Probing the Interaction of Protamine with Zn-insulin through Biophysical and Molecular Docking Studies

manoj munde[1], Soumya Aggarwal[1], and Neetu Tanwar[1]

[1]JNU

August 28, 2020

Abstract

There is a successful use of protamine-insulin formulation (Zn+insulin+protamine) to treat diabetes in which protamine is added to the stable form of hexameric insulin (Zn-insulin). The biophysical characterization of Zn-insulin, which can dissociate to form biologically active monomers, is well reported. However, its interaction with protamine, which is at the core of the mode of action in many pharmaceutical formulations, is unresolved. Through biophysical characterization, we have tried to dissect the interactions driving the Zn-insulin-protamine complexation. Based on the thermal melting study, it was found that protamine indulges in the destabilization of Zn-insulin. Fluorescence results revealed that Zn-insulin experiences Tyr quenching in the presence of protamine, undergoing a significant conformational change. As shown by the molecular docking study, protamine disturbs the H-bonding network at the dimer interface by binding to the amino acid residues involved in the dimer stabilization. It may result in the freeying of B-chain, introducing conformational fluctuations in the insulin. This is well supported by the loss of helical content seen in circular dichroism. Further, the insulin-protamine complex formation was strongly dominated by hydrogen bonding and a few hydrophobic contacts. The endothermic heat and positive entropy observed in isothermal titration calorimetry in the dissociation of Zn-insulin-protamine is a reflection of that. Finally, the ANS binding study proposed the adaption of a flexible conformation by the Zn-insulin-protamine complex containing exposed hydrophobic residues, a potential arrangement for successful receptor binding.

Abstract

There is a successful use of protamine-insulin formulation (Zn+insulin+protamine) to treat diabetes in which protamine is added to the stable form of hexameric insulin (Zn-insulin). The biophysical characterization of Zn-insulin, which can dissociate to form biologically active monomers, is well reported. However, its interaction with protamine, which is at the core of the mode of action in many pharmaceutical formulations, is unresolved. Through biophysical characterization, we have tried to dissect the interactions driving the Zn-insulin-protamine complexation. Based on the thermal melting study, it was found that protamine indulges in the destabilization of Zn-insulin. Fluorescence results revealed that Zn-insulin experiences Tyr quenching in the presence of protamine, undergoing a significant conformational change. As shown by the molecular docking study, protamine disturbs the H-bonding network at the dimer interface by binding to the amino acid residues involved in the dimer stabilization. It may result in the freeying of B-chain, introducing conformational fluctuations in the insulin. This is well supported by the loss of helical content seen in circular dichroism. Further, the insulin-protamine complex formation was strongly dominated by hydrogen bonding and a few hydrophobic contacts. The endothermic heat and positive entropy observed in isothermal titration calorimetry in the dissociation of Zn-insulin-protamine is a reflection of that. Finally, the ANS binding study proposed the adaption of a flexible conformation by the Zn-insulin-protamine complex containing exposed hydrophobic residues, a potential arrangement for successful receptor binding.

Keywords : Insulin, Protamine, ITC, Binding interaction**Introduction**

Insulin is a polypeptide hormone (Fig. 1) that modulates glucose levels in the blood. Deficiency in insulin's secretion causes diabetes[1, 2], and therefore it is also used as a medical compound that is widely prescribed for the treatment of diabetes. Insulin is stored in the pancreas in complex with Zn ions as a hexamer, which upon released into the bloodstream readily dissociates into the monomer[3-7]. This is an extremely essential step as only a bioactive monomer can bind and activate receptor to regulate blood glucose levels [3, 7].

In vitro study indicates that in solution at low concentration and physiological pH, insulin exists as a monomer or dimer[7]. Numerous biophysical studies of insulin under variable conditions helped to understand its structure and function[2, 5, 6, 8-15]. The active monomeric form consists of two polypeptide chains, chain-A 21 residues, and chain-B 30 residues (Fig. 1). These chains are held together by two interchain disulfide bonds (A7-B7 and A20-B19) and one intrachain disulfide bond in chain-A (A6-A11). Chain-B plays an important role in the association of insulin monomer into dimer and hexamer [6]. The hexamer (Zn-insulin), which is the most stable form of insulin[2, 10] is well characterized around neutral pH values [6, 8]. The hexamer is capable of transitioning among distinct conformational states, which have been given the nomenclature T_6, T_3R_3, and R_6 [16] depending on the conformations of the monomer subunits. The binding of anions and phenols to allosteric sites of insulin is needed to bring the conformational change from the T- to the R-state.

Figure 1

The clinical application of insulin is often challenged by changes in environmental conditions that can induce precipitation, chemical degradation, and/or fibrillation. The development of an insulin-protamine formulation is an important advancement in circumventing some of these problems[17]. In 1946, Hagedorn and coworkers introduced crystalline neutral protamine Hagedorn (NPH) insulin-containing insulin and protamine in "isophane" amounts (no excess of insulin or protamine) at neutral pH, also having small amounts of zinc and phenol[18]. There are several commercially available drugs based on NPH-insulin which are commercialized with the brand names (Humulin N, Novolin N, Novolin NPH, Gensulin N, SciLin N, and Insulatard). However, new formulations are still far from mimicking the original physiological role of insulin, and therefore there is a need to modify the existing ones to improve their medicinal value[19]. The key to addressing this problem is to cognize the interaction behavior among various constituents of the existing insulin-protamine formulation. Such a piece of information will not only provide guidelines to improve the existing formulation but also unwind different proteins or peptides, which can interact with insulin in a similar manner.

The use of strongly basic protein such as protamine, extracted from the nucleus of fish sperm combined with Zn-insulin (hexamer) was shown to prolong the effect of insulin. Although Zn-insulin interactions are widely studied, the interactions between protamine and zinc-insulin, which are at the core of the mode of action in the formulation, are unresolved. Therefore, understanding the molecular basis of such interactions is the major objective of this paper. Although several studies on insulin interaction with a variety of ligands have provided valuable insights into the possible ligand-binding mechanisms aimed at insulin [5, 20, 21], there are only a few reports on insulin-protamine complexes, including a crystallography study, which could not yield necessary atomic details due to poor resolution[17, 22]. There, it was concluded that the structure-based design is not a promising approach to improve on existing formulations[17].

In this study, with the careful use of experimental conditions, we investigated the insulin-protamine interaction in the presence of NaCl and Zn^{2+} ions around neutral pH [18]. Based on experimental results, it was concluded that protamine indulges in the destabilization of Zn-insulin. Fluorescence result revealed that Zn-insulin experiences Tyr quenching with a significant conformational change in the presence of protamine. The energetics of the dissociation of Zn-insulin in the presence of protamine indicated that the dissociation was endothermically as well as entropically driven phenomena. Molecular docking studies explained how protamine would enable the dissociation of insulin by binding to the amino acid residues at the dimer interface of insulin. Finally, based on ANS binding study, it was proposed that the protamine might play a role in promoting the conformation of insulin suitable for receptor binding. The results here will help to provide a basic understanding of the potential role of protamine in insulin formulation.

Material and methods:

Materials:

Insulin human recombinant, expressed in yeast ([?]98% purity, MW 5.8 kDa), protamine sulfate salt from salmon (MPRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR) (MW 5.1 kDa), 8 anilino 1 naphthalenesulfonic acid ammonium salt (ANS) and zinc sulfate monohydrate ($ZnSO_4$) were purchased from Sigma Aldrich. Na_2HPO_4, NaH_2PO_4, NaCl and phenol were purchased from Merck-chemicals Ltd. All experiments were performed in phosphate buffer at pH 8.0, containing 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, and 100 mM NaCl. The concentration of insulin was measured using absorbance a 276 nm on the Cary-100 UV-Vis spectrophotometer. The molar extinction coefficient used for insulin was 6200 $M^{-1}cm^{-19}$.

Methods:

UV-Vis spectroscopy: The UV measurements were performed in Cary 100 UV-Vis spectrophotometer. Melting studies were done over a temperature range of 25 °C–95 °C and absorbance at 276 nm was recorded after 3 hr incubation at 4 ^{0}C. The concentrations of insulin and $ZnSO_4$ were 30 μM and 15 μM, respectively. The experiment performed with phenol uses insulin:phenol ratio as 1:3.

Circular Dichroism (CD): The CD measurements were recorded by the CD spectrophotometer (Chirascan, Applied Photophysics). Far UV (200–260 nm) CD was used to investigate the secondary structural changes in insulin with a cuvette cell of path length 0.1 cm. Spectra were recorded for only-insulin and Zn-insulin in the presence and absence of protamine. The concentrations of insulin and $ZnSO_4$ for far UV CD were 20 μM and 10 μM respectively. All the experiments we performed in doublets. Additionally, experiments were also performed in the presence of phenol using insulin:phenol ratio of 1:3. Changes observed in the helical content were calculated using online software BeStSel (Beta Structure Selection) as shown in Figure 3C and 8D.

Intrinsic fluorescence spectroscopy: The fluorescence studies were performed using a fluorescence spectrophotometer from Cary eclipse Varian. For steady-state experiments, Tyr was used as an intrinsic probe. Protamine was titrated into only-insulin and Zn-insulin, and samples were excited at 276 nm and emission spectra were recorded between 280 to 400 nm. In case of the binding of protamine to Zn-insulin, the Stern Volmer equation was found to be non-linear (upward curvature in the Stern Volmer plot), a modified Stern Volmer[23] equation was used:

$$\frac{f_0}{(f_0 - f)} = \frac{1}{[Q]\, f_a\, K_Q} + \frac{1}{f_a}$$

Where, f_0 and f are the relative fluorescence intensity of protein in the absence and presence of quencher (Q), respectively; [Q] is the quencher concentration, K_Q is the effective quenching constant for the accessible fluorophores, and f_a is the fraction of accessible fluorophore. The plot of $f_0/(f_0$ - f) versus 1/[Q] yields $1/f_a$ as the intercept, and $1/(f_aK_Q)$ as the slope.

For the dilution experiment, samples were excited at 276 nm with a slit width of 10 nm. The initial concentration of insulin, $ZnSO_4$ and protamine for dilution were 20 μM, 10 μM and 20 μM, respectively. In order to calculate the quenching constant in case of binding of protamine to insulin and Zn-insulin, Stern-Volmer equation was used-

$$\frac{f_o}{f} = 1 + K_{\mathrm{SV}}\,[Q]$$

Where, f_o represents fluorescence intensity of insulin in the absence of protamine, f represents fluorescence intensity in increasing the concentration of protamine, K_{SV}represents Stern-Volmer quenching constant and Q represents the concentration of protamine. When data was plotted f_o/f i.e emission intensity v/s Q i.e concentration then upon linear fitting (y=mx+c), the value of slope will give the quenching constant K_{SV}.

ANS binding study using fluorescence: The surface hydrophobicity of insulin and its complexes with protamine was determined by the measurement of their fluorescence emission by titrating ANS into the insulin under various conditions. Emission spectra were recorded in the range of 400 to 600 nm by exciting all the samples at 388 nm. The excitation and emission slits were set at 5 nm and the scan speed was 600 nm/min. Here, the concentration of insulin was 10 µM and $ZnSO_4$ was 5 µM.

Isothermal Titration Calorimetry (ITC): ITC measurements were performed to obtain thermodynamic parameters associated with the dilution of only-insulin, Zn-insulin and Zn-insulin-protamine in 10mM phosphate at 25 @C on a MicroCal iTC200 (Malvern Instruments Ltd. UK)[24]. The concentration of insulin, $ZnSO_4$, and protamine was 200 µM, 100 µM and 200 µM respectively. A total volume of 40 µl from the injection syringe was added to a sample cell containing 280 µl of a buffer. There was a total of 20 injections with each injection of 2 µl of sample titrated into the cell containing buffer and each injection was separated by 150-sec interval to allow the signal to return to baseline. The sample from the syringe was titrated into an ITC cell containing a working buffer. All the experiments were done in at least doublets. The non-linear data was fitted to the single set of binding site model using the MicroCal ORIGIN 7 software supplied by the manufacturer, yielding binding constant (K_{ass}= $1/K_{diss}$), enthalpy change (ΔH_{diss}) and entropy change (ΔS_{diss})[24].

Molecular Docking: In order to explain the binding interaction of insulin and protamine, molecular docking was performed by utilizing HADDOCK2.2 (High Ambiguity Driven protein-protein DOCKing) program[25]. The structure of the protamine sequence was converted to PDB format using I-TASSER online software from Zang lab[26]. The crystal structure of the insulin monomer (PDB 2JV1) was downloaded from the protein data bank. All other docking parameters were set as default. After obtaining the docked structure, the analysis was done by Accelrys Discovery Studio 4.5. After the careful analysis of the parameters obtained for all the four docked structures, the structure with the most accurate possibilities and minimum energy was taken for further analysis.

Results

Probing the stabilization and conformational changes in insulin upon protamine binding

The thermal stability experiments were performed using UV-Vis spectroscopy to elucidate the melting behavior of insulin in its various associative states such as only-insulin and Zn-insulin. Samples were incubated for 3 hours to equilibrate before performing the melting experiments. In Fig. 2, high T_m (temperature at which the protein is half denatured) values for only-insulin (82^0C) and Zn-insulin ($>92^0$C) helped to establish their respective dimer and higher-order status[9,6]. Free protamine did not give any melting transition as protamine is a randomly coiled structure (data not shown). The higher stability for Zn-insulin compared to only-insulin is in agreement with the literature studies. On the contrary, the Zn-insulin-protamine resulted in a downward curve with reduced T_m of 45 ^{0}C. As per the prior reports, based on experimental and simulated data, ligand stabilizers shift the protein melting temperature upward, whereas ligand destabilizers shift the T_m downward[27]. Thus, it is obvious that the downward curve in the presence of protamine has resulted in the destabilization of Zn-insulin.

Figure 2

Far UV CD was employed in order to monitor the changes in the secondary structure of insulin under variable conditions. The far-UV CD spectrum of only-insulin (Fig. 3A) shows two minima, at 209 nm and at 222 nm, which signifies the presence of α-helical content in an agreement with the literature reports[2]. The only-insulin and Zn-insulin show identical helical contents (Fig. 3A and 3C). Since only-insulin forms dimer and Zn-insulin forms hexamer, the presence of identical helical contents suggests the structure of the independent dimer is nearly identical to the dimer of the hexamer insulin. However, in the presence of protamine, the CD spectrum of insulin (Fig. 3B) resulted in the reduction in the intensity of peaks at 208 and 222 nm, suggesting the loss of helical content in insulin. Such conformational changes would disturb the local environment, especially of the four Tyr residues present in the insulin. Similarly, protamine binding to Zn-insulin also resulted in deepening in the peak at 208 nm and 222 nm, indicating the destabilization of

the helical content in Zn-insulin[28]. The results support the melting data where the protamine was found to destabilize the Zn-insulin.

Figure 3

Fluorescence of aromatic amino acids in proteins acts as a probe for investigating the conformational changes in proteins[29]. Monomer insulin consists of four tyrosine residues, out of which two are involved in the helix of chain-A (A14 and A19) and the other two are involved in monomer-monomer interaction (B16 and B26)[30]. This suggests that tyrosine fluorescence could be exploited for probing the binding effect of protamine on structural rearrangements in free insulin and Zn-insulin. As shown in Fig. 4, the gradual addition of protamine to free insulin or Zn-insulin resulted in a decrease in fluorescence intensity. The quenching portrays the change in the microenvironment near Tyr residues, which can be attributed to the local conformational changes[23]. Since no shift in the maximum emission wavelength was observed, the polarity of tyrosine environment cannot be explained. Generally, Tyr/Try fluorescence is quenched when protein is in the native state, however, in this case, insulin not only undergoes quenching but also destabilization (from Tm and CD results) upon protamine binding. This could be attributed to i) binding-induced structural disorder resulted from perturbation of intra-/intermolecular interactions such as H-bonding, electrostatic interactions and hydrophobic ii) ionization of the Tyr by polar amino acids of protamine.

Fig. 4C depicts the modified Stern-Volmer plot for protamine binding carried out with insulin and Zn-insulin. The Stern-Volmer constant (K_{SV}) value represents the binding between quencher and fluorophore, whereas f_a represents fractional accessibility of tyrosine residues (Table 1). Ksv for Zn-insulin-protamine (8.9 $x10^3$ M^{-1}) was lower than that of insulin-protamine (2.3 $x10^5 M^{-1}$). It was accompanied by an increase of fa from 0.4 (only insulin) to 2.1 (Zn-insulin). These results indicate that tyrosine residues in free insulin were comparatively less accessible to the protamine than those in Zn-insulin. Hence, in both conditions, tyrosine residues possess different microenvironments, and ultimately different structural states.

Figure 4

Table 1

Probing the dissociation of the Zn-insulin-protamine complex using fluorescecne

The Zn-insulin complex, stored in the pancreas, upon released into blood forms biologically active monomeric species, and the process is driven mainly by dilution. Herein, we attempted to study a similar phenomenon. To this end, the intrinsic fluorescence spectra for only-insulin, Zn-insulin, and Zn-insulin-protamine complex were monitored while their concentration being constantly diluted to as low as 1 μM from 20 μM (Fig. 5A-C).

In all the cases, a linear decrease was observed in the characteristic tyrosine peak at 304 nm with a decrease in the overall concentration. The dilution was the major driving force for Tyr quenching here. When we plotted the data as a function of decreased insulin concentration (Fig. 5D), the slope value gives the quenching constant (K_{sv}). We found that the K_{sv} values for only-insulin (0.048 μM^{-1}) and Zn-insulin (0.033 μM^{-1}) were higher than the Zn-insulin-protamine complex (0.018 μM^{-1}). Since the starting concentration of insulin as well as the dilution prcedure was the same in each plot, the different K_{sv} values indicate differential Tyr environment in each case. In particular, the lowest K_{sv} in case of Zn-insulin-protamine suggests that Tyr residues in this case are least exposed.

Additionally, the fluorescence spectra of all the species (Top spectrun in Fig. 5A, B and C; before the start of dilution) were compared in Fig. 5E. The strength of the fluorescence intensity follows the order; only-insulin> Zn-insulin> Zn-insulin-protamine. The lowest intensity for Zn-insulin-protamine (Fig. 5E) further supports the burial of Tyr residues, suggesting that it may form a compact state.

Figure 5

Studying energetics of the dissociation of the Zn-insulin-protamine complex using ITC

Since the dissociation of Zn-insulin (hexamer) to biologically active species is an essential step, here, we

tried to obtain the quantitative thermodynamic parameters for the dissociation of Zn-insulin in the absence and presence of protamine using ITC (Fig 6). Herein, Zn-insulin and Zn-insulin-protamine samples were titrated from the syringe into an ITC cell containing a working buffer. As shown in Fig. 6A, as the sample concentration was titrated in the calorimeter cell with successive injections, the height of the endothermic peak decreased, and the magnitude of the heat uptake diminished accordingly. This gives rise to a typical heat of dilution curve, which can be fitted using the Origin software to obtain equilibrium dissociation constant (K_D=1/K_A), enthalpy change ($\boldsymbol{\Delta}$ H) and entropy change ($\boldsymbol{\Delta}$ S). The thermodynamic parameters are given in Table 2. The ITC analysis revealed that the K_D of Zn-insulin-protamine (223 M) is lower than Zn-insulin (333 M), suggesting the lower dissociation of Zn-insulin-protamine. The data further suggest that Zn-insulin-protamine dissociation is driven by higher endothermic heat and positive entropy than that of Zn-insulin. Endothermic heat indicates the loss of non-covalent interaction, whereas positive entropy indicates the increase in conformational freedom during the dissociation. The result helps to conclude that the protamine alters the way Zn-insulin undergoes dissociation, in agreement with the conclusions from the fluorescence experiments.

Figure 6

Table 2

Probing the conformational states of insulin through ANS binding

The extrinsic fluorescence dye 8-anilino-1-naphthalene sulfonate (ANS) is widely used for probing conformational changes in proteins[31, 32]. ANS has a tendency to fluoresce upon binding to solvent-exposed hydrophobic surfaces on proteins. Here, we used this property of ANS to understand the conformational changes in insulin under various conditions. In Fig. 7A and 7B, ANS binds very weakly to only-insulin (dimer) as well as to zinc-insulin (hexamer), suggesting that insulin under these conditions is intact with its hydrophobic regions buried inside the protein core. Also, since there is no blue shift in the wavelength maxima of ANS (dotted line), this is another indicator of no exposer of hydrophobic residues in the Zn-insulin and only-insulin states. On the other hand, the binding of ANS to the insulin-protamine complex is very strong (Fig 7C), implying that protamine-binding may have resulted in the dissociation and even partial unfolding of insulin, exposing large hydrophobic pockets to a solvent environment. This is supported by melting and CD studies where protamine was clearly shown to destabilize insulin without Zn. Interestingly, ANS binding to the Zn-insulin-protamine (Fig. 7D) resulted in intermediate intensity compared to the insulin-protamine complex and only-insulin or Zn-insulin. This suggests the exposure of a moderate hydrophobic region in the Zn-insulin-protamine complex, which may indicate the formation of a compact state. A similar compact state for chymopapain has been reported earlier using ANS as a probe[33]. The addition of ANS to protamine in the control experiment did not yield any increase in the intensity. This is because protamine does not have significant hydrophobic residues to bind to ANS (Material section). In conclusion, Zn-insulin-protamine gives highly altered, compact state, in agreement with fluorescence results (Fig. 4)

Figure 7

Probing insulin-protamine interaction under exact formulation condition

Apart from insulin, Zn and protamine, phenol is also an important ingredient of insulin formulation. Phenol is known to bind specific sites on the insulin hexamer, and induce different hexamer structures such as T and R by providing specific interactions at the dimer-dimer interface that stabilizes the B1-B8 R helices[16]. The major difference between T and R is that residues B1-B8 in the T-state are transformed from an extended conformation to an R-helix in the R-state[16]. Our results in CD (Fig. 8) also observed an increase in the helical content in Zn-insulin in the presence of phenol, which strongly supports the formation of more stable R_6hexamer[20, 34]. Here, our objective was to test the effect of phenol on the Zn-insulin-protamine mixture. To this end, we analyzed the UV melting and circular dichroism of Zn-insulin-protamine in the presence and absence of phenol (Fig. 8). The melting experiment shows a slight increase in T_m (by 7^0C) for Zn-insulin-protamine in the presence of phenol, suggesting that phenol acts as a moderate stabilizer of Zn-insulin even in the presence of protamine. Further, Far UV CD data (Fig. 8C-D) suggests that there is negligible

change in helical content in the presence of phenol. Since Zn-insulin-phenol attends R_6 state in formulation conditions, it is reasonable to say that, here, protamine acts on R_6 state of Zn-insulin.

Figure 8

Tracing the protamine binding site on insulin

In support of the quantitative experimental parameters, our goal was to find the most prefered binding site responsible for interaction between protamine and insulin. Earlier studies of the insulin-protamine complex based on X-ray and AFM studies provided very limited molecular level information[17, 22]. In view of this, we attempted molecular docking studies. Fig 9A displays the amino acid residues at the monomer-monomer interface in insulin (PDB: 4INS). Figure 9B-D shows the docking result of insulin-protamine complex, in which chain-B of insulin is exclusively involved in protamine binding (Fig. 9B). As shown in Fig. 9B-C, protamine binding to insulin was strongly dominated by hydrogen bonding interactions and few hydrophobic contacts (Table 3). The relevant details are further elaborated in the discussion section. Also, the significance of these interactions in the context of the insulin-receptor complex (Fig. 9E and F) are also discussed.

Figure 9

Table 3

Discussion

We have performed the first comprehensive biophysical analyses of the interaction between insulin and protamine in the presence and absence of Zn, which are all important components of several insulin formulations. In a solution, only-insulin exists as a dimer in the pH range 5-8 and, whereas in the presence of Zn^{2+}, the dimers form the hexamer assembly in the pH region 5-8 [6, 35]. In this study, we followed the experimental conditions that were used earlier in numerous insulin studies[14, 15, 17, 35]. In order to analyze interactions under equilibrium conditions, samples containing insulin and protamine were prepared at equimolar ratios (as used in formulation conditions) and allowed to equilibrate after the addition of Zn. All the samples were soluble under experimental conditions as we did not observe any precipitation.

The melting results (Fig. 2) indicate that Zn-insulin (hexamer) is thermally more stable compared to only-insulin (dimer). Based on structural evidence in the past, it is well known how several interactions play a vital role in the stabilization of insulin dimer. Specifically, the C terminus of the B-chain is accountable for the stabilization of the insulin dimers (Fig 9A) that are the building blocks of hexamers. As illustrated in Figure 9A, Tyr residues (B16 and B26) from each monomer stabilize insulin dimer through H-bonding interactions[30]. Apart from this, other residues of the B-chain (Gly^{B8}, Ser^{B9}, Val^{B12}, Glu^{B13}) and loop part of the B-chain (Phe^{B24}, Phe^{B25}, Thr^{B27}, and Lys^{B28})[7] are also involved in dimer stabilization. Subsequently, three such dimers combine to form hexamer, stabilized by residues (Ala^{B14}, Leu^{B17}, Gly^{B20} and, Leu^{A13}) in close-packed hydrophobic interactions at the central core (Fig 1D).

Since Zn-insulin is stored in the pancreas, interactions stabilizing this complex must be disrupted before it can undergo dissociation into active monomers during its endogenous delivery[7, 36]. As shown by T_m results, adding protamine has resulted in the destabilization of Zn-insulin (Zn-insulin-protamine), indicating that protamine successfully drives Zn-insulin dissociation. Also, as was seen in the fluorescence results (Fig 4), protamine binds more strongly to insulin than Zn-insulin, suggesting that the reaction favors dissociation of Zn-insulin. Here, molecular docking results (Fig. 9B-C) helped to dissect molecular-level details as to how protamine might be interacting with insulin to facilitate dissociation. As shown in Figure 9C, Tyr^{B26} of insulin is directly involved in H-bonding with the side chain of Arg^{15} as well as hydrophobic interaction with Arg^{14} of protamine. Also, Phe^{B24} with Arg^{14}, and Glu^{B13} with Arg^{30} are involved through H-bond interactions (Table 3). This may directly hamper the H-bonding interaction between Phe^{B24}-Tyr^{B26} of one monomer and Tyr^{B26}-Phe^{B24} of the second monomer, responsible for the stability of insulin dimer[37]. The fluorescence quenching observed in Fig. 4 during protamine binding may also have its origin in the polar interaction between Tyr^{B26} of insulin and Arg^{15} of protamine. Considering the vital role of Tyr^{B26} in the self-assembly of insulin, its direct interaction with protamine should contribute to the dissociation of dimer

assembly[38]. Apart from this, protamine is involved in direct H-bonding interactions with the other amino acid residues involved at the dimer interface (Ser^{B9}, Val^{B12}, Glu^{B13}, Tyr^{B16}, Leu^{B17}; Fig. 9B). Thus, the ability of protamine to block these key residues might trigger the loss of a strong interaction network at the insulin monomer-monomer interface, liberating the B-chain strands. Overall, this may result in higher mobility, thereby shifting the equilibrium toward the monomeric state of the insulin. The breaking of non-covalent interactions during dissociation is well reflected in the endothermic heat and positive entropy observed in ITC (Fig. 6).

When protamine selectively engages with (Fig. 9C) the active residues of insulin at the dimer interface, it actually results in the conformation of insulin with its hydrophobic residues exposed to the solvent (Fig. 9E), which is strongly supported by ANS binding studies (Fig 7). As per the reports, based on structural and mutational analysis (Fig 9F), hydrophobic amino acids (Gly^{A1}, Ile^{A2}, Val^{A3}, Tyr^{A19}, Gly ^{B8} and Leu^{B11} Gly^{B23} and Phe^{B25}) on the surface of insulin are highly essential for its interaction with insulin receptor (IR)[3, 13], and the protamine-binding here clearly helps to promote this kind of state in insulin (Fig 9D).

Figure 10

Moreover, as shown by CD in Fig. 3 and 8, upon protamine binding, loss of helical content supports the enhanced conformational fluctuations in Zn-insulin. This is important in the context of the earlier report, where insulin mutant (Gly^{B24}), despite the complete loss of B-chain character, is able to bind to receptor and maintain its nearly full biological activity[39]. Therefore, here, the protamine-binding induced flexible conformation of insulin containing exposed hydrophobic residues may possess all the signatures of the "active form" of insulin, which can strongly bind to receptor and perform its biological function (Fig 10).

Moreover, lower K_D (Fig. 6) value for Zn-insulin-protamine compared to Zn-insulin observed in ITC may explain its weaker dissociation. We know that Zn ions have a tendency to drive the insulin to an associative state (hexamer), whereas protamine is involved in the dissociation of Zn-insulin (T_m and CD). Additionally, prtoamine, being cationic in nature, can also masks negative charges on the surface of the insulin molecule, preventing the dissociated insulin from re-associating. The stronger binding of protamine with free insulin observed in fluorescence (Fig 4A) also supports this. Thus due to the opposing effects of Zn and protamine in formulation, insulin may adapt a conformtion, which upon delivered into blood undergoes controlled dissociation (into biologically active monomers) [40, 41]. This may explains the long-lasting effect of insulin formulation in controlling blood glucose levels. Overall, the role of protamine is to impair insulin's self-association ability and thermodynamic stability while at the same time promote flexible conformation with better receptor binding and therapeutic capability. Such an inverse correlation between stability and activity has been suggested in the modified analogues of insulin[42]. Such co-optimization highlights the multidimensional role of protamine in insulin formulation.

Further, it is well known that, active monomer insulin exacerbates the risk of undergoing fibrillation, which may likely trigger neurological disease-condition[43]. Since the Zn-insulin-protamine complex eventually leads to an active species, it was tested using ThT (Thioflavin T) to determine whether it can undergo fibrillation. ThT is very widely used to probe the protein fibrillation in various neurological disorders[44]. ThT, when excited at 440 nm, has characteristic emission maxima at 485 nm, which significantly increases upon binding with protein aggregates[44]. However, based on THT fluorescence data (elaborated in Supporting Information; Fig. S1) we could establish that insulin (in Zn-insulin-protamine complex) does not undergo fibrillation when monitored over a long period of time. The result may further suggests the role of protamine in promoting effective monomer concentration of insulin that can eventually help in controlling the glucose levels.

In conclusion, the studies presented here represent a first attempt at determining the binding of insulin with protamine using biophysical and computational methods. The results are expected to provide general guidelines about the binding mode of a peptide with insulin, which may open avenues for the use of new peptide sequences in insulin formulation.

Supplementary Information

Figure S1. ThT fluorescence studies of Zn-insulin-protamine.

Acknowledgment

This research work was supported by grants from DST-SERB (ECR/2016/000942), IRHPA-SERB (IPA/2020/000007), and DST-DPRP (VI-D&P/569/2016-1/TDT), DST-Purse, and UPE II- JNU. We also acknowledge DST FIST funding for ITC facility. NT acknowledges research grant from Council of Scientific and Industrial Research, Government of India. We also thank AIRF, JNU for the instrument facility.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this research article.

Reference:

1. Archontogeorgis, K.; Papanas, N.; Nena, E.; Tzouvelekis, A.; Tsigalou, C.; Voulgaris, A.; Xanthoudaki, M.; Mouemin, T.; Froudarakis, M.; Steiropoulos, P., Insulin Sensitivity and Insulin Resistance in Non-Diabetic Middle-Aged Patients with Obstructive Sleep Apnoea Syndrome. *Open Cardiovasc Med J* **2017,** *11* , 159-168.

2. Lisi, G. P.; Png, C. Y.; Wilcox, D. E., Thermodynamic contributions to the stability of the insulin hexamer. *Biochemistry***2014,** *53* (22), 3576-84.

3. Menting, J. G.; Whittaker, J.; Margetts, M. B.; Whittaker, L. J.; Kong, G. K.; Smith, B. J.; Watson, C. J.; Záková, L.; Kletvíková, E.; Jiráček, J.; Chan, S. J.; Steiner, D. F.; Dodson, G. G.; Brzozowski, A. M.; Weiss, M. A.; Ward, C. W.; Lawrence, M. C., How insulin engages its primary binding site on the insulin receptor. *Nature***2013,** *493* (7431), 241-5.

4. Petersen, M. C.; Shulman, G. I., Mechanisms of Insulin Action and Insulin Resistance. *Physiol Rev* **2018,** *98* (4), 2133-2223.

5. Carpenter, M. C.; Wilcox, D. E., Thermodynamics of formation of the insulin hexamer: metal-stabilized proton-coupled assembly of quaternary structure. *Biochemistry* **2014,** *53* (8), 1296-301.

6. Dunn, M. F., Zinc-ligand interactions modulate assembly and stability of the insulin hexamer – a review. *Biometals* **2005,***18* (4), 295-303.

7. Brange, J.; Owens, D. R.; Kang, S.; Vølund, A., Monomeric insulins and their experimental and clinical implications. *Diabetes Care***1990,** *13* (9), 923-54.

8. M. Sklepari; A. Rodger; A. Reason; S. Jamshidi; I. Prokes; Blindauer, C. A., Biophysical characterization of a protein for structure comparison: methods for identifying insulin structural changes.*Analytical Methods* **2016,** (41).

9. Huus, K.; Havelund, S.; Olsen, H. B.; van de Weert, M.; Frokjaer, S., Thermal dissociation and unfolding of insulin. *Biochemistry***2005,** *44* (33), 11171-7.

10. Mukherjee, S.; Mondal, S.; Deshmukh, A. A.; Gopal, B.; Bagchi, B., What Gives an Insulin Hexamer Its Unique Shape and Stability? Role of Ten Confined Water Molecules. *J Phys Chem B* **2018,***122* (5), 1631-1637.

11. Brems, D. N.; Alter, L. A.; Beckage, M. J.; Chance, R. E.; DiMarchi, R. D.; Green, L. K.; Long, H. B.; Pekar, A. H.; Shields, J. E.; Frank, B. H., Altering the association properties of insulin by amino acid replacement. *Protein Eng* **1992,** *5* (6), 527-33.

12. Ahmad, A.; Millett, I. S.; Doniach, S.; Uversky, V. N.; Fink, A. L., Stimulation of insulin fibrillation by urea-induced intermediates.*J Biol Chem* **2004,** *279* (15), 14999-5013.

13. Mayer, J. P.; Zhang, F.; DiMarchi, R. D., Insulin structure and function. *Biopolymers* **2007,** *88* (5), 687-713.

14. Attri, A. K.; Fernández, C.; Minton, A. P., pH-dependent self-association of zinc-free insulin characterized by concentration-gradient static light scattering. *Biophys Chem***2010,** *148* (1-3), 28-33.

15. Correia, M.; Neves-Petersen, M. T.; Jeppesen, P. B.; Gregersen, S.; Petersen, S. B., UV-light exposure of insulin: pharmaceutical implications upon covalent insulin dityrosine dimerization and disulphide bond photolysis. *PLoS One* **2012,** *7*(12), e50733.

16. Berchtold, H.; Hilgenfeld, R., Binding of phenol to R6 insulin hexamers. *Peptide Science* **1999,** *51* (2), 165-172.

17. Norrman, M.; Hubálek, F.; Schluckebier, G., Structural characterization of insulin NPH formulations. *Eur J Pharm Sci***2007,** *30* (5), 414-23.

18. Krayenbuhl, C.; Rosenberg, T., Crystalline protamine insulin.*Rep Steno Mem Hosp Nord Insulinlab* **1946,** *1* , 60-73.

19. Gualandi-Signorini, A. M.; Giorgi, G., Insulin formulations–a review. *Eur Rev Med Pharmacol Sci* **2001,** *5* (3), 73-83.

20. Smith, G. D.; Ciszak, E.; Magrum, L. A.; Pangborn, W. A.; Blessing, R. H., R6 hexameric insulin complexed with m-cresol or resorcinol.*Acta Crystallogr D Biol Crystallogr* **2000,** *56* (Pt 12), 1541-8.

21. Mohanty, J.; Shinde, M. N.; Barooah, N.; Bhasikuttan, A. C., Reversible Insulin Hexamer Assembly Promoted by Ethyl Violet: pH-Controlled Uptake and Release. *The Journal of Physical Chemistry Letters* **2016,** *7* (19), 3978-3983.

22. Yip, C. M.; Brader, M. L.; Frank, B. H.; DeFelippis, M. R.; Ward, M. D., Structural studies of a crystalline insulin analog complex with protamine by atomic force microscopy. *Biophys J* **2000,***78* (1), 466-73.

23. Lehrer, S., Solute perturbation of protein fluorescence. Quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry* **1971,** *10* (17), 3254-3263.

24. Gupta, S.; Tiwari, N.; Munde, M., A Comprehensive Biophysical Analysis of the Effect of DNA Binding Drugs on Protamine-induced DNA Condensation. *Sci Rep* **2019,** *9* (1), 5891.

25. van Zundert, G. C. P.; Rodrigues, J. P. G. L.; Trellet, M.; Schmitz, C.; Kastritis, P. L.; Karaca, E.; Melquiond, A. S. J.; van Dijk, M.; de Vries, S. J.; Bonvin, A. M. J. J., The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. *J Mol Biol* **2016,** *428* (4), 720-725.

26. Roy, A.; Kucukural, A.; Zhang, Y., I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc***2010,** *5* (4), 725-38.

27. Cimmperman, P.; Baranauskiene, L.; Jachimoviciūte, S.; Jachno, J.; Torresan, J.; Michailoviene, V.; Matuliene, J.; Sereikaite, J.; Bumelis, V.; Matulis, D., A quantitative model of thermal stabilization and destabilization of proteins by ligands. *Biophys J* **2008,***95* (7), 3222-31.

28. Clack, B. A.; Gray, D. M., A CD determination of the alpha-helix contents of the coat proteins of four filamentous bacteriophages: fd, IKe, Pf1, and Pf3. *Biopolymers* **1989,** *28* (11), 1861-73.

29. Hussain, F.; Birch, D. J.; Pickup, J. C., Glucose sensing based on the intrinsic fluorescence of sol-gel immobilized yeast hexokinase.*Anal Biochem* **2005,** *339* (1), 137-43.

30. Antolíková, E.; Žáková, L.; Turkenburg, J. P.; Watson, C. J.; Hančlová, I.; Šanda, M.; Cooper, A.; Kraus, T.; Brzozowski, A. M.; Jiráček, J., Non-equivalent role of inter- and intramolecular hydrogen bonds in the insulin dimer interface. *J Biol Chem* **2011,***286* (42), 36968-77.

31. Ahmad, A.; Uversky, V. N.; Hong, D.; Fink, A. L., Early events in the fibrillation of monomeric insulin. *J Biol Chem* **2005,***280* (52), 42669-75.

32. Schonbrunn, E.; Eschenburg, S.; Luger, K.; Kabsch, W.; Amrhein, N., Structural basis for the interaction of the fluorescence probe 8-anilino-1-naphthalene sulfonate (ANS) with the antibiotic target MurA.*Proc Natl Acad Sci U S A* **2000,** *97* (12), 6345-9.

33. Qadeer, A.; Rabbani, G.; Zaidi, N.; Ahmad, E.; Khan, J. M.; Khan, R. H., 1-Anilino-8-naphthalene sulfonate (ANS) is not a desirable probe for determining the molten globule state of chymopapain. *PLoS One***2012,** *7* (11), e50633.

34. Smith, G. D.; Pangborn, W. A.; Blessing, R. H., The structure of T6 bovine insulin. *Acta Crystallogr D Biol Crystallogr***2005,** *61* (Pt 11), 1476-82.

35. Blundell, T.; Dodson, G.; Hodgkin, D.; Mercola, D., Insulin: The Structure in the Crystal and its Reflection in Chemistry and Biology by. In *Advances in Protein Chemistry* , Anfinsen, C. B.; Edsall, J. T.; Richards, F. M., Eds. Academic Press: 1972; Vol. 26, pp 279-402.

36. DeFelippis, M. R.; Chance, R. E.; Frank, B. H., Insulin self-association and the relationship to pharmacokinetics and pharmacodynamics. *Crit Rev Ther Drug Carrier Syst* **2001,***18* (2), 201-64.

37. Jørgensen, A. M.; Olsen, H. B.; Balschmidt, P.; Led, J. J., Solution structure of the superactive monomeric des-[Phe(B25)] human insulin mutant: elucidation of the structural basis for the monomerization of des-[Phe(B25)] insulin and the dimerization of native insulin.*J Mol Biol* **1996,** *257* (3), 684-99.

38. Pandyarajan, V.; Phillips, N. B.; Rege, N.; Lawrence, M. C.; Whittaker, J.; Weiss, M. A., Contribution of TyrB26 to the Function and Stability of Insulin: structure-activity relationships at a conserved hormone-receptor interface. *J Biol Chem* **2016,** *291*(25), 12978-90.

39. Mirmira, R. G.; Tager, H. S., Role of the phenylalanine B24 side chain in directing insulin interaction with its receptor. Importance of main chain conformation. *J Biol Chem* **1989,** *264*(11), 6349-54.

40. Lindholm, L. H.; Ibsen, H.; Dahlöf, B.; Devereux, R. B.; Beevers, G.; de Faire, U.; Fyhrquist, F.; Julius, S.; Kjeldsen, S. E.; Kristiansson, K.; Lederballe-Pedersen, O.; Nieminen, M. S.; Omvik, P.; Oparil, S.; Wedel, H.; Aurup, P.; Edelman, J.; Snapinn, S.; Group, L. S., Cardiovascular morbidity and mortality in patients with diabetes in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet***2002,** *359* (9311), 1004-10.

41. Heller, S.; Kozlovski, P.; Kurtzhals, P., Insulin's 85th anniversary–An enduring medical miracle. *Diabetes Res Clin Pract* **2007,** *78* (2), 149-58.

42. Hua, Q. X.; Nakagawa, S.; Hu, S. Q.; Jia, W.; Wang, S.; Weiss, M. A., Toward the active conformation of insulin: stereospecific modulation of a structural switch in the B chain. *J Biol Chem* **2006,***281* (34), 24900-9.

43. Brange, J.; Andersen, L.; Laursen, E. D.; Meyn, G.; Rasmussen, E., Toward understanding insulin fibrillation. *J Pharm Sci***1997,** *86* (5), 517-25.

44. Sun, C. S.; Lee, C. C.; Li, Y. N.; Yao-Chen Yang, S.; Lin, C. H.; Chang, Y. C.; Liu, P. F.; He, R. Y.; Wang, C. H.; Chen, W.; Chern, Y.; Jen-Tse Huang, J., Conformational switch of polyglutamine-expanded huntingtin into benign aggregates leads to neuroprotective effect.*Sci Rep* **2015,** *5* , 14992.

Table 1: Parameters obtained from the Stern-Volmer plot

System	**Ksv**	$\mathbf{f_a}$	**R**
Insulin-protamine	8.9 x 10^3	0.4	0.98786
(Zn-Insulin)-protamine	2.3 x 10^5	2.1	0.99680

Table 2. Thermodynamic parameters obtained using ITC experiment.

Parameters	Zn-Insulin	Zn -Insulin-protamine
KA (M^{-1})	3.03 X 10^3	4.47 X 10^3
KD (M)	33.00 X 10^{-5}	22.37 X 10^{-5}?¿?
H (kcal/mol)	3.5410 X 10^3	3.9300 X 10^3
ΔS (kcal/mol)	11.9	13.2
TΔS (kcal/mol)	3.5479 X 10^3	3.9355 X 10^3

Table 3. Docking results of insulin monomer with protamine, displaying the type of interactions and bond distances between residues of insulin and protamine.

Residues of Insulin	Residue of protamine	Residue of protamine	Type of bonding	Bond distance
Glu B21 (COO^-)	Arg 10 (NH_2)	Arg 10 (NH_2)	Hydrogen bond	1.92
Glu B13 (COO^-)	Arg 30 (NH_2)	Arg 30 (NH_2)	Electrostatic	4.23
Phe B24 (CO)	Arg 14 (NH_2)	Arg 14 (NH_2)	Hydrogen bond	2.13
Tyr B26 (OH)	Arg 15 (NH)	Arg 15 (NH)	Hydrogen bond	2.55
Leu B17 (CO)	Arg 23 (NH_2)	Arg 23 (NH_2)	Hydrogen bond	2.43
Ser B9 (CO)	Arg 30 (NH_2)	Arg 30 (NH_2)	Hydrogen bond	2.09
Val B12 (CH_3-CH-CH_3)	Pro 18 (ring)	Hydrophobic	Hydrophobic	4.97
Tyr B16 (Aromatic ring)	Val 12 (CH_3-CH-CH_3)	Hydrophobic	Hydrophobic	5.11
Tyr B26 (Aromatic ring)	Arg 14 (CH_2 -CH_2)	Arg 14 (CH_2 -CH_2)	Hydrophobic	4.54
Phe B24 (Aromatic ring)	Val 12 (CH_3-CH-CH_3)	Val 12 (CH_3-CH-CH_3)	Hydrophobic	4.33

Figure legends

Figure 1: (A) Amino acid sequence of insulin containing B-chain (upper) and A-chain (lower) with disulfide bridges as indicated, (B) insulin monomer (chain A in red and chain B in green; PDB 2JV1), (C) insulin dimer, showing tyrosine residues in yellow sticks (PDB 2OMI).

Figure 2: Thermal stability curve of (A) only-insulin, (B) Zn-insulin and (C) Zn-insulin-protamine in 10 mM phosphate buffer containing 100 mM NaCl at pH 8.0. The insulin:Zn ratio was 1:0.5.

Figure 3: Far-UV CD of (A) only-insulin and Zn-insulin, (B) insulin-protamine, Zn-insulin-protamine and protamine. Experiments were performed in 10 mM phosphate buffer containing 100 mM NaCl at pH 8.0 and 25@C (C) % helical content of insulin and Zn-insulin with and without protamine.

Figure 4: Fluorescence titration of increasing concentration of protamine into (A) insulin, and (B) zinc-insulin; (C)) plot of [Log (fo/f -1)] vs Log [Q] using modified Stern-Volmer equation to determine binding constants for binding of protamine with only-insulin and Zn-insulin. Intensity was recorded at 304 nm. All the experiments were performed in 10 mM phosphate buffer at pH 8.0 and 25@C. The insulin:Zn ratio was 1:0.5.

Figure 5: Fluorescence spectra for dilution of (A) only-insulin, (B) Zn-insulin, (C) Zn-insulin-protamine. Each sample was diluted from 20 µM to 1 µM (D) Plot of emission intensity recorded at 304 nm against decreasing concentration for only-insulin, Zn-insulin, and Zn-insulin-protamine. Similar slit width was applied in all the experiments. The data was fitted using linear equation y = C + mx where, m is slope, (E) comparison of spectra of only-insulin, Zn-insulin, and Zn-insulin-protamine at (before the start of dilution) 20

μM each. All the experiments were performed in 10mM phosphate buffer at pH 8.0 and room temperature. The insulin:Zn ratio was 1:0.5.

Figure 6: Thermodynamic analysis using ITC dilution experiment. A) Dissociation isotherms of (A) Zn-insulin (Inset: control experiment in which buffer is titrated into buffer) and (B) Zn-insulin-protamine (Inset: control experiment in which protamine is titrated into buffer); All the experiments were performed in 10 mM phosphate buffer at pH 8.0 at 25@C on a MicroCal iTC200 (Malvern Instruments Ltd. UK)[24]. Wherever used, concentration of insulin, $ZnSO_4$, and protamine was 200 μM, 100 μM and 200 μM, respectively.

Figure 7: Titration of increasing concentration of ANS to (A) only-insulin, (B) Zn-insulin, (C) insulin-protamine and (D) Zn-insulin-protamine. The dotted line indicates only ANS excited at 388 nm. All the experiments were performed in 10mM phosphate buffer containing 100mM NaCl at pH 8.0 at 25@C.

Figure 8: (A) U.V-Vis melting of Zn-insulin-protamine with and without phenol at pH 8.0, temperature ranges 25-95 ^{0}C. Far UV-CD of (B) Zn-insulin with and without phenol, (C) Zn-insulin-protamine with and without phenol. Experiments were performed in 10 mM phosphate buffer at pH 8.0 at 25@C (D) % helical content of Zn-insulin and Zn-insulin-protamine with and without phenol. The insulin:phenol ratio was 1:3.

Figure 9: (A) Insulin dimer interface showing residues (in pink) of chain-B **(** from each monomer) responsible for monomer-monomer interaction (PDB: 4INS) (B) Docking result of insulin (PDB: 2JV1) with protamine (yellow surface) (C) Highlighting the interactions at the binding interface of insulin and protamine (D) Hydrophobic interactions between residues of insulin and protamine, (E) insulin showing exposed hydrophobic residues (sticks) that are not interacting with protamine (yellow surface) and (F) complex between insulin and receptor (αCT; 704–719 amino acids), displaying the crucial residues of insulin involved in receptor binding; grey shading shows αCT surface[3] (PDB: 4OGA). It can be seen that the hydrophobic residues, which are not involved in the binding in Fig. E are involved in receptor binding in F.

Figure 10: Model depicting how the protamine-binding to Zn-insulin resulted in insulin conformation suitable for receptor binding.

Figures:

Figure 1:

Hosted file

`image1.emf` available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Figure 2:

Hosted file

`image2.emf` available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Hosted file

`image3.emf` available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Figure 3:

Figure 4:

Hosted file

image4.emf available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Figure 5:

Hosted file

image5.emf available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Figure 6:

Hosted file

image6.emf available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Figure 7:

Hosted file

image7.emf available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Figure 8:

Hosted file

image8.emf available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Figure 9:

Hosted file

image9.emf available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies

Figure 10:

Hosted file

image10.emf available at https://authorea.com/users/354526/articles/478063-probing-the-interaction-of-protamine-with-zn-insulin-through-biophysical-and-molecular-docking-studies