J Mol Model (2014) 20:2496
DOI 10.1007/s00894-014-2496-x

ORIGINAL PAPER

# A molecular simulation study of the protection of insulin bioactive structure by trehalose

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Received: 16 July 2013 / Accepted: 9 October 2014 / Published online: 28 October 2014


**Abstract** Biopharmaceuticals are proteins with a crucial role in the treatment of many diseases. However, these protein medicines are often thermally labile and therefore unsuitable for long-term application and storage, as they tend to lose their activity under ambient conditions. Desiccation is one approach to improving protein stability, but the drying process itself can cause irreversible damage. In the current study, insulin was chosen as an example of a thermally sensitive biopharmaceutical to investigate whether the disaccharide, trehalose, can prevent loss of structural integrity due to drying. The experiment was performed using replica exchange molecular simulation and Gromacs software with a Gromos96 (53a6) force field. The results indicate that trehalose preserves the bioactive structure of insulin during drying, consistent with the use of trehalose as a protectant for thermally sensitive biopharmaceuticals. For instance, at the water content of 1.77 %, insulin without any protectants yields the highest RMSD value as 0.451 nm, then the RMSD of insulin in presence of trehalose only ca. 0.100 nm.

**Keywords** Drying · Insulin · Molecular simulation · Preservation of bioactivity · Thermally sensitive protein medicines · Trehalose

**Electronic supplementary material** The online version of this article (doi:10.1007/s00894-014-2496-x) contains supplementary material, which is available to authorized users.

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## Introduction

Increasingly, biotechnology is used to produce novel and effective vaccines and therapeutic drugs to treat a broad spectrum of diseases including diabetes, tuberculosis, malaria, and HIV/AIDS [1, 2]. Most such biopharmaceuticals, e.g., insulin, are thermally sensitive and are prone to lose their bioactivity at ambient or elevated temperature [3]. An effective method of preserving such medicines is low-temperature drying [4], but the drying process itself can lead to denaturation of biopharmaceuticals [5] and therefore a protectant is usually required to maintain protein stability [6].

Insulin is a peptide hormone, produced by pancreatic β-cells, which regulates carbohydrate and fat metabolism in the body. The failure of insulin to control blood glucose levels can result in diabetes mellitus [7]. The active form of insulin is the monomer, although it can also occur as a dimer or hexamer, and its diffusion rate is inversely related to its particle size. As insulin is turned over rapidly within the body, diabetes patients require repeated injections throughout the day. Therefore, to avoid the need for frequent injections, both inhaled insulin [8] and oral insulin [9] have been developed. Delivery by these routes requires insulin to be formulated as a dry powder, and this can be achieved by freeze drying, spray drying, and foam drying [10], as long as a suitable protectant is used to preserve bioactivity.

Disaccharides such as sucrose, trehalose, and lactose are widely used as protectants for various medicines and biological tissues/organisms during drying. Leslie et al. [11] used both sucrose and trehalose to assess the tolerance of cells dried with or without the protectants. These authors drew the same conclusion as Elbein et al. [12], namely that trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside), the only naturally occurring, non-reducing disaccharide of glucose is a multifunctional protectant. Trehalose can directly interact with protein surfaces via H-bonds, and can also enter internal or

confined regions of the protein [13]. Furthermore, trehalose can effectively decrease the mobility of adjacent water molecules, again through H-bond formation [14], resulting in high hydration levels. A power-law relationship between the relaxation time of H-bond network and the structural relaxation time of the protein was found [15], and protein-trehalose [16] and protein-glycerol [17] H-bonds can slow down the protein dynamics. Both the above results confirmed the dynamic and protective behavior of the hydration layer. Therefore, trehalose was chosen as a candidate protectant in the current research.

To gain insight into drying-induced structural changes in insulin, and the efficacy and mechanism of protection by trehalose, a molecular simulation (MD) study was performed to uncover a molecular level of detail for these processes [18].

## Theories and methods

The native structure of human insulin was downloaded from Protein Data Bank (PDB: 3inc [19], Fig. 1). Insulin is a globulin consisting of two chains with a total of 51 amino acids. Chain α has 21 residues forming two helix fragments at its N- and C-terminus, respectively. Chain β has 30 residues forming a larger, central helix fragment. These chains are linked to each other by two disulfide bonds. Like other globular proteins, the exterior of the insulin molecule is mainly hydrophilic, whereas the interior is largely hydrophobic. Folding into an overall compact structure is driven by the hydrophobic effect. When the water in which insulin is dissolved is removed during drying, the hydrophobic effect is no longer sufficient to maintain the protein's native structure. Consequently, insulin monomers will undergo a series of physicochemical reactions during drying [20, 21], including deformation [22], denaturation [23], aggregation [24] (insoluble fibrillation and precipitation) and hydrolysis, each of which can result in loss of bioactivity.

Various research models containing different numbers of trehalose and water molecules for simulation were built to investigate the protection of insulin bioactivity by trehalose. In detail, two sodium ions were used to balance the electrostatic charge of the initial model containing an insulin molecule. Zero, 30, 60, 120, and 180 trehalose molecules were added to the above model as the control system and four experimental systems, respectively.

The residual water content of freeze-dried biological agent usually is controlled at the range of 1 to 3 wt % [25], and many commercialized freeze-dried biological agents are controlled to ca. 2 wt%. Therefore, we chose three water contents in typical range to investigate the interaction between insulin and trehalose. According to crystal structure analysis of insulin (PDB: 3inc [19]), there are five interstitial water molecules in each insulin monomer. Three interstitial water molecules can form water-bridge by H-bonding. The other two can only form single H-bond, which are linked to residuals VAL2 and PHE25, respectively, at chain B of insulin. In order to safely keep the interstitial water molecules in each initial model, we manually removed water molecules step by step from the outer side to inner when we built models from high to low water content. Detailed components of each model are listed in Table 1.

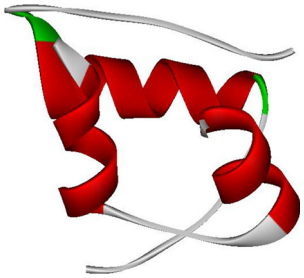

**Fig. 1** Native crystal structure of insulin (3inc) shown in the solid ribbon style with helices in *red*, turns in *green*, and coils in *gray*

The Gromacs 4.5 software package [26], the Gromos96 (53a6) forcefield [27], and the tip3p water model [28] were used for all simulations in this paper. Molecular simulations were performed in the isothermal-isometric (NVT) ensemble with a time step of 2 fs. Periodic boundary conditions with a box size of 10.00×10.00×10.00 $nm^3$ were used in all molecular dynamics simulations. According to the Gromacs manual (v4.5) [29], the short-range and long-range cutoff distances for Lennard-Jones potentials were chosen to be 1.0 and 1.4 nm, respectively, while the short-range cutoff distance for electrostatic interaction was set at 1.0 nm [29]. For long-range correction of the electrostatic interaction, the particle mesh Ewald (PME) method [30] was used in the simulation with the Fourier spacing being 0.12 and the PME interpolation order being 4. Initial velocities were taken from a Maxwell distribution at each temperature [31]. The velocity rescale method was applied for temperature coupling [32].

All bond lengths were constrained using the LINCS method [33] after 1000-step minimization, after which a MD simulation for 4 ns pre-equilibration at 300 K was performed. Subsequently, MD simulations for 10 ns equilibration were performed for temperatures of 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, and 310 K, respectively. Finally, a replica exchange molecular dynamics (REMD) simulation for 50 ns was performed. RMSD alteration of insulin in the driest and largest system indicates that the structure of insulin is completely equilibrated at almost 30 ns (Fig. S1). Conformations were exchanged every 1000 steps (per 2 ps). At the same time with the exchange, the corresponding conformations were saved as trajectories in order to sample as many significant conformations as possible. Trajectories of the last 5 ns were analyzed to obtain the results presented.

**Table 1** Research models for simulation and their detailed components

| Water content, wt% | Number of water molecules | | | | |
|---|---|---|---|---|---|
| | An insulin, 2 $Na^+$ (The control) | An insulin, 30 trehalose, 2 $Na^+$ | An insulin, 60 trehalose, 2 $Na^+$ | An insulin, 120 trehalose, 2 $Na^+$ | An insulin, 180 trehalose, 2 $Na^+$ |
| 0.89 % | 3 | 8 | 13 | 23 | 34 |
| 1.77 % | 6 | 16 | 26 | 47 | 67 |
| 4.30 % | 15 | 40 | 66 | 117 | 168 |

## Results and discussion

### Effect of drying on the 3D structure of insulin

The 3D structures of insulin with different numbers of trehalose molecules at various water contents are shown in Fig. 2. The global structure of the insulin monomer in the absence of trehalose deforms significantly after drying at the temperature range (280–310 K) mentioned in the methods part. The effect on secondary structure is particularly notable, with a clear decrease in α-helical content. In contrast, insulin structures are completely preserved in the presence of trehalose. The tertiary structure of dried insulin with different numbers of trehalose molecules is remarkably similar to the native structure. These results indicate that, as trehalose maintains insulin structure in the dry state, it should also prevent the protein from losing its bioactivity.

### The root mean square deviation of insulin after drying

The root mean square deviation (RMSD) value of insulin, which is used to investigate the tertiary structural deviation of dried insulin from its native crystal structure from Protein Data Bank (PDB ID: 3inc [19]), is useful for assessing structural stability. At the same water content, insulin without any protectants yields the highest RMSD value, consistent with the greatest structural alteration occurring during drying in the absence of protectants (Fig. 3).

All RMSD values for insulin dried with trehalose are lower than the control (i.e., without trehalose), which confirms a protective role for trehalose. This protection is least effective where only 30 trehalose molecules are used, because there are too few molecules to cover the whole surface of insulin. The other three structures show that, as the number of trehalose molecules increases, so does the protective effect, but the relationship is not linear. This could be because, once trehalose has completely covered the surface of the insulin, there is likely to be little improvement in level of protection. Thus, particularly at the lowest water content (0.89 %), the protective effect of 60, 120 or 180 trehalose molecules on insulin is essentially the same and therefore the most efficient mole ratio of insulin to trehalose is 1:60.

### Changes in insulin secondary structure after drying

The secondary structure of dried insulin was determined using an algorithm reported by Kabsch and Sander [34, 35]. Compared to the secondary structure of native insulin, the content of the amino acids in α-helix in insulin dried without trehalose greatly decreased, with the proportion of the amino acids in turns and random coil increasing correspondingly (Fig. 4).

In contrast, for insulin dried with trehalose, the numbers of the amino acids in helical conformation are maintained perfectly (Fig. 4a). The content of the amino acids in random coil is slightly reduced (Fig. 4c), while the proportion of the amino acids in turns increases (Fig. 4b): the average number of the amino acids in turns in insulin protected by trehalose is reduced by about one-third compared to insulin without protectant, and is increased by a factor of two compared to native insulin.

The above results are consistent with the experiment that the addition of carbohydrates to the formulations inhibited the protein secondary structure rearrangement. Trehalose appeared to be one of the most efficient excipients in preventing secondary structure changes [22].

These results indicate that trehalose can preserve both the tertiary (Fig. 3) and the secondary structure of insulin (Fig. 4) during drying. Since maintenance of protein activity correlates well with the degree of retention of native structure during drying [36], we may conclude that, as trehalose maintains insulin structure during drying, it should also preserve the protein's bioactivity in the dry state.

It can also be seen from Fig. 4 that the average values for the secondary structure of insulin with trehalose are closest to those of the native structure for a water content of 1.77 %, which suggests this may be the optimum water content for dried insulin.

### Alteration of hydrophobic-to-hydrophilic area ratios after drying

To further examine the protective efficacy of trehalose, the hydrophobic-to-hydrophilic area ratio was used. It can be seen

from Fig. 5 that the hydrophobic-to-hydrophilic area ratio of insulin dried without trehalose shows an increase compared to the native structure. This is consistent with the increased exposure of hydrophobic regions, normally buried within the insulin structure, after drying. There is a corresponding reduction in the hydrophilic area exposed, and indeed many hydrophilic residues are positioned in the center of the dried insulin monomer. Thus, for the control insulin (i.e., without trehalose), a hydrophilic-to-hydrophobic structural reversal takes place as a result of desiccation. This is accompanied by a deformation of the tertiary structure of insulin and the exposed hydrophobic residues are likely to cause different insulin molecules to aggregate, and ultimately to form insoluble fibrils that may precipitate [37]. On the other hand, when trehalose is used, the processes of aggregation and fibrillation are less likely to occur [38].

The hydrophobic-to-hydrophilic area ratio of insulin dried with trehalose is close to that of the native structure: while insulin with 30 trehalose molecules (water content: 1.77 %) is most similar to native insulin, the hydrophobic-to-hydrophilic area values of the other insulin: trehalose ratios are slightly lower.

It should be emphasized that in all cases the solvent-accessible surface area of insulin, both hydrophobic and hydrophilic portions, decreases sharply after drying (Fig. S2): the average decrease is about one-third of the hydrophobic and hydrophilic area of the native structure, suggesting that, as might be expected, insulin shrinks on drying. However, there is a significant difference between insulin with or without trehalose, i.e., insulin alone shows both a hydrophilic-to-hydrophobic structural reversal as well as shrinkage when dried, whereas in the presence of trehalose, insulin undergoes only structural shrinkage (Fig. 5).

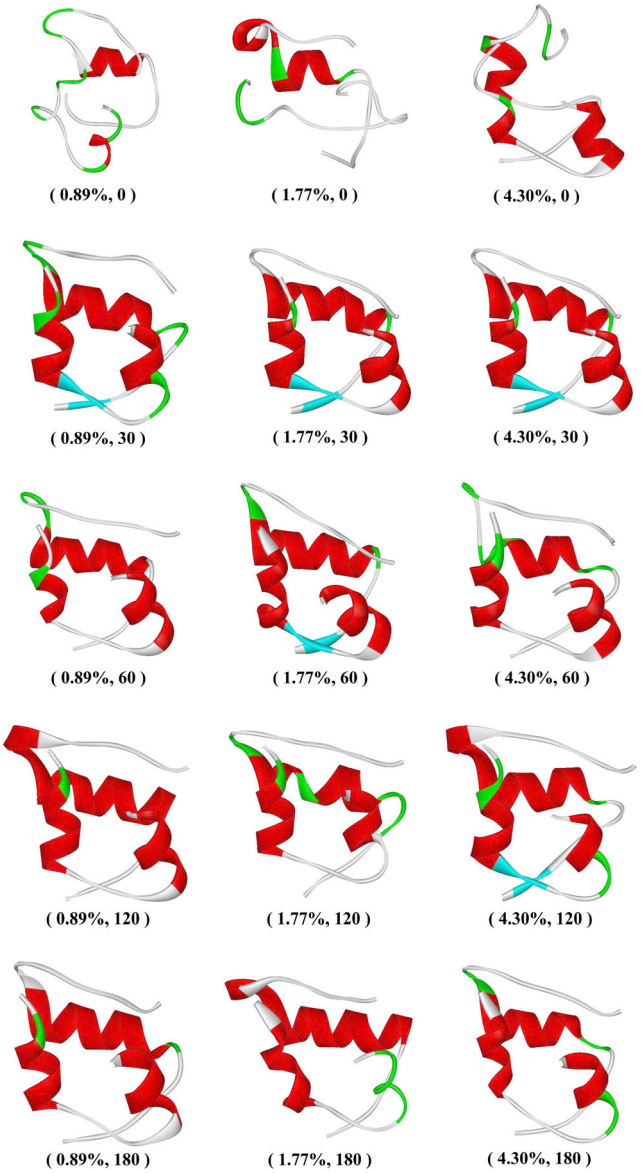


**Fig. 2** 3D structures of dried insulin: the numbers in parentheses are water content and number of trehalose molecules, respectively, with helices in *red*, sheets in *cyan*, turns in *green*, and coils in *gray*

## H-bond interactions after drying

It has been reported that the three-dimensional structure of biological macromolecules depends on the stabilizing effect of a molecular layer of water that interacts with surface residues via hydrogen bonding and electrostatic–polar interactions [39]. In addition, protein secondary structure is stabilized by H-bonding within or between strands of the polypeptide backbone. It can be seen from Fig. 6a that main-chain H-bond numbers for insulin dried without protectant are reduced compared to its native structure, while the total numbers of H-bonds within insulin increase (Fig. 6b). This suggests that both the secondary structure and the tertiary structure of insulin deform on drying. However, with the protection of trehalose, H-bond numbers are essentially identical to those of native insulin.

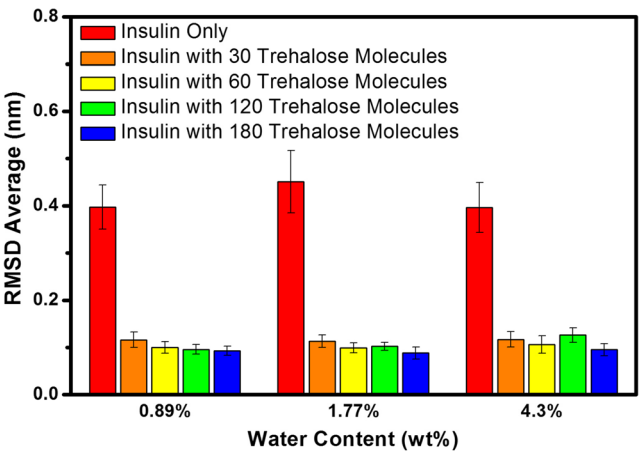


**Fig. 3** RMSD of insulin preserved by trehalose at different water contents (wt %)

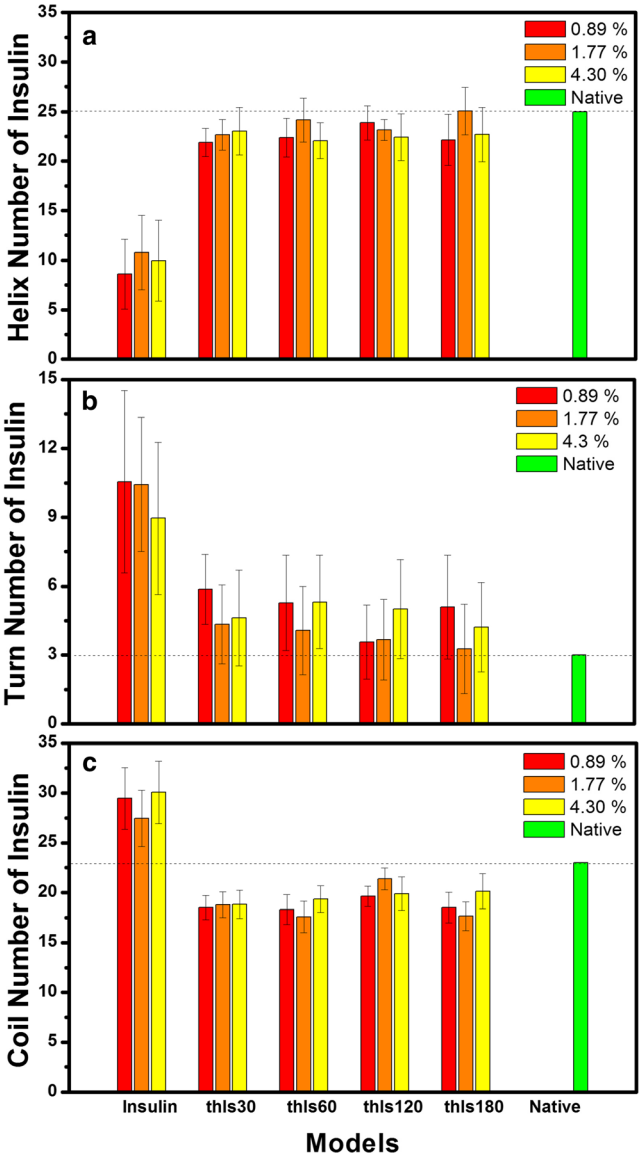


**Fig. 4** Alteration of secondary structure in different insulin models before and after drying: helix (**a**), turn (**b**), and coil (**c**)

Although the number of water molecules directly adsorbed on the surface of insulin decreases with drying (Fig. S4), the structure of dried insulin, when protected by trehalose, is perfectly preserved. Thus, trehalose may be acting as a water-replacement molecule, as suggested by the water replacement hypothesis [40]. That is, in the dry state, trehalose molecules form H-bond interaction with insulin that normally occur in solution between water molecules and the protein. Thus, trehalose molecules form large three-dimensional networks of H-bonds both between the trehalose molecules themselves and with insulin, thereby stabilizing the insulin structure [38]. Those results agree well with the conclusion that the preservative properties of the sugars, in terms of their ability to prevent protein denaturation, are directly linked to their ability to take part in hydrogen bond interactions with the protein [41].

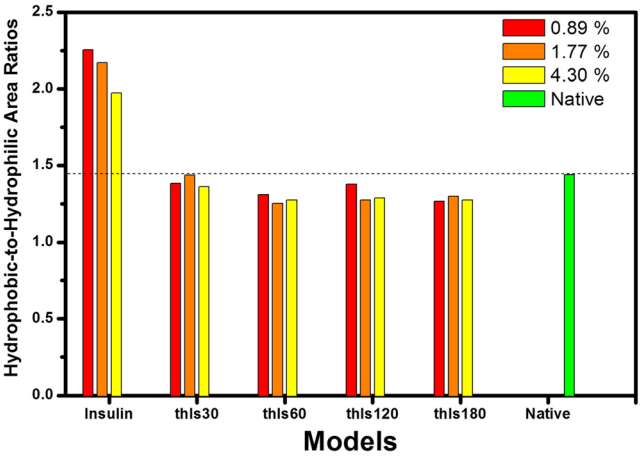


**Fig. 5** Alterations of hydrophobic-to-hydrophilic area ratios in different insulin models before and after drying

In addition to its role as a water-replacement molecule, trehalose is known to form organic glasses with a high glass

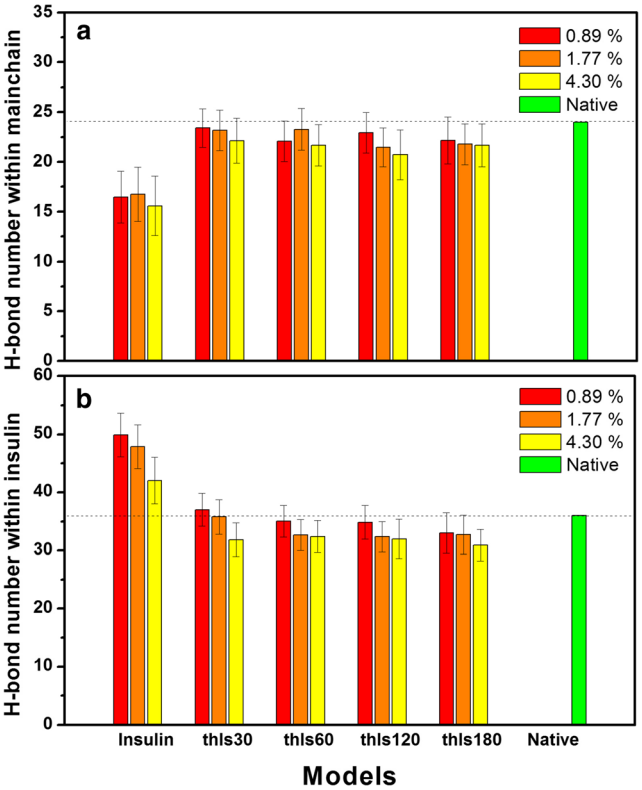


**Fig. 6** H-bond numbers within main chain of insulin (**a**) and within insulin (**b**): It can be seen from **a** that main-chain H-bond numbers for insulin dried without protectant (pure insulin model) are reduced compared to its native crystal structure (the *green* histogram), and those of other models protected by trehalose are essentially identical to those of native insulin. Obviously these results indicate the deformation of tertiary structure and the alteration of secondary structure of insulin dried without protectant. Furthermore, the ones protected by trehalose are preserved well. Likewise, the increasing of total H-bond numbers within insulin dried without protectant and the almost constant total H-bond numbers within insulin protected by trehalose (**b**), also prove the protection of trehalose on insulin

transition temperature, low molecular mobility [42], and low fragility [43, 44]. This vitrification effect is probably also important for preserving insulin structure and activity, as the protein is trapped in space, with a beneficial effect on its stability [45].

Experimental studies are consistent with our results: when insulin is lyophilized in the presence of trehalose, it does indeed retain more of its bioactivity and native-like structure in the solid state than in the absence of additives [23]. Just like the discussion on the influence of trehalose, maltose, and sucrose on some structural and dynamical properties of lysozyme [46], beta-relaxation is an important determinant of the stability of lyophilized insulin containing trehalose. Compared to other disaccharides, trehalose inhibits the beta-relaxation of insulin at low humidity as a result of insulin-trehalose interactions [21]. Trehalose can also increase the number of H-bonds formed by proteins in the dry state and make the H-bonds formed by water with proteins stable on long time scales [47]. Thus, in general, insulin powders prepared with trehalose are characterized by good physicochemical properties [20]. For the above reasons, increasing numbers of insulin or other biopharmaceutical products are formulated with trehalose [20, 23, 24, 48, 49].

## Conclusions

Molecular simulation studies show that, when insulin is dried in the presence of trehalose, it does not suffer the damaging structural changes experienced by insulin dried without the protectant disaccharide. Thus, compared to the native structure of insulin, the alteration of 3D structure, RMSD values and secondary structures, as well as the hydrophobic-to-hydrophilic surface area ratio, of dried insulin protected by trehalose are minimal. Numbers of H-bonds both within insulin as a whole and within its polypeptide backbone are also maintained. This is consistent with a role for trehalose as a protectant that can efficiently preserve the bioactive structure of insulin during and after drying (at 1 atm and below 310 K). As long as it can completely cover the surface of insulin, trehalose will prevent the protein from denaturation. These results support both the water-replacement and vitrification hypotheses for trehalose function, i.e., that trehalose can replace structural water around insulin and effectively restrain the structural alteration of insulin during drying, thereby preserving its activity.

**Acknowledgments** This project is supported by the National Natural Science Foundation of China (Grant No. 51076108), Key Disciplines Program of Shanghai, China (Grant No. T0503 and P0502), the Innovation Funds for International Cooperation of Science and Technology Commission of Shanghai Municipality, China (Grant No. 12430702000), and the Natural Science Foundation of Science and Technology Commission of Shanghai Municipality, China (Grant No. 12ZR1420400), the Innovation Funds of the Shanghai Municipal Education Commission (Grant No. 14YZ092) as well as the Alliance Program in Shanghai, China. We are grateful to Prof. Alan Tunnacliffe at University of Cambridge and to Prof. Chen Chenglung at National Sun Yat-Sen University for their insights into the protection mechanisms of trehalose on insulin.

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