ppm signal. Since the micromagnetic environment of the His(B5) C2 proton is unaffected by aggregation, it seems unlikely that the C4 proton would be shifted from 6.78 to 7.33 ppm. 8 According to the assignments of Roy et al. (1990a), in monomeric insulin Ser(B9)→Asp, Thr(B27)→Glu, the set of doublets due to ring protons of Tyr(A19) has decreased and, most evident, the dispersion of chemical shifts, and coupling constants similar to those of ring protons of Tyr(B16). At pH 9.3, ring protons 2.6 and 3.5, of Tyr(A19) resonate at 7.32 and 6.77 ppm, respectively, and those of Tyr(B16) resonate at 7.31 and 6.85 ppm, respectively. Thus, according to our present assignment of the ring protons of Tyr(B16), the chemical dispersion of doublets due to the ring protons of Tyr(A19) has decreased and, most evident, the resonance due to protons 2.6 of Tyr(A19) has shifted upfield (see phase-sensitive COSY spectrum in Fig. 5D) in the spectrum of associated insulin species. This result implies that the interaction responsible for the large dispersion of the ring proton resonances of Tyr(A19) in the monomer is perturbed or destroyed upon association. Protons interacting with aromatic rings generate large effects in chemical shifts due to ring current effects (Wuthrich, 1986). The x-ray crystal structure of the T6 insulin hexamer shows that the ring of residue Phe(B25) makes contact with the ring of Tyr(A19) in molecule B of the asymmetric monomer in the dimer and that the aromatic ring of Tyr(B26) makes contact with the ring of Tyr(B16) (Roy et al., 1990a). It is therefore quite compelling to attribute the large dispersion of the doublets due to ring protons of Tyr(A19) and Tyr(B16) to the above cited ring-ring interactions. Our results thus suggest that, in the native structure of associated insulin, the ring of Phe(B25) does not make contact with the ring of Tyr(A19). Such a change in conformation is conceivable since the x-ray crystallographic data show two different conformations of the Phe(B25) ring in the two different monomers of the dimer.
of a Ser residue that are shifted to lower field by the ring current from an adjacent aromatic residue. In the R-state conformation, the Ser(B9) β protons are located below the aromatic ring of the Tyr(B16) residue. Together, these NOESY cross-peaks are consistent with the assignment of an R-like structure to the NaCl-induced aggregate.

The T to R transition (Kaarsholm et al., 1989) was first described in structural detail in two different crystalline insulins, T3R3 and R6 (Smith et al., 1984; Derewenda et al., 1989). Phenol was found to induce a T₆ to R₋ conversion (all six subunits are affected) (Kaarsholm et al., 1989; Derewenda et al., 1989), whereas lyotropic anions, such as SCN⁻, I⁻, Br⁻, and Cl⁻, convert only three of the six subunits, giving a crystalline T₃R₃ species (Smith et al., 1984; DeGraaff et al., 1981). It was later found that the two conformational changes could also be induced in solutions of various metal-substituted hexameric insulin species (Roy et al., 1989; Kaarsholm et al., 1988; Brader and Dunn, 1990; Brader et al., 1990, 1991). A metal-free insulin hexamer, the B13Glu → Glu mutant, also was found to undergo the T to R transition, thus establishing that metal ions were not a prerequisite for the conformational equilibrium toward the higher associated species. Hence, NaCl ions may also affect the association by interacting specifically with insulin at the Glu(B13) sites in a fashion similar to Ca²⁺, Cd²⁺, Pb²⁺, and other divalent metal ions (Hill et al., 1991; Coffman and Dunn, 1988; Storm and Dunn, 1984; Sudmeier et al., 1981). NMR (Palmiere et al., 1988) and kinetic studies (Kaarsholm and Dunn, 1987; Coffman and Dunn, 1988) have provided evidence that the binding of Cd²⁺ or Ca²⁺ to the B13 site (Baker et al., 1988; Hill et al., 1991) drives the assembly of the hexamer.

Herein, we demonstrate that sodium chloride induces a T to R conformation change. This transformation requires the disruption of a salt bridge at the dimer-dimer interface of the T-state between the α-NH₁ of PheB₁ and the carboxylate of GluA₁. This salt bridge contributes to the association of dimers into aggregates of the T-state conformation (tetramer or hexamer). As evidenced by the presence of peak 1 in Fig. 1 (spectrum 2) and by the absence of cross-peak b in Fig. 5 (inset D), assembly of dimers into T-state tetramers takes place at high insulin concentrations in the absence of NaCl. The conversion to the R-state destroys the Phe(B₁)-Glu(A₁) ion pair bridge. Indeed, fairly low concentrations of salt (below 0.1 M) are sufficient to provide electrostatic shielding between ion pairs (Dill, 1990). Accordingly, we conclude that one effect of NaCl is to lower the stability of the salt bridge. The experimental evidence of a simultaneous conformation change and a shift of equilibrium toward the aggregated form suggests that NaCl shifts the equilibrium via stabilization of the R-state. It follows that coulombic ion-pair interactions must be decreased in the R-state aggregate, and this destabilization of the salt bridge shifts equilibrium toward the formation of associated R-state species.

CONCLUSIONS

Experimental evidence from ring current-induced chemical shifts, integration of C2 histidine resonances, saturation transfer data, and COSY and NOESY spectra establish the existence of the insulin monomer at very low concentration (Roy et al., 1990b), and the dimer and the tetramer in metal-free insulin solutions at higher concentrations and at alkaline pH (9–9.4) (viz. Figs. 1 and 3).

The organization of insulin subunits in the zinc-free tetramer in solution appears to resemble that of the T-state zinc-free insulin hexamer in the crystal, particularly with respect to the disposition of the His(B10) rings (Hill et al., 1991) (Fig. 2). The two His(B10) zinc sites are partially formed in the tetramer. Anisotropic ring current effects between adjacent His(B10) rings cause one of the His(B10) C₂ protons of each dimeric unit to be shifted upfield. The micromagnetic environment of His(B5) is essentially unperturbed by aggregation. Under the experimental conditions employed, formation of the metal-free hexamer is thermodynamically unfavorable. We postulate this change in stability is due to electrostatic repulsions between the B13 glutamyl carboxylates, which make close contacts at the center of the hexamer.

The presence of an NOE between the C₂ ring proton of His(B5) and the ring protons of a Tyr residue in sodium chloride solutions is most reasonably explained as resulting from an NaCl-induced transformation to an R-like conformation. If this NOE arises from an R-state conformation,
then the Tyr resonances almost certainly must belong to the Tyr(B16) ring protons, the only aromatic structural candidates known to be able to interact with His(B5) ring protons. We conclude that NaCl acts by disrupting the salt bridge at the dimer-dimer interface between Phe(B1) and Glu(A17) in the T-state via electrostatic screening of this ion pair interaction, and this effect shifts the T- to R-conformational equilibrium in favor of the R-state.

REFERENCES


Friederich, E. (1956) Arch. Biochem. Biophys. 65, 218-228


