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Original Research Article

Explicit Solvent Molecular Dynamics Simulation Studies of the Dissociation of Human Insulin Hexamer into the Dimeric Units

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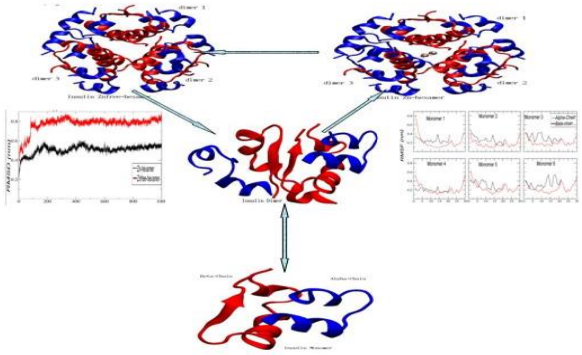
ABSTRACT

The molecular dynamics (MD) simulation of two structures of human insulin hexamers (Zn-hexamer and Znfree-hexamer) in explicit solvent was performed and the role of the Zn^{2+} ions in the hexamer's cavity as it may affect the propensities of the dissociation of insulin hexamer into dimers and monomers investigated. The starting structure of the Zn-hexamer contains two cavity water molecules and two Zn^{2+} ions in addition to the amino acids residues of the insulin hexamer while the starting structure Znfree-hexamer is made up of only the amino acids residues of the insulin hexamer. The MD simulation was performed for 1 μs under the isothermal-isobaric conditions (NPT) conditions using the GROMACS software, amber ff99-SB force field, TIP 3P water model with periodic boundary conditions imposed on x-, y- and z- directions. Structural analyses of the two experimental structures were carried out and root mean square deviations (RMSD) and root mean square fluctuations (RMSF) computed from the referenced initial structures. The average RMSD value of the backbone atoms for the Zn-hexamer is 0.293 nm and Znfree-hexamer is 0.785 nm. The structural analysis revealed that, there were dimer's dissociation and pronounced fluctuations of the amino acids residues in the Znfree-hexamer compared to the Zn-hexamer during the MD simulation. This is evident in the higher inter-dimer distances of the six glutamate-13(β) residues in the Znfree-hexamer which confirmed the influence of the removal of the Zn^{2+} ions and cavity water molecules on dissociation of the insulin hexamer.

GRAPHICAL ABSTRACT



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Introduction

Molecular dynamics (MD) is one of the principal tools in the theoretical study of the biological molecules. It is possible to investigate the internal motion of the biomolecules as it affects their biological functions and obtain information that are not accessible from experiments with molecular dynamics simulations [1]. Insulin is an important biomolecule known to be a key hormone regulating glucose content in the human body serving as an important drug for treatment of diabetes, that many people all over the world [2]. The insulin molecule consists of two peptide chains, the α-chain (A) and the *β*-chain (B), which are linked by two di-sulphide bonds. The *α*-chain also contains an intramolecular di-sulphide bond. The *α* -chain consists of 21 amino acids containing two α-helices in A1-A8 and A12-A20 positions connected by a loop, whereas the *β*-chain consist of 30 amino acids containing a central α-helix in position B9-B19 flanked by two turns and flexible regions in both termini [3]. Insulin forms higher oligomeric states, dimers at micromolar concentration and hexamers at millimolar concentration with addition of zinc ions. Binding of small aromatic alcohols, such as phenol and *m*-cresol in the hexameric form, can facilitate a conformational change of the *N*-terminal end of the *β*-chain extending the central helix to include the positions B1-B8 [4]. The oligomeric states of insulin stabilize the molecule; however, it is the monomeric state of insulin which facilitates its binding to the insulin receptor. The three native di-sulphide bonds have been conserved in the insulin structure for a long time and are of major importance for the stability of the molecule [5].

At the *β*-cell level, the propensity of insulin to self-associate into hexamers is crucial for the hormones' processing and storage. The insulin molecules formed dimers at high concentrations and neutral pH and the aggregation of the dimers in the presence of the Zn^{2+} ions that leads to an insulin hexamer. In a Zn^{2+} ions rich environment, His-10 residues from β-chains of the six monomers get coordinated to two Zn^{2+} ions (3 His-10(*β*) coordinate each Zn^{2+} ion) leading to the formation of a hexameric assembly. The additions of zinc ions and phenolic compounds into the human insulin not only prevent the undesirable chemical degradation and promote the hexamer conformation, but also prevent physical denaturation [7]. The formation and crystallization of the insulin hexamers from the monomeric units stabilizes insulin molecule and prevents its degradation in the storage vesicle [8]. Dissociation rate of the hexamer to release the insulin monomer has been an important research area [9-11]. This property of the insulin molecule has been exploited in pharmaceutical formulation to produce stable solution preparations and microcrystallines used for diabetes treatment [7].

Albumin or any Zn^{2+} chelator has been reported to improve Zn^{2+}-insulin disintegration into subunits [11]. Therefore, the *in-vitro* insulin determination in insulin-release experiments is clearly sensitive to the presence of serum albumin in the solution since insulin seems to be released as a hexamer. The vital role played by the insulin hexamer as the storage bank of insulin in human body, has necessitated the need to clearly understand the dynamic equilibrium between the insulin hexamer and the dimer that partly controls the release of the monomer in response to the human body's glucose level.

Conformational dynamics studies on the insulin molecule have been severally approached using experimental techniques [10-14]. However, computational approaches to the study of insulin dynamics have improved on the experimental studies providing better understanding of the conformational activities of the biomolecule [15-18].

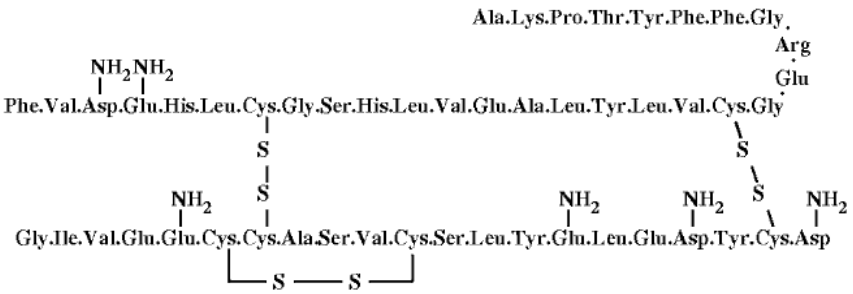

Figure 1. Amino acid sequence of human insulin monomer [6]

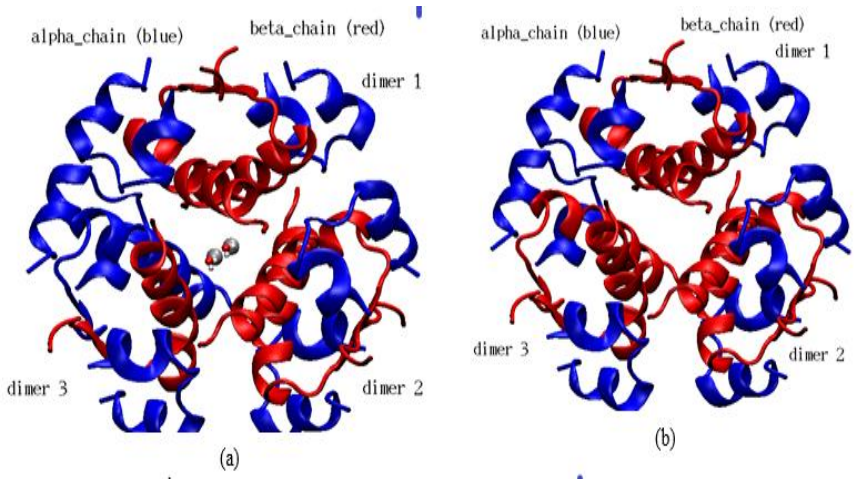

Figure 2. Starting structures insulin Zn-hexamer and Znfree-hexamer for the MD simulations drawn using the VMD package [29]

This present study employs plain molecular dynamics simulation at a long time scale (1 μs) to further gain insight better into the influence of the zinc ions and the cavity water molecules on the dissociation tendencies of the insulin hexamer to the protein's biologically active monomeric unit. The study also aimed at identifying the residues actively enhancing the solvent accessibility and the structural transitions.

Experimental

The X-ray structure of the insulin hexamer used for the study was downloaded from the repository of the Protein Data Bank (PDB) (http://www.wwpdb.org) with PDB code 1 ai0 [19]. The X-ray structure was edited appropriately to prepare two insulin hexameric structures; Zn-hexamer and Znfree-hexamer. The Zn-hexamer consists of the amino acids residues of the protein and two Zn^{2+} ions and cavity water molecules while the Znfree-hexamer is the protein molecule without the two Zn^{2+} ions and cavity water molecules. After the initial system preparations, the starting structures were solvated completely in an explicit cubic box of water molecules to mimic the natural physiological environment of the protein. All the simulations were carried out using the GROningen MAchine for Chemical Simulations (GROMACS 2016) software [20-22]. The Amber force field 99-SB [23] and the TIP3P water model [24] were used. The LINCS [25] algorithm was used to constrain all the bonds; the full electrostatic interactions were computed at every step using the particle-mesh Ewald summation algorithm [26,27]. Short range repulsive and attractive interactions were computed by Lennard-jones potential with a cut off of 10.0 Å. The Van der waals interactions were treated with twin range cut-offs, an atom-based non-bonded cut-off of 10.0 Å and neighbour list cut-off of 10.0 Å. Periodic

boundary conditions were imposed on the x, y- and z- directions. The MD simulation studies were performed for 1 μs under isothermal-isobaric (NPT) conditions using the previously reported MD protocol for multimeric proteins [28].

Results and discussion

Root mean square fluctuation (RMSF)

The variability in the localized structural changes in the two insulin hexamers was investigated by plotting the root mean square fluctuation against number of residues from the MD Trajectories. The relative RMS fluctuations of the backbone atoms of the two hexamers and the two chains (α and β) in each monomer were compared (Figure 3).

Figure 3a reveals that the fluctuation of the backbone atoms in Znfree-hexamer is more pronounced than in Zn-hexamer as evident in the larger RMSF values during the entire simulation. This implies higher dynamic activities of the residues in the Znfree-hexamer and hence, the structure from MD simulation is remarkably different from starting structure as the hexamer has dissociated into the dimers. The smaller RMSF values in the Zn-hexamer during the simulation corroborates the fact that the presence of Zn^{2+} ions in the insulin hexamer conferred extra structural stabilities and limits the amplitudes of the fluctuations of its residues.

The comparison of the RMSF values for the α- and β-chains residues in both hexamers during the simulations are as shown in Figures 3b and 3c. It is evident that the α-chains have larger RMSF values (which is essentially from the non-polar residues) than the β-chains. This indicates that the α-chain residues have high influence on the protein dynamics in both insulin hexamers. However, the terminal residues of the β-chains displayed pronounced RMS fluctuations indicate that the terminal residues in the β-chain of each monomeric unit of the hexamers are free and have high solvent accessibility.

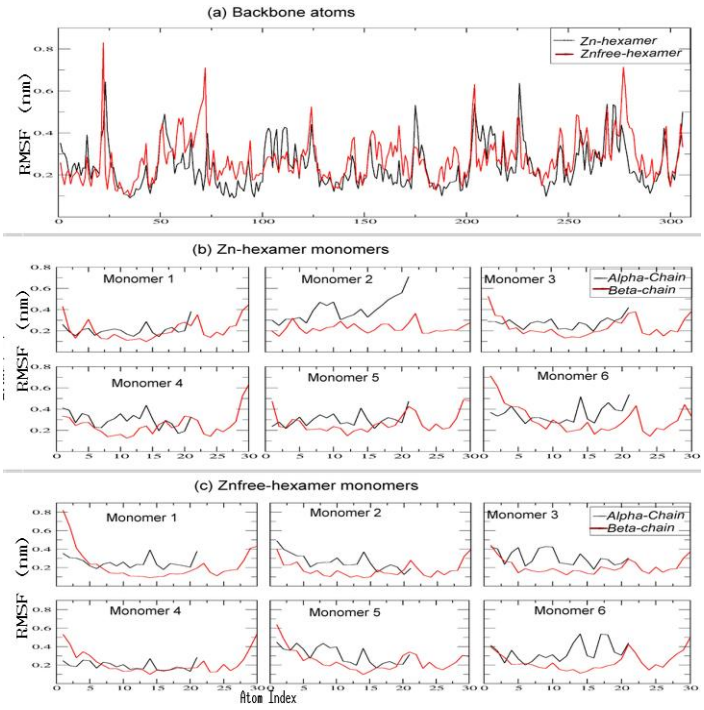

Figure 3. RMSF comparative plots of (a) Residues of both insulin hexamers, (b) α- and β-chains of each monomer in the Zn-hexamer, (c) α- and β-chains of each monomer in the Znfree-hexamer

Root mean square deviation (RMSD)

The RMSD of the MD simulation structures from the starting structures were computed for all the frames in the trajectories for the backbone atoms, dimers, monomers and also the glutamate 13(β) residues. These RMSD plots were compared for the two insulin hexamers to investigate the effect of the removal of the Zn^{2+} ions on the dynamics of the proteins (Figure 4).

The effect of the removal of the Zn^{2+} ions and the cavity water molecules on the insulin hexamer was evident during the MD simulation as the overall RMSD values of the backbone atoms in the Znfree-hexamer was higher (with average RMSD value of 0.785 nm) than that of the Zn-hexamer with the average RMSD value of 0.293 nm (Figure 4a). This high RMSD value from the Znfree-hexamer simulation implied that, the structure obtained from the MD simulation is that in which the hexamer has dissociated into the dimers as a result of the removal of the Zn^{2+} ions and the cavity water molecules. However, the low RMSD value from the Zn-hexamer simulation implies the hexamer structure still remain intact without undergoing any dissociation throughout the MD simulation. Our result agrees with the studies of Pertusa *et al.,* (2017) [18] which revealed that insulin secretion rate increases due to higher zinc ion chelation and that this is accompanied by dissociation of the hexamer into monomers and subsequent dissolution in blood.

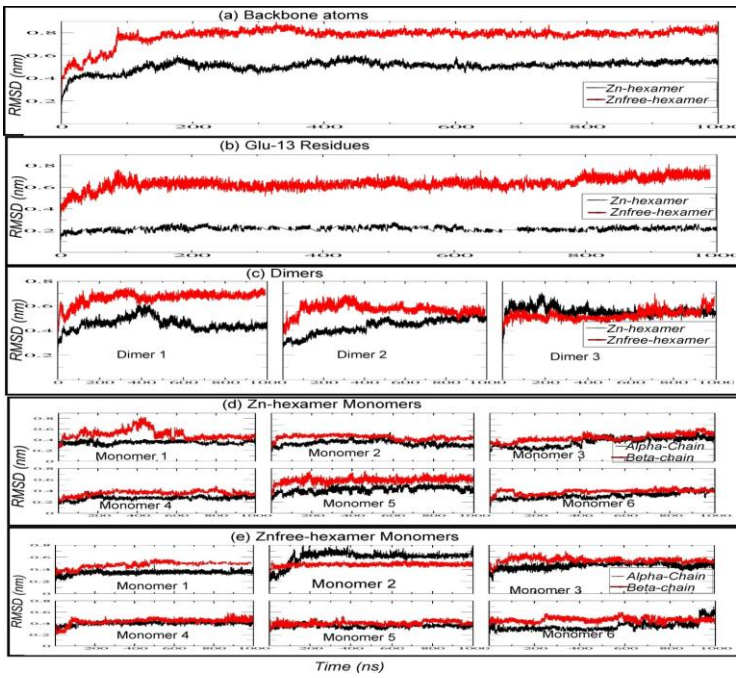

Figure 4. RMSD comparative plots of (a) Backbone atoms of both insulin hexamers, (b) Glu-13 residues of both insulin hexamers, (c) Backbone atoms of the 3 dimers in both insulin hexamers, (d) Backbone atoms of α- and β-chains of each monomer in the Zn-hexamer, (e) Backbone atoms of α- and β-chains of each monomer in the Znfree-hexamer

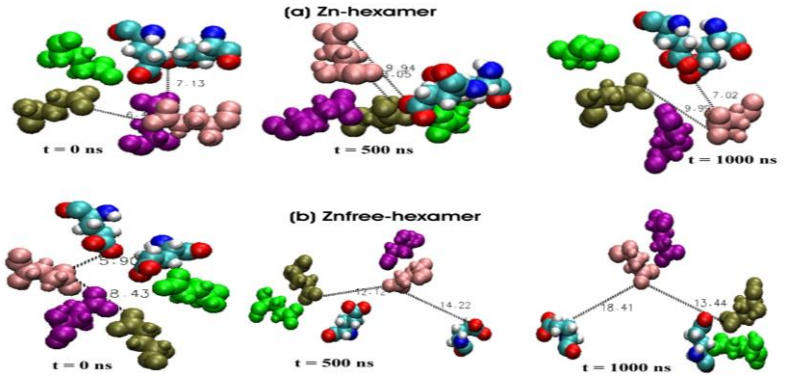

Figure 5. Inter-dimer distances of the six glutamate-13(β) residues of (a) Zn-hexamer and (b) Znfree-hexamer during simulation, drawn using the VMD package [29]

Investigation of the increased dissociation and robustness of the insulin hexameric unit focusing on the effects of the Zn^{2+} removal on the dimers and Glu-13(β) residues confined in the cavity formed at its center is better understood. Blundell *et al.,* (1972) [30] reported that, the repulsion among the negative charges on the Glu-13 side chains causes destabilization of the hexamer leading to dissociation in an environment where Zn^{2+} ions are absent. This suggests that the repulsion of the six Glu-13(β) residues present at the cavity partly facilitates the hexamer dissociation process. This was also confirmed from our MD simulations with the higher RMSD values of the Glu-13(β) residues (with average RMSD value of 0.633 nm) in Znfree-hexamer compared to Zn-hexamer (with average RMSD value of 0.227 nm) (Figure 4b) and the remarkable change in the inter-dimer distances of the Glu-13(β) residues of the Znfree-hexamer during the MD simulation as compared to that of the Zn-hexamer (Figure 5).

Figure 4c revealed that, the dimers of the Zn-hexamer have smaller RMSD values and are therefore within a close interacting distance throughout the simulation indicating that the presence of the Zn^{2+} ions stabilizes the insulin hexamer structure. However, for the Znfree-hexamer, there is a gradual dissociation tendency of the dimers within few nano-seconds of the simulation due to the collapse of the hexamer cavity leading to separations of the dimers as evident in the higher RMSD value when compare to that of the Zn-hexamer. Consequently the separations of the dimers into the monomers further into the simulation time. Figure 4d reveals that the monomers 1 and 2 of dimer 1 in the Znfree-hexamer have distinctly higher RMSD values than other monomers. These higher RMSD values of the monomeric units of the dimer 1 structure represent the tendency of the dimer to further dissociate into its monomers.

The measured distances between the Glu-13(β) residues of the dimers in both the Zn-hexamer and the Znfree-hexamer before, during and after the MD simulations (Figure 5) revealed the stability of Zn-hexamer as evident in the insignificant changes in the inter-dimer distance values of the Glu-13(β) residues throughout the MD simulation. The Glu-13(β) residues are held in place by the combined effects of coordination sphere of the Zn^{2+} ions and the central water cavity of the insulin hexamer which shielded them from the expected repulsion and subsequent destabilization of the hexamer. In contrast, the absence of the Zn^{2+} ions and the subsequent collapse of the central water cavity in the Znfree-hexamer results in repulsion between the negatively charged side-chains of the Glu-13(β) residues and remarkable difference in the inter-dimer distances of the Glu-13(β) residues which is an indication of the dissociation of the insulin hexamer into dimers.

This MD simulations result confirms the previously reported findings [11,31] that the insulin hexamer dissociation is enhanced by removing the Zn^{2+} by ligands that are present in the hormone storage pancreatic-cells. The firmness and the close distance interactions of Glu-13(β) residues in the Zn-hexamer throughout the MD simulation implies that the structure is stabilized by the presence of the Zn^{2+} ions and the cavity water molecules. These findings corroborate the potential physiological aspect of the stabilizing effect of the two Zn^{2+} ions and cavity water molecules present in the insulin showing the Zn-hexamer conformer as the storage species of insulin and the removal/chelation of the Zn^{2+} ions to form the zinc free species (Znfree-hexamer) as the better route to the dissociable hexamer → dimer → monomer equilibrium.

Thus, under physiological conditions, the insulin aggregates more in a zinc rich system, the removal or inhibition of these ions enhance the dissociation of the hexamer

thereby releasing the monomeric units known to be the bioactive form of insulin in human. The relevance of this is that the zinc ions form an electrostatic interaction by coordinating six histidines residues (His-10(β)) to form the insulin hexamer. Similarly, the conserved water molecules play crucial role in screening the electrostatic field due to the carboxylate groups of six Glu-13(β) residues in the vicinity of hexameric cavity which indeed effect reduction in repulsion among the negative charges of Glu-13(β) side chains. The combination of these two factors stabilizes the hexameric assembly for a long period of dynamics. During the MD simulation of the Znfree-hexamer, the cavity collapses within a few ns as the Glu-13(β) perturbs the empty coordination environment of the Zn^{2+} ions in the hexamer. Dehydration and masking of this histidine residues ligand with stronger ligand is a recognized factor that will govern insulin hexamer release. These observations support the notion that the Zn^{2+}-binding properties of albumin improve the dissociation of Zn^{2+}-insulin hexamer into subunits, which might be useful in insulin determination, or insulin pharmacokinetic assays.

Hydrogen bonds (H-bonds)

The numbers of intramolecular hydrogen bonds present in the insulin hexamers during the MD simulations is shown in Figure 6. The core of most protein structures is composed of secondary structures such as α-helix and β-sheet which are strengthened by *H*-bonds. Hydrogen bond is formed by interaction of a hydrogen atom that is covalently bonded to an electronegative atom (donor) with another electronegative atom (receptor). These intramolecular *H*-bonds contribute to the structural stability and compactness of the proteins. Three types of intramolecular *H*-bonds have been identified to contribute to protein stability [32], these are; the *H*-bonds between (i) two amino acids residues of the protein, (ii) a protein residue and a water molecule and (iii) two water molecules surrounding the protein. The number of the intramolecular H-bonds formed in Zn-hexamer and Znfree-hexamer are not significantly different (Figure 6) indicating that the secondary structures (α-helix and β-sheet) of two hexamers are well conserved during the MD simulation. However, it was observed that hydrogen bonds formed in the Zn-hexamer are of shorter length when compared with those of the Znfree-hexamer (Table 1). The implication of this is that the H-bonds of the Zn-hexmer are more conserved/stable than that of Znfree-hexamer. Therefore, the insulin Zn-hexamer is more structurally stabilized by intramolecular H-bonds during the MD simulation than the Znfree-hexamer. For instance the most abundant *H*-bonds formed between two amino acids residues in each of the hexamer are presented in Table 1.

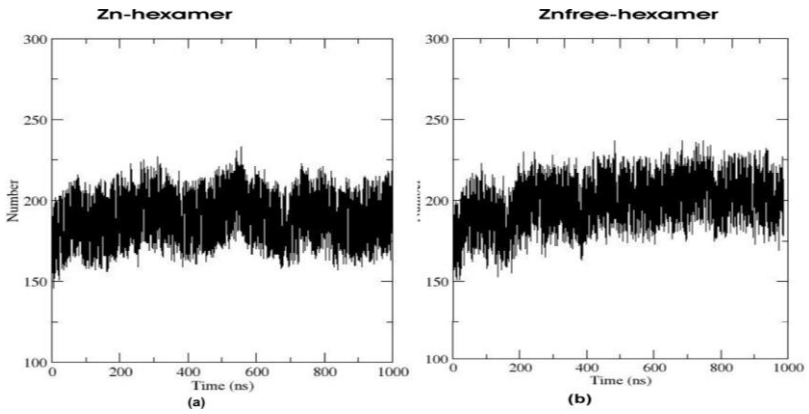

Figure 6. Intramolecular H-bond plots of insulin (a) Zn-hexamer and (b) Znfree-hexamer

Table 1. The major Hbonds formed in both Zn-hexamer and Znfree-hexamer

No	Major *H*-bonds	Zn-hexamer		Znfree-hexamer	
		%occupancy	Bond length (Å)	%occupancy	Bond length(Å)
1	Tyr-26(β) (Main) - Phe-24(β) (main)	245.45%	2.75	154.55%	3.52
2	Glu-17(α) (Main) - Leu-13(α) (Main)	118.18%	2.61	150.00%	3.37
3	Glu-13(β) (Main) - His-10(β) (Main)	154.55%	2.44	10.00%	3.07
4	Ser-9(α) (Main) - Gln-5(α) (Main)	100.00%	2.84	90.00%	3.18
5	Thr-8(α) (side) - Glu-4(α) (Main)	100.00%	2.57	60.00%	3.12

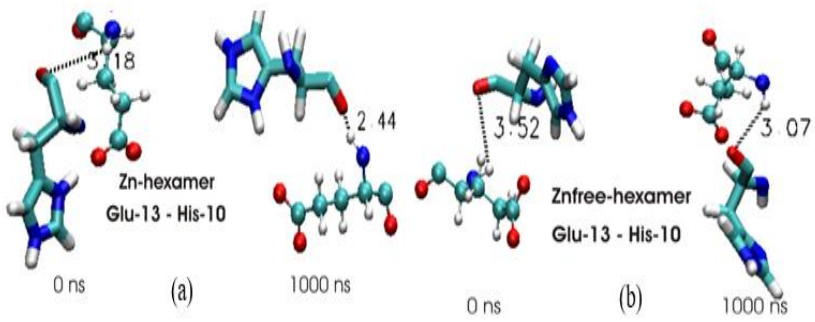

Figure 7. Glu-13(β) - His-10(β) H-bond conserved in both MD structures of (a) Zn-hexamer and (b) Znfree-hexamer, drawn using the VMD package [29]

Table 2. Zn-hexamer and Znfree-hexamer salt-bridges

No	Salt-bridges	Zn-hexamer Bond length (Å)	Znfree-hexamer Bond length (Å)
1	Glu-4(chain A) - Lys-29(chain B)	4.0	15.5
2	Glu-4(chain C) - Lys-29(chain D)	4.0	15.5
3	Glu-4(chain G) - Lys-29(chain H)	4.2	10.5
4	Glu-17(chain A) - Arg-22(chain B)	3.3	11.42
5	Glu-17(chain C) - Arg-22(chain D)	3.3	11.42
6	Glu-17(chain E) - Arg-22(chain F)	4.0	9.8
7	Glu-17(chain G) - Arg-22(chain H)	4.2	4.24
8	Glu-17(chain I) - Arg-22(chain J)	4.2	10.5
9	Glu-17(chain K) - Arg-22(chain L)	4.2	13.5
10	Glu-17(chain K) - Arg-22(chain F)	4.0	15.0
11	Glu-17(chain H) - His-5(chain F)	3.5	10.8

Salt-brigdes

The number of salt-bridges present in the insulin hexamers before and after the MD simulation was analyzed to underscore effect of the dynamics on interactions between the charged species of the different residues within close proximity in the protein. As seen in Table 2, 11 salt-bridges were present between the chains of the six insulin monomers in Zn-hexamer and the bond lengths of these salt-bridges in Zn-hexamer are closer to the maximum value of 4 Å, however, the bond lengths of these salt-bridges are very high (10 -15 Å) for the Znfree-hexamer. Therefore, the 11 salt bridges formed in Zn-hexamer are broken in Znfree-hexamer. This is due to the fact that, the bond lengths between the two residues that formed each salt-bridge in Znfree-hexamer are significantly greater than 4 Å maximum which means that the hexameric structure in the absence of the Zn^{2+} ions tends to dissociates to the dimeric units during dynamics. This also supports the fact the insulin hexamer is only structurally stable in the presence Zn^{2+} ions.

Conclusion

Insulin hexamer serves as the natural storage state for the physiologically relevant insulin monomers. We employed the MD simulation technique to investigate the role of Zn^{2+} ions regulating in modulating the propensity for dissociation of the insulin hexamer → dimer → monomer states. The results confirmed that the absence/ presence of the Zn^{2+} ions triggers the dissociation or otherwise of the insulin hexamer into the more physiologically useful monomers.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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