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NMR STRUCTURE NOTE

Biosynthetic engineered $B28^{K}$–$B29^{P}$ human insulin monomer structure in water and in water/acetonitrile solutions

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Biological context

Insulin engineering is a process intended to obtain desired therapeutic properties by rationally designed sequence mutations of a wild type insulin (HI) which originally consists of two chains, having 21 and 30 amino acids (AA) in chain A and B, respectively, held together by two disulfide bridges, namely $A7^{C}$–$B7^{C}$ and $A20^{C}$–$B19^{C}$ and intra strand bridge in chain A; $A6^{C}$–$A11^{C}$. It is known that human insulin (HI) circulates in the bloodstream as a monomer and in such a form passes effectively through the capillary membrane and binds to the insulin receptor (DeFelippis et al. 2001). An increase in insulin concentration causes formation of noncovalent dimers and higher oligomers. These entities are present in pharmaceutical preparations. Natural propensity of insulin to self-association has meaningful implication on pharmacokinetic behaviour upon hormone administration. Insulin diffusion into tissues entails progressive dissociation of hexamers into dimers and monomers. This is revealed as an increase in a serum insulin concentration reaching a maximum at approximately 2–3 h after subcutaneous injection and followed by slow regression within 9 h. Such pharmacokinetic behaviour causes inconvenience to patients forcing them to adapt their schedules to a medicine, what adversely affects their quality of life.

Therefore, two therapeutic strategies have emerged with the potential to mimic physiological insulin secretion. First, rapid-acting insulin analogues were designed to have a more rapid onset of action than HI preparations. These analogues are suitable for mealtime blood glucose control as they simulate pulsatile insulin secretion during meals. Secondly, long acting insulin analogues characterized by delayed absorption and peakless activity profile were developed to mimic basal insulin secretion between meals and through the night.

An example is provided by Lantus, a medicinal product containing ArgB31–ArgB32, A21 → Gly or recently published recombinant insulin LysB31–ArgB32. The pharmaceutical motivation for the addition of a dibasic tag at the end of the B-chain is to shift the isoelectric point from 5 to 7, thus leading an acidic formulation (at pH 4.0) to undergo isoelectric precipitation at physiological pH in the subcutaneous tissue. Such precipitation is accompanied by zinc insulin hexamer and provides a ‘long-acting depot’, which is redissolved slowly and absorbed into the bloodstream lasting for about 24 h (Lepore et al. 2000).

Another way of protraction hypoglycaemic activity of insulin is to attach fatty acids to insulin, which forms a reversible complex with serum albumin. The binding occurs through the fatty acid moiety. As an example, insulin detemir has been developed by the removal of threonine from the position B30 and acylation of the C14 fatty acid chain (myristic acid) at position 29 on the B

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chain. Detemir complexes slowly dissociate from albumin and therefore prolong activity of the drug is achieved (Kurtzhals 2004).

Design strategy for rapid-acting insulin analogs is targeted against insulin propensity to dimerization. It is well known that the formation of insulin dimer requires β-strand in the dimer interface, B24–B28 motif in C terminus of chain B. This is observed in a dimer embedded in a crystal structure of T6 Zn native HI hexamer (Smith et al. 2003), in solution structure of R6 native HI hexamer, and in a crystal structure of T3R3f hexamer of the KP biosynthetic HI (also designated insulin lispro) (Ciszak et al. 1995). Within last two decades several groups have investigated sequence mutation of HI which would favor the monomer structure in solution and allow understanding mechanisms of fibrillation (Hua and Weiss 2004; Mauro et al. 2007). It was already demonstrated that insulin self-association can be drastically altered by substitution of one or two key AA responsible for dimer formation. Significant disruption of association was achieved by replacing LysB29 with Pro and placing the Lys amino acid at B28, as in the KP HI (Birnbaum et al. 1997; Brems et al. 1992). This amino acid reversal at B28–B29 decreases association by primarily disrupting the formation of a dimer. Such amino acid substitutions also substantially reduces the Zn-induced insulin hexamer formation. The formation of monomeric insulins through amino acid replacements was accompanied by conformational changes at C-terminus of B chain that may be the cause for decreased association.

We have recently shown that the water/acetonitrile solvent allows to retain tertiary structure exactly matching the native structure of a monomer embedded in a crystal of hexamer and prevents the aggregation of a species having native-like conformation (Borowicz et al. 2009, 2011, 2012). Unlike the water solutions, where an extent of oligomerization is concentration dependent, in this water/organic solvent, insulin is monomeric in a wide range of concentrations (Bocian et al. 2008a, b). Use of this solvent therefore potentially allows to study the effect of various factors on misfolding process of a monomer under classical conditions leading to amyloid formation. This property of a water/acetonitrile solvent also allows to compare the results of various techniques at the same concentration of a monomer. This seems to be of interest in view of recent suggestion that the model of thermal denaturation may be different for the zinc bound insulin hexamer versus monomer or dimer (Huus et al. 2005). Our recent results show that at 3 mM and pH 3.6, in water, HI exists in complex equilibrium also involving higher than hexamer oligomers, but is primarily monomeric at ca. 20 μM (Bocian et al. 2008a, b). Therefore, kinetic results obtained by various techniques, applied at vastly different concentrations of a solute, may be prone to concentration biased differences (Dzwolak et al. 2005).

Thus the effect of water-acetonitrile solvent may bring about similar situation to that observed in insulin KP (Howey et al. 1994), in which disruption of natural β-strand, as in HI, results in dramatic (300 fold) decrease of the monomer–monomer association. Although the KP insulin was launched on diabetic pharmaceutical market several years ago, its monomer structure in solution and aggregation (self-association) behaviour are not known. Here we show that tertiary structure of a monomer is essentially the same in water and in water-acetonitrile solution. We show that network of hydrogen bonds (HB) is the same in both solvents. Furthermore, nearly the same set of HBs is found in a crystal structure of insulin KP what serves as evidence that solution tertiary structures closely much the one established by X-ray.

Methods

Insulin source and preparation

Insulin used for measurements was zinc insulin lispro CRS (Ph. Eur. Reference Standard; code no. Y0000348; batch 1.0 ca 5.93 mg) which was purified on Sephadex G25 fine column using 1 % acetic acid as an eluent, followed by overnight incubation with Chelex 100 BIORAD in 1 % acetic acid. Finally, insulin KP solution was lyophilized with deuteriated acetic acid and stored at −20 °C. Human insulin standard (HI) was purchased in Sigma-Aldrich and purified as above.

Sample preparation

The preparation of the NMR samples was conducted by the similar procedure, as described by Hua et al. (1995) except that different solvent was used. The solvent used in present work was $H_2O(D_2O)/CD_3CN$ (73:27 vol%). The insulin lispro solutions of 2.9 ± 0.1 mM concentration, pH 2.5 ± 0.1 (adjusted by adding the aliquots of HCl and NaOH solutions) at ambient temperature, 25 °C, were used for measurements in $H_2O(D_2O)/CD_3CN$ (73/27 vol%). In this solvent two samples of similar concentration (1.85 mM) were measured, H_2O/CD_3CN was used to run NOESY and TOCSY whereas HSQC was run in D_2O/CD_3CN. The spectra of KP insulin in water solution were run as follows; NOESY spectrum was measured in H_2O/D_2O (90/10 vol%), at concentration of 0.5 mM whereas carbon spectrum was measured in neat D_2O, also at 0.5 mM concentration. The concentration of samples was measured by UV absorption spectroscopy at 280 nm. The ^{1}H and ^{13}C NMR spectra were calibrated *vs*. TSPA signal in a studied solvent.

NMR spectra

The spectra in H_2O/D_2O (90/10 vol%) and in neat D_2O were acquired on Varian 700 MHz and in $H_2O/(D_2O)/CD_3CN$ (73:27 vol%), on Varian 500 MHz instruments. The exhaustive experimental details of *NOESY, TOCSY, COSY, HSQCAD, HSQCAD–TOCSY* and *PFGSE* spectra are given in Supplementary Information.

Calculation procedures and refinement protocol details are given in Supplementary Informations.

Results

Establishing an aggregation state in solution

During dilution, self-associated insulin aggregates tend to dissociate until the concentration characteristic for a monomer is reached. This process can be monitored by measurements of translational diffusion coefficients, Di, using PFGSE technique. Our earlier results on HI aggregation (D_2O, 30 °C, pH 3.6) suggested that at 0.5 mM concentration there still is significant amount of tetramer present in a solution. In this work, aggregation states of HI and insulin KP were studied comparatively at the same conditions. Figure 1 shows that the concentration dependence of the diffusion coefficient for HI and insulin KP differs significantly indicating a very different dissociation pattern.

Human insulin at pH 2.5 even at low concentrations forms higher aggregates in contrast to the KP insulin which at concentration of 0.5 mM has the Di value of the order of 1.25×10^{-10} m^2/s. It is worth noting that the concentration dependence of the KP insulin shows exponent which can be indicative of equilibrium. The fit of a dilution curve (Fig. 1S) to an isodesmic model, assuming nonspecific aggregation, revealed that at 0.5 mM concentration there is over 75 % of monomer, 18 % of dimer and 5, 1.4, 0.4 and 0.1 % of higher aggregates. This distribution was calculated using value of 1.37×10^{-10} m^2/s as a diffusion coefficient for monomer (see to Supplementary Information). These results agree with the literature data claiming that the KP insulin is mainly monomeric in water solution at concentration up to 0.5 mM (Ciszak et al. 1995; Brems et al. 1992). Our experiments also point to a complex equilibrium of monomer/dimer/tetramer/hexamer/higher aggregates at concentration of ca. 3.0 mM. It is judged from the fact that at 3 mM concentration diffusion coefficients, Di, are similar for both insulins. At the same time it does not exclude the situation that distribution and shape of higher aggregates of the KP insulin can be different from that established earlier for HI (Bocian et al. 2008a, b). This in fact happens, as indicated by experimental results

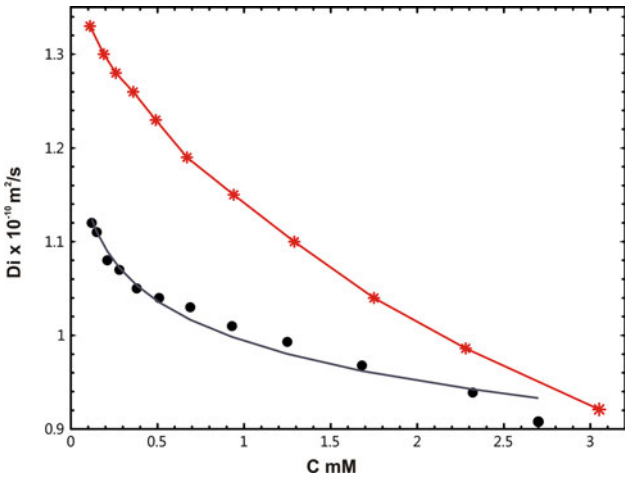

Fig. 1 Comparison of translational diffusion coefficient, Di $\times$ 10^{-10} (ms^2/s), versus concentration in D_2O of insulin KP (*upper curve*) and human insulin (*lower curve*), at pH 2.5 and 25 °C

published to date. It was presented that monomer–monomer association of insulin KP is ca. 300 times weaker than that of HI (Brems et al. 1992; Birnbaum et al. 1997). Another published data suggests that insulin KP in pharmaceutical formulations, existing as a hexamer in the presence of Zn and phenol, disscociates directly to monomer after subcutaneous injection (Ciszak et al. 1995; Brems et al. 1992). Both these facts seem suggest that dimerization of insulin KP does not proceed via specific association using a dimer interface as in HI, rather it associates nonspecifically, using oppositely charged surface faces of a monomer and only presence of Zn and phenol can induce formation of a hexamer. Furthermore, fast exchange of species in NMR time scale does not warrant long enough contact time to observe intermolecular cross peaks between exchanging species and therefore they are characteristic for a monomer as a dominant form in solution, most probably entering the higher, poly dispersed oligomers in its monomer conformation.

According to the above reasoning it was observed during this study that the shape of ^1H NMR spectrum does not change below 0.5 mM concentration, although the value of diffusion coefficient is still rising. Taken together, all these experimental observations strengthen our confidence that monomer conformation is dominating in the solution characterized by the high value of Di, 1.25×10^{-10} m^2/s, and therefore we have chosen this concentration for study of the monomer of the insulin KP in water at pH 2.5, 25 °C using 700 MHz NMR.

The discussion of dilution experiments for both insulins reveals the main difference in their behaviour, i.e. weaker propensity for dimerization of insulin KP in comparison to native human insulin. The intuitive cause of this is disruption of the dimer interface, B24–B28, due to mutual displacement of B28, B29 AA.

In water/organic solvent the situation is different, from that described above, for both insulins (see Fig. 4S in Supplementary Information). Dilution of insulin KP in D_2O/CD_3CN (73/27 vol%) solution gives a linear concentration dependence of diffusion coefficient, D_i. A small, linear decrease of D_i with increasing concentration is expected because of friction, as a result of crowding of the monomeric species. Such a situation is observed in Fig. 4S which stands for the evidence of existence of the insulin KP monomer in D_2O/CD_3CN (73/27 v/v %) solution at pH 2.5 over an NMR accessible concentration range. It is important to note that the dependence of diffusion coefficient versus concentration in Fig. 4S is linear, without exponential curvature present, what is an evidence of absence of the equilibrium. Therefore, one can reason that there is a single species present in solution. Accordingly, the signals in threonine region in Fig. 3S do not change lineshape or chemical shifts. Furthermore, the diffusion coefficient found for the insulin KP (see Fig. 1) at lowest concentration is corresponding to protein of ca. 6 kDa (Ilyina et al. 1997) which points to the monomer. Several spectral features indicate that this species is a monomer. The ^1H NMR spectrum has sharp lines and shows dispersion of NH chemical shifts characteristic for structured insulin (Figs. 2S, 3S). The NOE's characteristic for a dimer are not found (Jørgensen et al. 1992). CSI analysis of C-terminus of chain B shows the lack of the β-strand, which is observed in the dimer interface of human insulin in a crystal structure. This is another independent prove of a monomer structure.

Secondary structure motifs

The comparison of CSI graphs for both chains for H^{α} protons in both solvents (Figs. 5S, 6S) show close similarity in the α-helices. Some differences are observed in unstructured motives, as for example for AA's $A20^C$, $B7^C$ or $B24^F$. There are two important conclusions from these data, namely, α-helices are placed in the same regions of the chain and begin and terminate at the same AA unit. Furthermore, the C terminus of chain B in water shows random coil pattern which confirms the monomer structure as found in H_2O/CD_3CN solution. Figures 7S and 8S allow also comparison of secondary structure motifs in KP insulin versus HI. It is seen that structure motifs are placed in the same places. The differences of Δδ for individual AA are very similar what could be a hint that tertiary structures are also very similar. This suggestion finds confirmation in a tertiary structure of HI insulin established earlier in water/acetonitrile solution (Bocian et al. 2008a, b). The only significant difference is observed at positions B27 and B28. This however can be expected as this is the site which is close to structural modification differentiating both insulins. The similarity of the chemical shift values in C terminus of B chain also suggests that the dynamics of this motif in both compared insulins should not differ very much in a given solvent. This suggestion finds confirmation in RCI values established here (vide infra).

Tertiary structures

The overall inspection of tertiary structures established in both used solvents (Fig. 2) shows close similarity of ensembles with higher flexibility at the very end of the B chain in H_2O/CD_3CN solution. The more detailed analysis is presented in Table 1. The two structures are solved with the same precision using comparable number of experimental restraints. The structure ensembles shown in Fig. 2 are uniformly dispersed around mean as judged from the similar values of RMSD. It is also worth mentioning that the rmsd values from X-ray of human insulin HI and insulin KP are very close in the two solvents strengthening our confidence that water/acetonitrile solvent can be used with success instead of water for establishing the native tertiary structure in physiological conditions.

The very important features which largely determines the biological properties of insulin are the dynamics of a flexible motifs of a molecule and hydrogen bond network (HB net) which is crucial for the shape of the backbone, overall tertiary structure and hydrophobic core of a protein.

In a following we discuss these features for insulin KP studied in water and in water/organic solvent in comparison to its X-ray. Figure 9S presents the dynamics of the KP insulin in both solvents versus human insulin in a form of RCI graph. This type of data analysis is recently proposed as a means of studying the protein segment flexibility. It relies essentially on the tenet that ensemble of CSI (Chemical Shift Index) (Wishart et al. 1995) values reflects the dynamic state of a motif. Displaced in Fig. 9S analysis was based on CSI values for $^1H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, N^1H nuclei using algorithm given in web server at http://wishart.biology.ualberta.ca/rci. It is seen that the shape of RCI plot for both insulins, KP versus HI, in H_2O/CD_3CN solution is the same although the KP insulin is more flexible at the very end of chain B. Insulin KP in H_2O/D_2O solution is more flexible along motif B25–B30 than human insulin, HI, and insulin KP in H_2O/CD_3CN. At the very end of C terminus of B chain, i.e. motif B27–B30, it is seen that insulin KP is more dynamic than human insulin HI, irrelevant of the solvent. This can be assigned to the structural difference of both insulins which results in disruption of a dimer interface, B24-B28 in insulin KP.

Interesting conclusions can be drawn from the analysis of HBs in liquid media versus X-ray structure of insulin KP

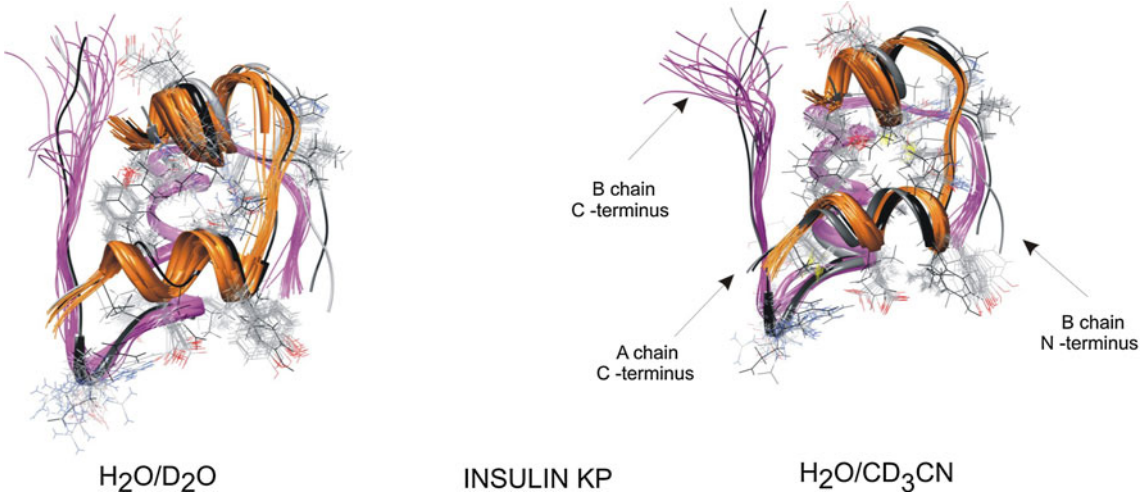

Fig. 2 Comparison of tertiary structure of insulin KP in H_2O/D_2O (90/10 vol%) and in H_2O/CD_3CN (73:27 vol%). *Black string* in both cases represents backbone from X-ray structure (**1lph**)

Table 1 Structural statistics of insulin KP for 100 structures, in solvent H_2O/CD_3CN[a] (PDB ID **2m1d**, BMRB entry **18858**) and H_2O/D_2O[b] (PDB ID **2m1e**, BMRB entry **18859**)

	AMBER_GB[a] $B28^{Lys}$-$B29^{Pro}$ Human insulin (insulin KP)	AMBER_GB[b] $B28^{Lys}$–$B29^{Pro}$ Human insulin (insulin KP)	AMBER_GB[c] Human insulin standard (HI)
Experimental NOE's[d]	2,118	2,194	1,251
Experimental restraints			
Total inter-proton	922	779	680
Intra-residue	268	247	177
Sequential	276	215	251
Medium range	222	162	142
Long-range (/j–i/>5)	156	155	110
RMSD from mean structure[e]			
All atoms in ensemble	1.95 (0.85)	1.92 (0.99)	1.828 (1.265)
Backbone heavy atoms	1.38 (0.26)	1.27 (0.37)	0.991 (0.482)
Long range restraints			0.8883
Ramachandran statistics (% residues included in)			
Most favored regions	86.9	84.3	92.4
Additionally allowed regions	11.6	14.5	6.9
Generously allowed regions	0.6	0.5	0.2
Disallowed regions	0.9	0.7	0.5

Comparison with human insulin, HI[c], in solvent, H_2O/CD_3 CN (73/27 vol%), structure deposited in PDB—**2jv1**, BRMB accession no. **15464**. Detailed comparisons with X-ray structures are given in Table 3S in Supplementary Informations

[a] Insulin KP in H_2O/CD_3 CN (73/27 vol%)

[b] Insulin KP in H_2O/D_2O (90/10 vol%)

[c] Human insulin, HI, in solvent, H_2O/CD_3 CN (73/27 vol%)

[d] The numbers of manually assigned NOE cross peaks. Only unambiguous cross peaks were considered in input for insulin KP and HI. The number of automatically assigned cross peaks for insulin KP[b] from total of 2,194 experimental cross-peaks is 1,714

[e] 20, 20 and 50 low energy structures are compared for insulin KP[a], insulin KP[b], and HI, respectively. Values in parentheses refer to statistic with excluded ultimate units in both chains, i.e.; residues 2–20 and 3–29 in chain A and chain B, respectively for HI but 3–20 and 5–20 residues in insulin KP[a,b]

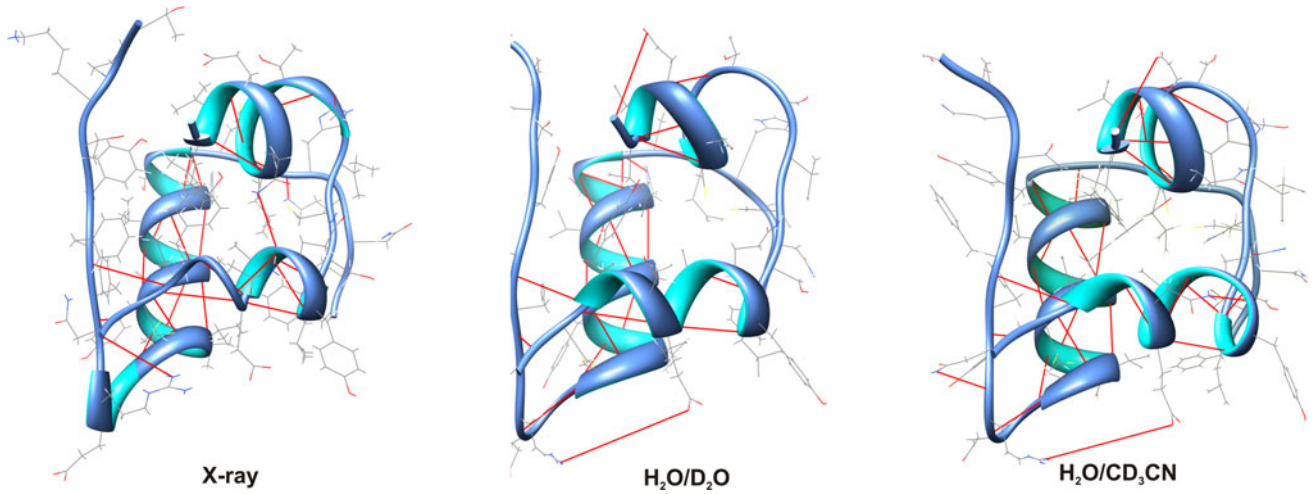

Fig. 3 Hydrogen bond network (*red lines*) in insulin KP in H_2O/D_2O and H_2O/CD_3CN solutions compared with that in X-ray derived structure. In solutions backbone represents mean of 20 discrete structures ensemble therefore the HB distances can be distorted

(**1lph**) (Fig. 3). Detailed data are given in supplement Tables 4S, 5S and 6S. The analysis of 20 structure ensembles reveal 97 HBs in both solvents whereas only 30 HBs are found in an X-ray structure. Inside the set of 97 HBs there are 23 HBs which are found in both solvents and they appear at least in 50 % of descrete 20 structures containing ensembles. This is evident than that both solvents allow establishing the tertiary structure in a very similar manner, but the adventage of using of water/organic solvent is that it allows elimination of protein aggregation. The 19 of these HBs are found in a crystal structure what can be treated as evidence that all three structures are essentially the same.

Conclusions

The tertiary structure of insulin KP monomer was established in water (H_2O/D_2O) and in organic (H_2O/CD_3CN; 73/27 vol%) solution at pH 2.5 and natural abundance of isotopes used in NMR analysis. These covered NOESY, TOCSY, $^{1}H/^{13}C$-HSQC and PFGSE techniques. The latter technique allowed gathering evidence of monomer structure in both solvents, at ca. 0.5 mM concentration in water and at higher concentrations in organic solvent. The aggregation in water was discussed in more detail based on measurements of translational diffusion coefficients at different concentrations of protein. It was concluded, on the basis of applying the isodesmic model in simulation the concentration dependence of translational diffusion coefficient, that insulin KP can aggregate to some extent forming polydispersed aggregates rather than specific forms of dimers or tetramers. This is explained by the fact that monomer/monomer association constant is ca. 300 times weaker for KP insulin as compared to human insulin, HI, and therefore specific aggregation is not abundant. The analysis, in a form of CSI graphs, of established ^{1}H and ^{13}C chemical shifts, revealed the same secondary structure motifs in both solvents. The detailed analysis of HB found in helices and in the side chains allows conclusion that both structures are essentially identical and are very close to the X-ray structure. This result strengthens our confidence that organic cosolvent can be successfully used instead of a physiological water solution in cases where intensive aggregation has to be eliminated. The conformational space of a tertiary structure defines its biological activity in interaction with insulin receptor (IR). This is essentially restricted, inter alia, via HBs in residue's side chains, either to donors or acceptors in helices or, in a long range interactions, to functionalities in other side chains. In the analysis of HBs there were found 5 such long range interactions in both solvents, appearing in 50 % of descrete structures forming final ensemble. However, there were total of ca. 60 HBs found in a whole ensemble but appearing less frequently. Establishing the HBs existing in solution is a challenge which is attempted in ongoing work.

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