Understanding the effects of two bound glucose in Sudlow site I on structure and function of human serum albumin: theoretical studies

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Human serum albumin (HSA) is the most abundant protein found in blood serum. It carries essential metabolites and many drugs. The glycation of HSA causes abnormal biological effects. Importantly, glycated HSA (GHSA) is of interest as a biomarker for diabetes. Recently, the first HSA structure with bound pyranose (GLC) and open-chain (GLO) glucose at Sudlow site I has been crystallised. We therefore employed molecular dynamics (MD) simulations and ONIOM calculations to study the dynamic nature of two bound glucose in a pre-glycated HSA (pGHSA) and observe how those sugars alter a protein structure comparing to wild type (Apo) and fatty acid-bound HSA (FA). Our analyses show that the overall structural stability of pGHSA is similar to Apo and FA, except Sudlow site II. Having glucose induces large protein flexibility at Sudlow site II. Besides, the presence of glucose causes W214 to reorient resulting in a change in W214 microenvironment. Considering sugars, both sugars are exposed to water, but GLO is more solvent-accessible. ONIOM results show that glucose binding is favoured for HSA (−115.04 kcal/mol) and GLO (−85.10 kcal/mol) is more preferable for Sudlow site I over GLC (−29.94 kcal/mol). GLO can strongly react with K195 and K199, whereas K195 and K199 provide slightly repulsive forces for GLC. This can confirm that an open-chain GLO is more favourable inside a pocket.

**Keywords:** human serum albumin; glycated human serum albumin; glucose; molecular dynamics simulations

Introduction

Human serum albumin (HSA) is the most abundant transport protein and constitutes up to 60% of total proteins in blood. HSA is well known for its astonishing binding capacity for metabolites and a wide range of drugs (i.e. warfarin, ibuprofen and indomethacin) (Fasano et al., 2005; Petersen, Ha, Mandel, & Bhagavan, 1995; Petitpas, Bhattacharya, Twine, East, & Curry, 2001). Physiological and pathological changes can induce an alteration of protein conformation and efficiency of ligand binding resulting in the function impairment in HSA. Glycation is one of the basic impairments found in HSA. Previous studies revealed that the high level of glycated HSA (GHSA) was observed in diabetic patients (Anguizola et al., 2013; Roohk & Zaidi, 2008). Many studies are therefore devoted to investigate GHSA as a diagnostic marker for diabetes mellitus (Cao, Chen, & Shi, 2015; Mohamadi-Nejad, Moosavi-Movahedi, Hakimelahi, & Sheibani, 2002; Raghav & Ahmad, 2014; Roohk & Zaidi, 2008).

HSA has a heart-like shape composed of 585 amino acids with 3 domains (I, II and III) and each domain is subdivided into 2 subdomains (A and B) (Figure 1(A)). HSA also contains two drug sites (Sudlow sites I (warfarin–azapropazone binding site) and II (indole–benzodiazepine binding site)) as shown in Figure 1(A). In the presence of glucose, a free amino group on HSA interacts with an aldehyde group on glucose and finally forms an Amadori product (Rohovec, Maschmeyer, Aime, & Peters, 2003; Shaklai, Garlick, & Bunn, 1984; Thornalley, Langborg, & Minhas, 1999). Earlier studies with mass spectrometry suggested many glycation sites on HSA (K12, K51, K93, K195, K199, K205, K233, K276, K281, K286, K378, K414, K439, K525, K538 K545) (Barnaby, Cerny, Clarke, & Hage, 2011; Lapolla et al., 2004; Wa, Cerny, Clarke, & Hage, 2007). K195 and K199 were also computationally found to interact with glucose (Nasiri, Bahrani, Zahedi, Moosavi-Movahedi, & Sattarahnady, 2010). The glycation on HSA was found to show the low ligand-binding capacity and different properties than...
normal HSA (Lim, Cheng, & Yang, 2007; Shaklai et al., 1984), but how GHSA is different from normal HSA remains unclear. Recently, the first crystal structure of GHSA was solved (Wang et al., 2013). Two isomers of glucose molecules (pyranose (GLC) and open-chain (GLO) forms in Figure 1(B)) were found to sit in a Sudlow site I within subdomain IIA (Wang et al., 2013). GLO is located at the entrance of Sudlow site I near K195, K199, R218, R222 and A291, whereas GLC is at the bottom surrounded by Y150, L238, R222, H242, R257 and A291, respectively (Figure 1(A)). This study also suggested the glycation mechanism of K195 by GLO. Recently, Szkudlarek and co-workers (Baraka-Vidot et al., 2015; Szkudlarek, Maciazek-Jurczyk, Chudzik, Rownicka-Zubik, & Sulkowska, 2016; Szkudlarek, Sulkowska, Maciazek-Jurczyk, Chudzik, & Rownicka-Zubik, 2015) have found that the binding of galactose sugar and some fluorescent markers has more impact on subdomain IIIA than IIA. The clear microscopic pictures for structural and functional changes that cause differences between glycated HSA and normal HSA remain unclear. However, at this early state, the pre-glycated HSA (pGHSA) is studied here. Both sugars in this study are non-covalently bound to HSA, although a previous X-ray work suggested a covalent link between K195 and GLO. We are interested in the influence of sugars before a glycation reaction takes place and how HSA dynamically responds to these guests at an early stage.

This study is aimed at understanding structural and dynamical properties of pGHSA comparing to normal and FA-bound HSA. Importantly, the effect of bound glucose on protein structure and dynamics is investigated here. In order to capture dynamic properties at microscopic level, molecular dynamics (MD) simulations were performed. MD simulations have been widely used in a number of earlier studies to investigate structure and function of HSA (Castellanos & Colina, 2013; Pongprayoon & Gleeson, 2014; Rahnama, Mahmoodian-Moghaddam, Khorsand-Ahmadi, Saberi, & Chamani, 2015). MD simulations can successfully reveal key interactions for drug binding and ligand recognition in HSA (Abou-Zied, Al-Lawatia, Elstner, & Steinbrecher, 2013; Aghaei, Ghasemi, Manouchehri, & Balalaie, 2014; Li, Jiang, & Zhu, 2015; Meneghini et al., 2014; Yu, Fang, Lu, Liu, & Zhang, 2014). We thus performed MD simulations on pGHSA to observe how HSA responds to two bound GLO and GLC molecules and compare protein’s structure and function to a native HSA (Apo) and HSA with seven bound fatty acids (FA) from our previous study (Pongprayoon & Gleeson, 2014). Moreover, we employed a quantum calculation (ONIOM method) to better understand the energetic properties of two glucose isomers inside a pocket.

**Methods**

**Molecular dynamics simulations**

The crystal structures of native (Apo), myristic acid-bound (FA), and pre-glycated HSA (pGHSA) (PDB ID: 1E78 (Bhattacharya, Curry, & Franks, 2000), 1E7G (Bhattacharya, Grüne, & Curry, 2000), and 4IW2 (Wang et al., 2013)) were downloaded from the Protein Data Bank (www.rcsb.org). The protonation states of all charged amino acids were set at physiological pH. Simulations were performed using the GROMACS 4.5.4 simulation package (www.gromacs.org) (Lindahl, Hess,
& van der Spoel, 2001) with AMBER99SB forcefield (Hornak et al., 2006). pGHSA was placed in a cubic simulation box solvating with water and counter ions. To relax steric conflicts generated during setup, all energy minimizations used up to 1000 steps of steepest descent. Long-range electrostatic interactions were treated using the particle mesh Ewald (PME) method (Darden, York, & Pedersen, 1993) with a short range cut-off of 1 nm, a Fourier spacing of 0.12 nm, and fourth-order spline interpolation. All simulations were performed in the constant number of particles, pressure and temperature (NPT) ensemble. The temperature of the protein, solvent and ions were each coupled separately using the v-rescale thermostat (Bussi, Donadio, & Parrinello, 2007) at 300 K with a coupling constant $\tau_\text{p} = 1$ ps. The pressure was coupled using the Berendsen algorithm at 1 bar with a coupling constant $\tau_\text{p} = 1$ ps. The time step for integration was 2 fs. Coordinates were saved every 2 ps for subsequent analysis. The 10-ns equilibration runs were performed and followed by the 60-ns production runs. The simulations were repeated twice. All results provided here are the average values from two simulations. The data were analysed by GROMACS and locally written code. Molecular graphic images were prepared using VMD (Humphrey, Dalke, & Schulten, 1996). RMSD and RMSF calculations were computed using an initial structure from each production run as a reference. To better understand how glucose influences structure and function of HSA, the results were compared to native HSA and HSA with seven bound fatty acids. The methods for setting up these two systems can be seen from our previous work (Pongprayoon & Gleeson, 2014).

**ONIOM calculations**

In order to investigate the energetic properties of bound glucose molecules, the binding energy (BE) and residue interaction energy (INT) were calculated from snapshots extracted from every 10 ns of MD trajectories. All energies were calculated by ONIOM(M062X/6-31 g(d):UFF) method using Gaussian 09 package (Gaussian 09 et al., 2009). Glucose molecules and their surrounded amino acids within 4 Å were treated as a quantum region. The binding energies (BE) were calculated as shown below.

$$BE_{\text{GLO+GLC}} = E_{\text{protein + GLO + GLC}} - E_{\text{protein}} - E_{\text{GLO+GLC}}$$

$$BE_{\text{GLO}} = E_{\text{protein + GLO}} - E_{\text{protein}} - E_{\text{GLO}}$$

$$BE_{\text{GLC}} = E_{\text{protein + GLC}} - E_{\text{protein}} - E_{\text{GLC}}$$

$BE_{\text{GLO+GLC}}$ is the binding energy of GLO and GLC in the binding site where $BE_{\text{GLO}}$ and $BE_{\text{GLC}}$ are the binding energies of GLO and GLC, respectively. Residue interaction energy (INT) between glucose and its neighbours was computed using M062X/6–31 g(d) method with correction for basis set superposition error (BSSE).

$INT$ was defined as follows: $INT_{\text{residue + GLO or GLC}} = E_{\text{residue + GLO or GLC}} - E_{\text{residue}} - E_{\text{GLO or GLC}}$.

**Results and discussions**

Overall, the decreased C-alpha RMSDs and RMSFs in Figure 2 indicate that bound ligands (in FA and pGHSA) clearly promote the global protein’s rigidity. Both bound fatty acids (FA) and glucose (pGHSA) obviously reduce the protein flexibility. Nonetheless, pGHSA appears to enhance structural flexibility in Sudlow sites I and II (Figure 2(C)–(F)). Especially, the large RMSD and RMSF fluctuations in pGHSA demonstrate that the presence of bound glucose significantly promotes the large fluctuation of Sudlow site II in subdomain IIA (Figure 2(E) and (F)). This finding is in a good agreement with a recent fluorescent study of galactose-bound HSA (Szkudlarek, Maciazek-Jurczyk, Chudzik, Rownicka-Zubik, & Sulkowska, 2015). Clearly, binding two glucose molecules increases the flexibilities of not only Sudlow site I in subdomain IIA, where both glucose molecules reside, but also Sudlow site II. Similar distances between key residues at Sudlow site II of Apo and pGHSA in Table 1 can imply the existence of usual interactions among key amino acids at Sudlow site II, whereas the shorter distances found in FA indicate more packed protein structure at this region in order to hold bound fatty acids. Furthermore, to observe the motion of subdomains IB and IIIB, distances between H510 and V116 at the interface were measured (Table 1 and Figure 4(C)). Our previous study shows that subdomains IB and IIIB move apart when binding to FA (long distance of 2.63 nm in FA), but here both subdomains in pGHSA appear to move towards each other (short distance of 1.83 nm in pGHSA). Considering interactions between K195 and D451 located at the interface between Sudlow sites I and II, K195 and D451 seem to form a consistent hydrogen bond in FA, whereas this bond is occasionally occurred in Apo and especially pGHSA (Figure 3(A)). This hydrogen bond helps to tether subdomain IIA to IIA forcing subdomains IIIB and IB to move apart in FA (see graphical views in Figure S1 in Supplementary information).

Furthermore, the properties of important tryptophan at position 214 (W214) located at subdomain IIA are also studied here. Usually, drug-binding affinities in HSA can be observed by quenching of W214 fluorescence (Helms, Petersen, Bhagavan, & Jameson, 1997). Binding to drug/ligand causes a change in W214 environment resulting in different fluorescence decay (Kamal & Behere, 2005; Sengupta & Sengupta, 2002). However, it is no clear evidence about how drug/ligand induces a change in W214 environment. Recently, Szkudlarek and co-workers (Szkudlarek et al., 2015) have reported that glycation can induce a change in W214 environment. To
better understand how ligand binding affects W214, interactions of W214 with neighbours and its conformations inside a pocket are captured here. In Figure 3(B), the favourable rotameric states of W214 in all simulations are captured. The Cβ-Cγ torsion angles (chi) are calculated. The typical torsion angles for most W214 in Apo, FA, and pGHSA are 80°, 85° and 90°, respectively. However, some W214 in Apo can adopt −60° (Figure 3(B)). The cartoon views in Figure 3(C) also display the orientations of W214 indole ring inside a pocket. An indole ring in FA seems to be more rigid, while those in Apo and pGHSA appear to escape from its original position. Especially, in pGHSA, the W214 ring is initially aligned perpendicular to a backbone axis before aligning parallel to a backbone axis (Figure 3(C)). Aligning parallel to a backbone axis seems to be the most favourable conformation for an indole ring in all cases. Furthermore, the W214 reorientations observed in Apo and pGHSA also reflect more volume in this region. W214 rings in Apo and pGHSA have a free space with an average volume of .38 nm³, while that of FA occupies

Table 1. Distances between centres of mass of each residue.

| Residue   | Distance (nm) |
|-----------|---------------|
|           | Apo* | FA* | pGHSA |
| H510-V116 | 2.12  | 2.63 | 1.83  |
| E383-R485 | 0.74  | 0.78 | 0.73  |
| K195-D451 | 0.81  | 0.69 | 0.76  |
| V343-E450 | 0.89  | 0.66 | 0.81  |
| V344-E450 | 0.62  | 0.67 | 0.60  |
| R348-E450 | 0.91  | 1.18 | 0.90  |

*The results from our previous study.

Figure 2. C-alpha RMSDs and RMSFs of protein (A and B) and Sudlow sites I and II (C-F) in Apo, FA, and pGHSA systems. All RMSD and RMSF data are computed using an initial structure in each production as a reference.
smaller room of 0.37 nm³ (Table 2). W214 in Apo and pGHSA have protein contacts of 67.71 and 63.45, whereas the more compact region in FA results in higher number of protein contacts (77.44 contacts) (Table 2).

Both smaller area and higher protein contacts explain why the frozen orientation of W214 ring in FA is observed. To better understand what affects W214 orientation, a set of hydrogen bonds is calculated. Generally, backbones of W214 in all systems can hydrogen bond to A210 and R218 with various degrees of existence. Typically, W214 in pGHSA can form moderate interactions with A210 (57.64%) and R218 (51.12%) (Table 2 and the locations of all amino acids are in Figure 3(C)). For Apo, apart from a moderate hydrogen bond with A210 (54.12%), W214 can permanently interact with R218 (73.14%) (Table 2). Unlike Apo and pGHSA, W214 in FA can sustain hydrogen bonds with both A210 (86.65%) and R218 (98.97%) where very weak extra interactions with F211 and A211 are also observed. Sugar binding seems to make W214 the most flexible among the three. In contrast, the presence of fully bound FAs causes W214 to become more stiff. The hydrogen bonds between W214 and ligands (Table 2) are not observed, but the rotameric states of W214 depend on ligand binding. Types of ligands activate different change in W214 rotamers resulting in different fluorescence decay. The hydrogen bonds between W214 sidechain and its neighbours are not seen here.

Considering the behaviour of glucose inside Sudlow site I (location of GLO and GLC is in Figure 4(C)), both sugars remain exposed to water. The number of water and protein contacts suggests that GLC is more buried, while GLO is more water exposed (Figure 4(A) and (B) (top)). This leads to more mobile GLO. High GLO flexibility therefore induces more protein contacts and consequently more GLO–protein hydrogen bonds (Figure 4). Despite the fact that GLO is more solvent accessible,
both sugars can approximately form ~4.5 hydrogen bonds with water (Figure 4). Different numbers of protein contacts and interactions appear to play a role in sugar binding affinity. To get an in-depth detail, a set of interaction energies is computed using ONIOM method. The negative protein–sugar interaction energy of $-115.04$ kcal/mol indicates that glucose binding is favourable for HSA. Furthermore, different individual interaction energies of two sugars display that Sudlow site I prefers GLO ($-85.10$ kcal/mol) over GLC ($-29.94$ kcal/mol). This set of energies agrees well with a higher number of hydrogen bonds found in GLO (Figure 4). Despite higher number of hydrogen bonds and interaction energy, our two simulations reveal the delocalisation of GLC, whereas GLC seems to be more buried and quieter inside Sudlow site I (Figure 5). Nonetheless, sugars in both simulations, especially GLO, are found to shift away from positions observed in a crystal structure (Figure S2 in supplementary information). This is due to a solvation effect. GLO appears to move away from its origin in the first simulation (Sim1) and towards GLC in our second simulation (Sim2) (Figure 5). These results reflect the excessive volume of Sudlow site I for sugars. Additionally, this excess space is confirmed by a previous study where drugs were found to bind Sudlow site I in a presence of sugars (Matsuda, Li, Zheng, & Hage, 2015). Besides, the large area in Sudlow site I and GLO displacement suggest that the GLO glycation is not a very fast process. Adjusting to the right GLO orientation is required before enabling a glycation.

To better understand how both sugars reside in Sudlow site I, a number of hydrogen bond and interaction energies between sugar and key amino acids are computed. For interaction energies (INT), only interactions with key amino acids are shown in Table 3. Key hydrogen bonds between sugars and their neighbours are calculated as a function of time in Figure 5. Mainly, a trapped GLC is found to permanently hydrogen bond to R257 (INT = $-5.60$ kcal/mol), H242 (INT = $-1.30$ kcal/mol) and Y150 (INT = $-3.25$ kcal/mol) and transiently bind to S287 (INT = $-2.00$ kcal/mol) and R222 (INT = $-3.10$ kcal/mol) (Table 3 and Figure 5). Other weak attractive interactions with R218 (INT = $-1.70$ kcal/mol), E292 (INT = $-1.30$ kcal/mol), K444 (INT = $-1.90$ kcal/mol) and D451 (INT = $-0.50$ kcal/mol) are also observed (Table 3). Interestingly, GLC appears to be slightly disfavoured by K195 (INT = $0.80$ kcal/mol) and K199 (INT = $1.30$ kcal/mol). In case of GLO, its high flexibility enables various hydrogen bonding networks. GLO in both simulations can interact with K195, K199, R218, R222, E292 and D451 (Figure 5). Since GLO is more water accessible, this permits GLO to move inside a pocket. In Sim1, GLO is stationary and interacts with K195, K199, R218, R222, E292 and D451 before moving to the other side of Sudlow site I (Figure 5).
After 30 ns, only R218, R222 and D451 can maintain their interactions, especially permanent interaction with R218 (Figure 5). The rest are diminished, but new interactions with H440 and K444 are formed. For GLO in Sim2, it translocates to the other side of Sudlow site I before moving to an area near GLC and forming hydrogen bonds with K199 and H242 (Figure 5). This GLO can maintain almost all interactions even though some are transient. However, almost all are removed (expect with K199 and H242) when GLO sit next to GLC (Figure 5). Even though K195 and K199 have transient interactions with GLO, they provide high favourable interaction energies of −6.90 kcal/mol (K195) and −3.20 kcal/mol (K199). Besides, strong attractive interactions are observed for R218 (−6.40 kcal/mol), R222 (−3.20 kcal/mol), E292 (−7.50 kcal/mol), K444 (−4.10 kcal/mol) and D451 (−9.10 kcal/mol). Especially, strong attractive forces from R218, R222, E292, K444 and D451, located near an entrance of Sudlow site I, help to trap GLO inside. Since the high water exposure causes GLO more mobile, the different GLO relocations are observed in this work. Such different GLO displacement also implies that Sudlow site I is not specific for glucose. This finding is supported by many previous studies (Frost, Chaudhry, Bell, & Cohenford, 2011; Syrový, 1994; Szkudlarek et al., 2016; Wang et al., 2013) where the glycation by other monosaccharide are observed at this site (i.e. galactose and fructose).

Considering interactions with K195 and K199, an open-chain GLO appears to be favoured by both lysines (especially K195). The different interaction energies between two sugars and these lysines observed here support a proposed mechanism by Wang and co-workers (Wang et al., 2013) that K195 and K199 do not interact with pyranose GLC at the bottom of a pocket, but instead interact with a second open-chain sugar at the entrance. GLC is stabilised by strong interactions with R257 (−5.60 kcal/mol) and R222 (−3.10 kcal/mol) at the
bottom and experiences slightly repulsive forces from K195 and K199. Such repulsive interactions by K195 and K199 may serve as a barrier to prevent a buried GLC from exiting. Our energetic and hydrogen bond results show that GLC is less favourable than GLO, but the X-ray structure with this bound GLC has been solved. This can imply a key role of GLC for glycation. Since GLC is unfavourable for K195 and K199, it can imply that this trapped GLC cannot react with both lysines. Firstly binding GLC may help HSA adopt a proper conformation to accept the next sugar for glycation. However, further studies are required to observe the role of this GLC on glycation at this site.

Conclusions

Overall, the presence of GLO and GLC has no serious effect on global protein structure. An overall structural stability of pGHSA is similar to those of Apo and FA. However, having two sugars in Sudlow site I seems to significantly increase the protein flexibility at Sudlow site II. Unlike Apo and FA, D451 in pGHSA is found to bond to GLC, therefore this causes the loss of K195-D451 interaction that fasten subdomain IIA to IIIA consequently resulting in the observed movement of subdomain IB and IIB. Moreover, this study, for the first time, reveals that the orientation of W214 is dependent on ligand binding. A change in rotameric conformations of W214 can completely answer why W214 fluorescence quenching can be employed for probing drug-binding affinity. For sugar binding, our evidences identify that GLO is more favourable in a pocket. An open-chain glucose can interact with K195, K199 and E153 as suggested by a previous study (Nasiri et al., 2010). However, there remain many gaps to be filled since only single docked open-chain GLO was studied by Nasiri and co-workers (Nasiri et al., 2010). Recently, the X-ray structure of HSA with two bound glucose isomers has been crystallised. Comparing with X-ray structure, the docked open-chain glucose by Nasiri and co-workers (Nasiri et al., 2010) was buried near the bottom of Sudlow site I which is not the same site as observed by an X-ray experiment (Wang et al., 2013). An X-ray open-chain glucose was found to sit near an entrance of Sudlow site I in a presence of buried GLC (Wang et al., 2013). So, a study of GLO binding in the presence of buried GLC is not covered by an earlier computational work. However, some of key interactions obtained by Nasiri and co-workers (Nasiri et al., 2010) still agree well with our findings. We also reveal that a trapped GLC does not favourably interact with K195 and K199 unlike GLO. Nonetheless, too large space and high water-accessible area in a pocket promotes a high GLO flexibility leading to the observed GLO displacement. This finding can signify the non-glucose-specific Sudlow site I. Our microscopic results can be used to support the existence of galactation and fructation observed by previous studies (Szkudlarek et al., 2015; Wang et al., 2013). Additionally, although a buried GLC does not form any strong interactions with HSA like GLO, it is interesting to understand a role of GLC on glycation and why this trapped GLC is not glycated upon arrival, but remains inside when a second sugar gets glycated. Since Sudlow site I is large and has high water exposure, it can accept different types of sugar. Does a glycation take place if different types of sugar are present inside a pocket? It appears that many questions on glycation process are still largely open. An insight into the nature of glycated HSA and glycation mechanism will play a vital role in controlling and monitoring diabetes and other metabolic diseases.

Table 3. Interaction energies of sugars with protein and sugars with key amino acids. Only interaction energies of key amino acids are shown here.

| Residue               | GLO  | GLC   |
|-----------------------|------|-------|
| 2 Glucose-bound Protein| -115.04 | -29.94 |
| Glucose-bound Protein  | -85.10 | -2.90  |
| Y150                  | .00  | -2.60 |
| E153                  | -2.70 | -2.60 |
| K195                  | -6.90 | .80   |
| K199                  | -3.20 | 1.30  |
| R218                  | -6.40 | -1.70 |
| R222                  | 3.20  | 1.30  |
| H242                  | .00  | -1.30 |
| R257                  | .00  | -5.60 |
| S287                  | .10  | -2.00 |
| E292                  | -7.50 | -1.30 |
| K444                  | -4.10 | 1.90  |
| D451                  | -9.10 | -5.50 |

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