1H NMR Spectrum of the Native Human Insulin Monomer

EVIDENCE FOR CONFORMATIONAL DIFFERENCES BETWEEN THE MONOMER AND AGGREGATED FORMS*

(Received for publication, August 28, 1989)

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The effects of high dilution on the 1H Fourier transform NMR spectrum of native human insulin at pH* 8.0 and 9.3 have been examined at 500 MHz resolution. The dependence of the spectrum on concentration and comparison with the spectrum of a biologically highly potent monomeric insulin mutant (SerB9→Asp) establish that at 36 μM (pH* 9.3) or 18 μM (pH* 8.0) and no added buffer or salts, human insulin is monomeric. Under these conditions of dilution, ionic strength, and pH*, human insulin and the SerB9→Asp mutant exhibit nearly identical 1H NMR spectra. At higher concentrations (i.e., >36 μM to 0.91 mM), native human insulin dimersize, and this aggregation causes a change in insulin conformation. Although there are many changes in the spectrum, the Tyr** ring H3,5 proton signals located at 6.63 ppm and the methyl signal located at 0.105 ppm (characteristics of monomeric insulin) are particularly distinct signatures of the conformation change that accompanies dimerization. Magnetization transfer experiments show that the 0.105 ppm methyl signal shifts downfield to a new position at 0.45 ppm. We conclude that the 0.105 ppm methyl signal is due to a conformation in which a Leu methyl group is centered over and in van der Waals contact with the ring of an aromatic side chain. Dimerization causes a conformation change that alters this interaction, thereby causing the downfield shift. Nuclear Overhauser studies indicate that the methyl group involved is located within a cluster of aromatic side chains and that the closest ring-methyl group interaction is with the ring of PheB**.

Insulin self-associates in solution in a complex and dynamic equilibrium that includes monomeric, dimeric, tetrameric, and hexameric forms, as well as hexamer aggregations. Coordination of Zn** to the HisB** sites of the hexamer and Ca** to the GhuB** sites of tetramer and hexamer strongly favor aggregation (Blundell et al., 1972; Sudmeier et al., 1981; Storm and Dunn, 1985; Dunn et al., 1987; Kaarsholm and Dunn, 1987; Coffman and Dunn, 1988). At the concentrations found in blood, the monomer is the predominant species and is almost certainly the physiologically relevant form (Pekar and Frank, 1972; Blundell et al., 1972). At neutral pH, ultracentrifugation, concentration difference spectroscopy, and circular dichroism studies of metal-free insulin (bovine and porcine) yield dimerization association constants (K12) in the range of 1×10^5 to 2×10^5 M^-1 (Strazza et al., 1985; Jeffrey et al., 1976; Goldman and Carpenter, 1974; Lord et al., 1973; Rupley et al., 1967). Circular dichroism studies by Pocker and Biswas (1981) gave a larger apparent value for K12 of 7.5×10^5 M^-1. Based on osmotic pressure measurements, earlier work indicates that moderately dilute solutions of metal-free insulin (0.18-0.43 mm) dissociate to monomers upon an increase in pH to 9.1-9.5, while more dilute solutions (70 mM) are predominately monomeric at pH 8.3 (Fredericq, 1956; Marcker, 1960a, 1960b). Low ionic strength lessens the degree of association (Fredericq, 1953, 1956), and studies in the acid pH range indicate that association is favored in solutions of high ionic strength (Fredericq and Neurath, 1950; Oncley et al., 1952). Although the aggregation phenomena are more complicated at alkaline pH, a similar relationship between increasing ionic strength and association has been reported (Fredericq, 1956). Previously, proton NMR studies have not been sufficiently sensitive to allow structure-function studies of the native monomer at a pH that is not either extremely acidic (i.e., pH 3) or basic (i.e., pH 11) (Footnote 1; Hsu et al., 1988; Bradbury and Ramesh, 1985).

Recently, we were able to assign the aromatic region of the 1H NMR spectrum of several genetically engineered monomeric human insulin mutants at neutral to basic pH. Most of these insulin mutants, including the SerB9→Asp mutant, retain high in vivo potency (Brange et al., 1988). Due to the increased sensitivity of high resolution Fourier transform NMR spectrometers, we are now able to work at concentrations sufficiently low to obtain an 1H NMR spectrum of the native insulin monomer at pH* 8.0 and above. By comparison to the sequence-specific assignments of the monomeric insulin mutants and of the native insulin monomer at high pH,1 we are able to propose sequence-specific assignments of the aromatic region of native monomer at pH* 9.3 and 8.0 and to characterize the 1H NMR spectral changes that occur as monomer associates to an aggregated form that is predominantly the dimeric species. These studies indicate the native insulin monomer has a conformation that differs in some respects from the conformations of higher aggregates.

EXPERIMENTAL PROCEDURES

NaOD (40% solution), DC1 (20% solution), and 2,2-dimethyl-2-silapentane-5-sulfonate-2,2,3,3-d4 were purchased from Sigma, D2O (99.8%) was purchased from Aldrich. Chemical shifts are reported in

* This work was supported by American Diabetes Association Grant ADACA/DUNN/88 and by a grant-in-aid (to M. R.) from the Novo Research Institute. 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.

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parts/million (ppm)\(^2\) relative to the methyl signals of 2,2-dimethyl-2-silapentane-5-sulfonate-2,2,3,3-d\(_4\). Human insulin and the SerB9→Asp mutant as the metal-free lyophilized powders (Orange et al., 1988) were dissolved in D\(_2\)O and adjusted to the desired pH* with NaOD for the \(^1\)H NMR studies reported herein. Insulin concentrations were determined from measurements of the 280 nm absorbance (\(c_{280} = 6200 \text{ M}^{-1} \text{cm}^{-1}\)) (Schlichtkrull et al., 1975). All quoted concentrations refer to the monomeric species. pH meter readings in D\(_2\)O solutions are given as pH* and are uncorrected for the isotope effect. All glassware was acid-washed and rinsed with metal-free distilled water prior to use.

NMR spectra were obtained on a GN-500 spectrometer and Nicolet 1280 computer with 226 K byte RAM. The proton probe was thermostated at 25.5 °C. One-pulse one-dimensional NMR spectra were run with the decoupler frequency set to the Hz0 frequency for a specified interval prior to data acquisition. At least 32 accumulations were collected for each signal-averaged free induction decay, and free induction decays were processed by exponential multiplication with line broadening typically set at 0.3 or 0.5 MHz. For the truncated NOE (TNOE) experiments, at least 1000 accumulations were collected with the following pulse sequence \(t_1-\text{II}-t_2-\text{II}/2\)-acquisition. The sum of \(t_2\) and \(t_3\) is set so that there is selective suppression of the water peak (due to differing \(T_1\) recovery times), while \(t_3\) is the standard relaxation delay. The value of \(t_2\) (decoupler on) ranged from 500 ms to 3.5 s and the value of \(t_3\) was adjusted accordingly. In order to avoid nonselective effects, the decoupler power was kept quite low (\(L_1 = 4 \times 15\)).

RESULTS AND DISCUSSION

Two important findings emerge from the data presented in this communication: 1) Monomeric human insulin (Fig. 1, spectrum a; Fig. 2, spectrum d) and the biologically active monomeric mutant insulin, SerB9→Asp (Fig. 1, spectrum b; Fig. 2, spectrum e), exhibit nearly identical \(^1\)H NMR spectra. 2) Aggregation of human insulin is accompanied by changes in the \(^1\)H NMR spectrum (compare spectra a and d of Fig. 2) that almost certainly arise from a change in conformation (Fig. 3). These findings strongly support the conclusion that monomeric human insulin and the monomeric AspB9 mutant have highly similar three-dimensional structures in solution, and that subunit interactions in higher aggregation states stabilize conformations which are different from that of the biologically active monomer. The evidence for these findings and conclusions is presented and discussed in the following paragraphs.

Comparison of the \(^1\)H NMR Spectra of Monomeric Native Human Insulin and B9Asp Mutant Human Insulins at pH* 9.3 and 8.0—The equilibrium constant for dimerization is more favorable at neutral than alkaline pH (Fredericq, 1956). Therefore, to compare the spectrum of native insulin monomer with that of the monomeric mutant at pH* 8.0 (Fig. 1, spectra a and b, respectively), it was necessary to record the spectrum of the native species at 18 \(\mu\)M. As the signal to noise ratio indicates, this protein concentration (Fig. 1a) is close to the sensitivity limitations of the GN 500 MHz spectrometer. For work on the native insulin monomer, scrupulously metal-free glassware and solvent are required. Studies were done in D\(_2\)O without any added buffer, and the minimum amount of NaOD was used to titrate samples to the desired pH. From the association behavior (determined via osmotic pressure measurements and by circular dichroism spectroscopy, Orange et al., 1988), the AspB9 mutant has been shown to be almost completely monomeric at a concentration of 1 mM 25 °C and pH ≥ 7. Complete, sequence-specific assignments for the aromatic region of the spectrum are given for the B9Asp mutant (Fig. 1, spectrum b).\(^3\) The close similarity between the linewidths and chemical shifts of resonances in the two spectra establish that human insulin is predominantly monomeric under these conditions and that the assignments for AspB9 also apply to the native species.

In Fig. 2, the spectral changes of native insulin at pH* 9.3 for the subunit concentration range from 0.91 mM to 36 \(\mu\)M...
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Fig. 3. TNOE intensities of 1 mM AspB9 insulin at pH* 9.3, 25.5 °C. A, comparison of the one-dimensional 1H NMR spectrum of the aromatic region (upper panel) with the one-dimensional TNOE difference spectrum (lower panel) generated by selective irradiation of the methyl signal at 0.1 ppm. Sequence-specific assignments of the aromatic region are indicated. Additional TNOE signals occur in the aliphatic region (data not shown). B, comparison of the one-dimensional 1H NMR spectrum of the aliphatic region (upper panel) with the one-dimensional TNOE difference spectrum (lower panel) generated by selective irradiation of the TyrBz6 H3,5 signal at 6.90 ppm. Note that irradiation at 0.1 ppm generates TNOEs from residues TyrBz6, PheBz5, and perhaps TyrBz6 (A), while only irradiation at 6.90 ppm give a TNOE at 0.1 ppm, the upfield methyl signal (B). To avoid nonselective effects, the decoupler power level was kept deliberately low for the TNOE studies. DSS, 2,2-dimethyl-2-silapentane-2,2,3,3-d4 (internal standard).

are presented (spectra a–d). The spectrum of the monomeric mutant SerB9→Asp at pH 9.3 and 1 mM concentration is shown in trace e for comparison. Comparison of the native human insulin spectra measured at 0.0 mM and at 36 μM insulin subunits (spectra a and d) indicates the emergence of a new doublet at 6.63 ppm in the 36 μM sample (spectrum d). The line widths and close similarity of spectrum d to spectrum e indicate that human insulin is predominantly in the monomeric state at 36 μM. The aromatic and aliphatic regions of the 1H NMR spectra (Figs. 1 and 2) indicate little change in monomer conformation as the pH* drops from 9.3 to 8.0.

1H NMR Signatures for the Interconversion of Monomeric and Oligomeric Forms of Insulin—Our unpublished 1H Fourier transform NMR studies strongly indicate that the changes in Fig. 2 (spectra a–d) correspond to dimer ↔ monomer interconversion. The molecular weight studies of Fredericq (1956) and of Goldman and Carpenter (1974) support this conclusion. The dimer is the predominant (metal-free) species of bovine insulin in the concentration range 0.1–10 mg/ml at pH 8.0, 23 °C, and an ionic strength of 0.1 (Goldman and Carpenter, 1974). Fig. 2 shows that the B26 tyrosine ring, H3,5 doublet at 6.63 ppm, is a particularly clear-cut marker of aggregation; as the fraction of monomer present increases (due to dilution), the signal for the B26 H3,5 doublet appears at 6.63 ppm. X-ray studies of 2Zn insulin have established that the TyrBz6 ring is located on the monomer-monomer interface. The peptide carbonyl O and amide N of TyrBz6 both are H-bonded in pleated sheet fashion to the corresponding amide N and carbonyl O atoms of PheBz5 across the interface (Baker et al., 1988). Clearly, since the spin system of the PheBz5 ring is also affected by changes in the extent of aggregation (Fig. 2, spectra a and d), the environment of PheBz5 is also altered by this interaction. Although the aromatic ring of PheBz5 has been postulated to be important in dimerization (e.g. Pullen et al., 1976; Blundell et al., 1972; Pocker and Biswas, 1981), the B25 resonances appear little affected by aggregation. In the hexamer x-ray structure, the PheBz5 ring makes contact with the neighboring B25 ring across a 2-fold axis (Baker et al., 1988). Because the PheBz5 spin system is not well resolved, subtle perturbations are impossible to perceive with 1H NMR, and this lack of resolution may be responsible for the apparent discrepancy between the x-ray structure and the NMR data.

The PheBz5 spin system is reasonably well resolved both in the aggregated species (probably the dimer, Fredericq, 1956), and in the monomer (cf. traces a and d, Fig. 2), assignments are given in Fig. 1b for the AspB9 mutant, and these signals undergo very little change. In the x-ray structure of the insulin hexamer, the PheBz5 ring extends out across the dimer-dimer interface and is inserted into a pocket on the adjacent dimer (Baker et al., 1988). The shift of this spin system as the dimer aggregates to tetramer and hexamer (data not shown) is in agreement with predictions drawn from the x-ray structure. Hence, PheBz5 resonances indicate the extent to which the dimer-dimer interface is involved in aggregation. Despite the possibility of aggregation along the dimer-dimer interface (Jeffrey, 1985), under the conditions of these experiments the AspB9 mutant does not associate along that interface.

Of the remaining aromatic amino acid side groups (in Fig. 2, spectra a–d), absolutely no change in chemical shift is seen in HisB5* or HisB1*. There is a slight upfield shift in the TyrBz6 signals. While the resolution of the TyrBz6 resonances is sufficiently poor that a small change would be missed, a larger perturbation would be noticeable. The ring of A14 is not involved in the formation of the monomer-monomer interface of the crystalline 2Zn insulin hexamer. Consequently, the 1H NMR spectral changes characterizing the transition from dimer to monomer are in reasonably good agreement with predictions based on the x-ray structure. However, in the disassociation transition, there is a noticeable upfield shift in the H3,5 doublet of the TyrBz6 ring. Although this residue has never been assigned a direct role in insulin dimerization, TyrBz6 has been postulated to be part of the extended receptor binding region (Pullen et al., 1976). Therefore, it is possible that the change in the local environment of TyrBz6 when dissociation to monomer occurs reflects a conformation change that is essential for the physiological activity of the monomer.

In the aliphatic region of the 1H NMR spectrum, the monomer is distinguished from higher aggregation states by perturbation of several signals located between 0 and 1.4 ppm and by changes in linewidth (Fig. 2, compare spectra a and d). The signal located at 0.105 ppm is particularly characteristic of the monomer state. Two-dimensional correlation spectroscopy and relay two-dimensional correlation spectroscopy experiments, as well as integration and classification of aliphatic proton spin systems3 are now underway to assign the aliphatic region of the spectrum of the SerB9→Asp mutant. Our preliminary classification work indicates the 0.105 ppm signal is due to a Leu methyl resonance. At 0.105 ppm, this signal is shifted far upfield from the normal spectral region of methyl groups in free amino acids and in random coil peptides (Wüthrich, 1986, p. 19) and from the median chemical shift of methyl resonances found in native proteins (Gross and Kalbitzer, 1988). When placed in close proximity to a methyl group, carbonyl groups and disulfide bonds can
One-dimensional truncated nuclear Overhauser effect (TNOE) was used to establish the proximity of aromatic ring protons to the protons of the 0.105 ppm signal (Fig. 3). The resonance at 0.105 ppm was selectively irradiated (see "Experimental Procedures"). The TNOEs resulting from this pre-irradiation are illustrated in the difference spectrum shown in Fig. 3A. The TNOE difference spectrum contains the resonances of the H2,6 and H3,5 doublets of TyrBz5, the H4 and H3,5 triplets and apparently the H2,6 doublet of PheBz4, and the H4 and H3,5 triplets of PheBz5. With selective irradiation at 6.90 ppm (the resonance of the B24 H3,5 triplet), the TNOE difference spectrum (Fig. 3B) includes the upfield methyl signal centered at 0.105 ppm. No NOE results at 0.105 ppm when either the resonance of the H3,5 doublet of B24 or that of the H4 triplet of B25 were pre-irradiated (data not shown). The closest aromatic ring to the upfield methyl appears to be that of PheBz4. Although it is unlikely, from the data available we cannot rule out the possibility that the TNOE effects on TyrBz5 and PheBz4 are due to spin diffusion. Two-dimensional NOE spectroscopy experiments confirm the presence of the strong NOE signal between the 0.105 ppm methyl resonance and the H2,6 doublet of PheBz4 (data not shown).

The position of the Leu methyl signal in the dimer was determined by magnetization transfer under conditions of concentration (0.3 mM insulin subunits) where there are similar concentrations of the monomer and the dimer. These studies (data not shown) establish that magnetization is transferred from the methyl resonance at 0.105 ppm in the monomer to a resonance located at 0.45 ppm in the dimer.

In the x-ray structure of the zinc-insulin hexamer, the LeuBz1 methyl group is 4.8 Å away (closest methyl C–C ring distance) from the B24 C2 ring (ring). The other methyl groups neighboring to B24 in the crystalline hexamer include ValBz4 CG1 (at 5.8 Å), ValBz4 CG1 (at 8.3 Å), HisBz4 CG2 (at 8.8 Å), and LeuBz2 CD2 (at 9.7 Å). COSY studies identify the 0.105 ppm signal as that of a Leu methyl (data not shown). If the x-ray structure is relevant to the solution conformation of monomeric insulin, then the 0.105 ppm methyl signal is most likely derived from the CD1 methyl group of LeuBz1.

As human insulin is diluted and dissociation to monomer occurs (Figs. 1, a and b, and 2), the general change in line widths appears to correlate with the change in molecular size expected for conversion of dimer to monomer. The area under the 0.105 ppm signal increases without undergoing much change in line width, and there appears to be no change in chemical shift. Assuming association and dissociation occur via second-order and first-order processes, respectively, the invariance of chemical shift and line width indicate that monomer and dimer are in slow exchange (Sudmeier et al., 1980) and that, in the oligomeric state, the methyl resonance is located downfield at 0.45 ppm. Therefore, the area of the 0.105 ppm signal provides a direct measure of the fraction of monomeric species present.

Assuming the aggregation process is dominated by dimerization at pH 7.3, analysis of the concentration dependence of the 0.105 ppm signal yields an estimate of  for 3 ± 1 × 10^9 M^-1. Owing to the sensitivity limitations of H NMR in the relevant concentration range, the value determined for  is not very precise. This value is measured at a higher pH and a much lower ionic strength than previously published values (viz., Goldman and Carpenter, 1974, Blundell et al., 1974), parameters that critically influence insulin association. Consequently, the magnitude of this value is considerably larger than previously reported values.

Evidence for a Conformational Transition between Monomer and Dimer—Ring currents from Phe or Tyr rings shield protons which reside within a cone (with an apex angle of ~65°) perpendicular to the plane of the ring, while protons outside this cone experience a downfield shift (Pople, 1956). The shift of the Leu methyl resonance from 0.105 ppm in the insulin monomer to 0.45 ppm in the dimer almost certainly establishes that insulin undergoes a change in conformation upon aggregation. In the monomer, the Leu methyl group must be in very close contact with, and orthogonal to, the plane of PheBz4 and not far away from the aromatic rings of PheBz4 and TyrBz5. This set of interactions is altered by the aggregation-induced conformational transition. Using the benzene ring as a model (Johnson and Bovey, 1958), we estimate that the spectral perturbation arises from a conformational change involving a minimum relative motion of 1–3 Å. A conformation change of this magnitude could give the reduced anisotropic effect. Of course, the change could result from a larger motion in which one of the perturbing aromatic rings (e.g. PheBz5) takes up an alternative conformation. Consequently, we conclude that the free monomer conformation is not the same as that in the aggregated state. There are prior indications of conformational differences between monomer and oligomeric forms of insulin. Circular dichroism data suggest that the dissociated monomer has less α-helical secondary structure than that found in the monomer subunit of a hexamer (Pocker and Biswas, 1960).

On the Relationship between Conformation and Biological Activity—Studies of semisynthetic insulin analogues (Nakagawa and Tager, 1986, 1987; Mirmira and Tager, 1989) indicate that the C-term region of the B-chain in the vicinity of residues B24 and B25 is important for the specificity and affinity of insulin-receptor interactions. The elegant studies of Mirmira and Tager (1989) give evidence indicating that flexibility in the main chain near B24 is important for achieving high affinity and that interaction of the aromatic ring of B24 with the receptor triggers conformational changes in insulin that are important for insulin-receptor recognition.

Since the monomer conformations within the hexamer are stabilized by interprotomer interactions, it is not obvious what the conformation of the insulin monomer should be in solution. The H NMR spectral changes that accompany dissociation from monomer strongly imply that the monomeric species takes up a conformation in solution that is different from that in the aggregated forms. The magnitude of the conformational change and the significance of this change to the interactions between insulin and its receptors must await further structure-function studies.

Acknowledgment.—We thank Dr. Niels Kaarsholm for helpful advice and encouragement.

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1H NMR spectrum of the native human insulin monomer. Evidence for conformational differences between the monomer and aggregated forms.

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J. Biol. Chem. 1990, 265:5448-5452.

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