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Structural Mechanism of Ring-opening Reaction of Glucose by Human Serum Albumin*

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Background: Glucose can glycate human serum albumin (HSA), but the mechanism is unknown.
Results: Crystal structures of rHSA in the presence of glucose show that glucose is linearized and covalently linked to rHSA.
Conclusion: The residues Lys-195 and Lys-199 of rHSA are involved in glucose ring opening.
Significance: This work provides a structural mechanism of protein glycation.

Glucose reacts with proteins nonenzymatically under physiological conditions. Such glycation is exacerbated in diabetic patients with high levels of blood sugar and induces various complications. Human albumin serum (HSA) is the most abundant protein in plasma and is glycated by glucose. The glycation sites on HSA remain controversial among different studies. Here, we report two protein crystal structures of HSA in complex with either glucose or fructose. These crystal structures reveal the presence of linear forms of sugar for both monosaccharides. The linear form of glucose forms a covalent bond to Lys-195 of HSA, but this is not the case for fructose. Based on these structures, we propose a mechanism for glucose ring opening involving both residues Lys-195 and Lys-199. These results provide mechanistic insights to understand the glucose ring-opening reaction and the glycation of proteins by monosaccharides.

Glucose is the ubiquitous monosaccharide and plays vital roles in energy metabolism in all life forms from bacteria to humans. Glucose is oxidized to CO_2 and water through glycolysis and later in the reactions of the citric acid cycle, yielding energy, mostly in the form of ATP. The glucose level in human blood is regulated by insulin and other mechanisms. Glucose is a commonly measured blood marker for human health. Higher glucose level (above 7 mmol/liter) is the indication of prediabetes or diabetes mellitus condition. Under physiological conditions, glucose reacts nonenzymatically with a wide variety of proteins, but not all proteins, to form stable adducts, which is named the glycation reaction (1). Glycated human serum albumin (HSA)[2] level is an important biomarker in clinic and used for glycemic control. The glycation reaction is typically between ϵ-amino groups of lysine (and sometimes arginine) residues in a protein and glucose, leading toward the formation of a Schiff base, which can rearrange chemically to more complex products (Amadori products) and eventually result in the formation of advanced glycation end products. Advanced glycation end products are implicated in a range of age-related chronic diseases, such as microangiopathy, retinopathy, and nephropathy (2–4). Glycation of proteins and its potential pathophysiologic significance have been a subject of extensive studies.

Human serum albumin is the most abundant protein (~640 μM) in blood plasma with a long half-life in plasma (18 days), and it plays critical roles in maintaining the colloid osmotic pressure of plasma and transporting endogenous and exogenous compounds (5–7). In healthy persons, albumin was found to be glycated at a level of 1–10% (5, 8). In patients with diabetes mellitus, the glycation level of albumin can be quite high (20–30%) (9, 10). The level of glycated HSA reflects shorter term glucose changes when compared with the long term glucose marker: glycated hemoglobin (11–13). Functional consequences of glycation are also observed in HSA; the glycated HSA from patients shows lower ligand binding capacity of HSA and different antioxidant properties than HSA (8, 14). HSA is a commonly used model protein to study nonenzymatic glycation.

Despite these evidences indicating that glycation takes place in HSA, the glycation sites on HSA remain controversial according to different studies. Early studies established that four lysine residues (Lys-199, Lys-281, Lys-439, and Lys-525) had been modified by nonenzymatic glycosylation (8, 15). Later, different groups identified other lysine residues (Lys-12, Lys-51, Lys-93, Lys-159, Lys-205, Lys-233, Lys-276, Lys-286, Lys-378, Lys-414, Lys-439, Lys-538, Lys-545) and some arginine residues as being glycated by using mass spectrometry *in vitro* (16–18).

The structural mechanism of the nonenzymatic glycation reaction and determinants for the specificity of glycation site have long been topics for intensive research (15, 19–21). Positively charged amino acids situated close to a lysine in either the

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The atomic coordinates and structure factors (codes 4IW2 and 4IW1) have been deposited in the Protein Data Bank (http://wwpdb.org/).
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[2] The abbreviations used are: HSA, human serum albumin; rHSA, recombinant HSA; SAL, salicylic.

primary or the three-dimensional structure were proposed to catalyze the glycation of that lysine (15). Johansen *et al.* (19) suggested from statistical analysis that glutamate and lysine residues catalyzed the glycation of nearby lysines, and the catalytic glutamates were located mainly C-terminally from the glycation site, whereas the catalytic basic lysine residues were found mainly N-terminally. However, little mechanistic or structural information concerning glycation is known.

Here we report the first three-dimensional structural evidence of glycation of HSA through the determination of the crystal structures of HSA in complex with monosaccharides (glucose or fructose) by x-ray crystallography. We found two different forms of glucose molecules: one in the cyclic form (pyranose) and another in the open form, with the open form covalent linked to the residue Lys-195 of HSA. Both forms of glucose are located in a major drug binding site of HSA (Sudlow site I). The structural conformations of glucose provide the structural mechanism to understand the glycation of HSA by glucose. These results may have broader implications enabling us to understand the nonenzymatic glycation and the specificity of other proteins by glucose.

EXPERIMENTAL PROCEDURES

Preparation and Crystallization of Recombinant HSA (rHSA) in Complex with Glucose—Defatted recombinant HSA, free of fatty acid, was kindly provided by Zhejiang Hisun Pharmaceutical Co. Ltd. D-Glucose and D-fructose were purchased from China National Medicine Corp. Ltd. The rHSA·glucose and rHSA·fructose complexes were prepared at a molar ratio of 1:15. The final HSA concentrations in the complexes were about 100 mg/ml, similar to previous studies (22–24). The complex was then crystallized by sitting drop vapor diffusion at room temperature by mixing 1 μl of the protein complex with 1 μl of the precipitant solution containing 25–30% (w/v) polyethylene glycol 3350 in 50 mM potassium phosphate at pH 7.5. Diffracting quality crystals were obtained after 3–4 weeks at room temperature.

X-ray Data Collection—X-ray diffraction data of the crystals were collected at 100 K on beam line 17U of Shanghai Synchrotron Radiation Facility at a wavelength of 1.04 Å. The crystals were soaked briefly in the cryoprotectant solution containing 10% dimethyl sulfoxide (DMSO) in the crystallization precipitant solution and frozen in the liquid nitrogen stream of the beam line. The diffraction data were scaled and processed using the HKL2000 program (25). The crystal structures of the rHSA·glucose and rHSA·fructose complexes were solved by the molecular replacement program MOLREP of the CCP4 package using Protein Data Bank (PDB) entry 1AO6. Only the HSA coordinates were used for molecular replacement, and the ligands were removed to minimize model bias (26). The electron densities for the glucose and fructose were clearly visible after the initial refinement step, and these molecules were manually positioned into this electron density, and then manual refinement and model building were performed with COOT (27). After several cycles of positional and B-factor refinement together with manual adjustments, the results were successfully refined to the final R and R_{free} values by CCP4. The structure were analyzed by PyMOL (28).

RESULTS

Ring Opening of Glucose in the Presence of HSA—Human serum albumin (Fig. 1*a*) is an often used model protein to study the pyranose ring-opening reaction of glucose and protein glycation by many techniques, notably mass spectrometry, to obtain information about glycation site (16, 18, 29, 30). To understand the mechanism and the structural determinant for glycation, we determined the crystal structure of rHSA in complex with glucose. We generated the molecular complexes of rHSA·glucose at a molar ratio of 1:15. The concentrations used were not too far from the physiological concentrations (rHSA at 0.64 mM and glucose at 7 mM). The complex was then crystallized at room temperature (22 °C) by the vapor diffusion method at pH 7.5. The initial crystals were optimized by the streak seeding method. The total time between crystallization and data collection was about 3–4 weeks. The rHSA·glucose crystals diffract to a relative high resolution of 2.40 Å with a synchrotron radiation source (Table 1).

We identified two glucose molecules in the structure of the rHSA·glucose complex. Both glucose molecules are found in Sudlow site I (31, 32) of rHSA, which is located in subdomain IIA of rHSA (Fig. 1). HSA has seven ligand binding sites for a variety of endogenous (fatty acid) or drug molecules (7); two of them are major drug binding sites and were first described by Sudlow *et al.* (31, 32) and named as Sudlow site I and II, accordingly. Sudlow site I is a large drug binding pocket and can be further divided into three nonoverlapping subsites (22): a salicylic (SAL) subsite at the bottom of Sudlow site I (33) and an indomethacin subsite (34) and a 3′-azido-3′-deoxythymidine subsite (22) near the entrance (Fig. 1*a*). Sudlow site I is also highly polarized with charged residues (Lys-195, Arg-218, and Glu-292) at its entrance (Fig. 1*b*) and with hydrophobic residues lining the bottom of the pocket. One glucose was found near the bottom of Sudlow site I (close to the SAL subsite), forming multiple hydrogen bonds to the rHSA residues, including Tyr-150, Arg-222, His-242, and Arg-257 (Fig. 2*b*). The pyranose ring of glucose is pinched between the side-chains of Leu-238 and Ala-291, a feature found for many drugs bound at Sudlow site I (Fig. 2*b*) (7).

Interestingly, the second glucose was found to be in the linear open form and located at the entrance of Sudlow site I. The presence of the linear form of glucose was supported unambiguously by the strong electron density in the $F_{obs} - F_{calc}$ map (Fig. 2*c*). This electron density had elongated shape and could not accommodate a glucose molecule at the pyranose form (cyclic form). In addition, we found that this density fused with the electron density of the Lys-195 residue located on the HSA subdomain IIA. Thus, we modeled the linear glucose covalently linking to the side chain amine group of Lys-195 on HSA (Fig. 2, *c* and *d*). At the current resolution, we are not able to distinguish whether the glucose·rHSA adduct is a Schiff base or an Amadori product.

As a negative control to confirm the glycation of residue Lys-195, we determined the HSA structure in the absence of any ligands (Table 1); we observed very weak electron density near the linear glucose binding around Lys-195. It was impossible to fit a glucose molecule to this density. In addition, the Lys-195

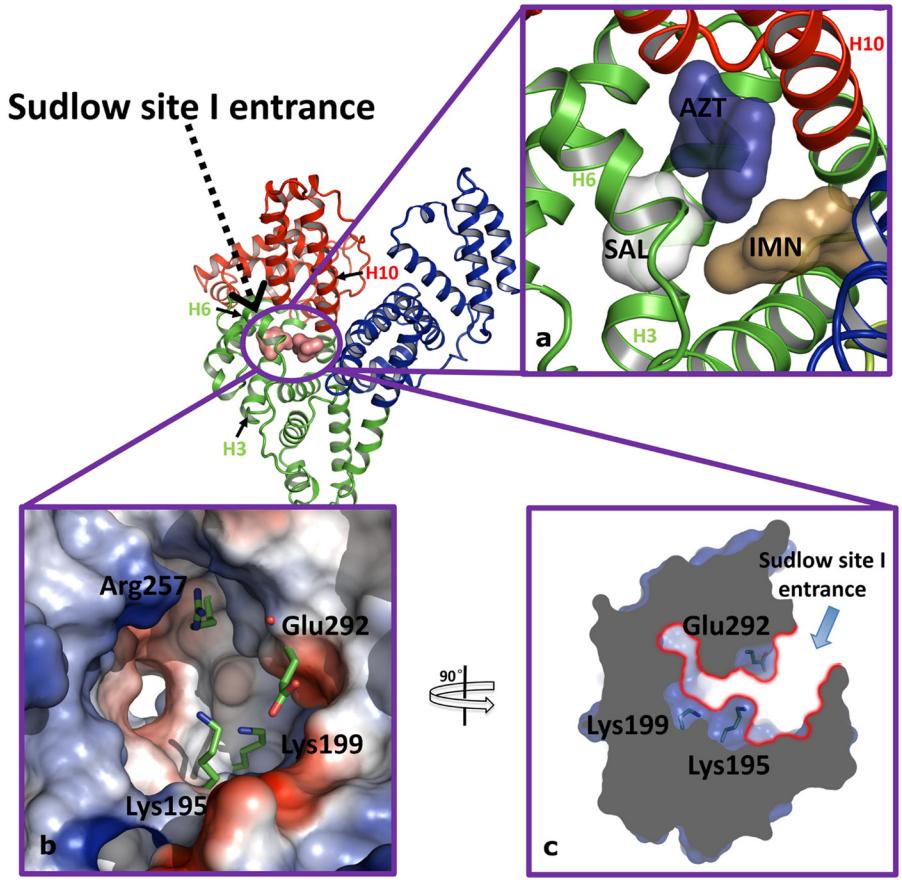

FIGURE 1. **Overall structure of HSA and the architecture of a major ligand binding site (Sudlow site I).** The structure of HSA is shown in graphic representation and is color-coded by its domains (domain I in *red*, domain II in *green*, and domain III in *blue*). Sudlow site I is surrounded by three helices (the long h10 of domain I and the h3 and h6 of domain II). The glucose molecule binding site (*pink*) is shown as semitransparent van der Waals surfaces (*a*). Sudlow site I is a large drug binding pocket and can be further divided into three subsites: SAL subsite (*SAL*), indomethacin subsite (*IMN*), and 3′-azido-3′-deoxythymidine subsite (*AZT*). *b*, view of the Sudlow site I pocket from its entrance (colored by charge). Selected residues Lys-195, Lys-199, Arg-218, and Glu-292 are shown in stick and color-coded by atom type: carbon in *green*; oxygen in *red*; and nitrogen in *blue*. *c*, cut-away view of Sudlow site I pocket with selected residues shown in sticks.

TABLE 1

Data collection and structural refinement statistics of rHSA·glucose and rHSA-fructose structure

	HSA	rHSA·glucose	rHSA·fructose
Data collection			
X-ray wavelength (Å)	1.04	1.04	1.04
Space group	P21	P21	P21
Cell dimensions (Å)	58.41, 179.89, 57.46, $\beta = 105.86°$	57.15, 87.06, 58.53, $\beta = 102.57°$	58.36, 87.99, 59.35, $\beta = 102.70°$
Independent reflections	18979	21137	18509
Resolution range (Å)	50.0–3.23 (3.30–3.23)[a]	50.0–2.4 (2.44–2.40)[a]	50.0–2.56 (2.59–2.56)[a]
Completeness (%)	90.8 (83.2)[a]	97.2 (90.6)[a]	97.7 (97.5)[a]
Multiplicity (redundancy)	2.7 (2.6)[a]	3.1 (2.3)[a]	3.4 (3.2)[a]
R_{sym} (%)[b]	5.1 (45.4)[a]	8.4 (45.9)[a]	8.7 (58.7)[a]
I/σ	12.9 (2.22)[a]	19.2 (2.48)[a]	20.12 (2.07)[a]
Refinement statistics			
R_{work} (%)[c]	21.2 (21.5)	24.2 (34.2)	23.9 (33.8)
R_{free} (%)[d]	26.5 (27.9)	30.1 (35.1)	31.5 (44.6)
r.m.s.d.[e] from ideal values			
Bond lengths (Å)	0.010	0.012	0.012
Bond angles (°)	1.5	1.7	1.7
% of residues in favored, allowed, and outlier regions	91.4, 6.4, 2.2	92.2, 5.9, 1.9	90.2, 6.9, 2.9
PDB ID	4K2C[f]	4IW2	4IW1

[a] Values in parentheses are for the outermost resolution shells.
[b] $R_{sym} = \Sigma|(Ihkl) - (I)|/\Sigma(Ihkl)$ where $Ihkl$ is the weighted mean intensity of a given reflection.
[c] $R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.
[d] R_{free} is the R_{work} calculated using a randomly selected 5% reflection data omitted from the refinement.
[e] r.m.s.d., root mean square deviation.
[f] From Ref. 53.

residue is usually disordered at its side chain, as shown by its electron density map, in a number of binary HSA-ligand structures (35). At the ring glucose binding site (SAL subsite) in the ligand-free HSA structure, there was no extra electron density. Thus, the extra electron density we observed for Lys-195 was indeed caused by the glycation by glucose.

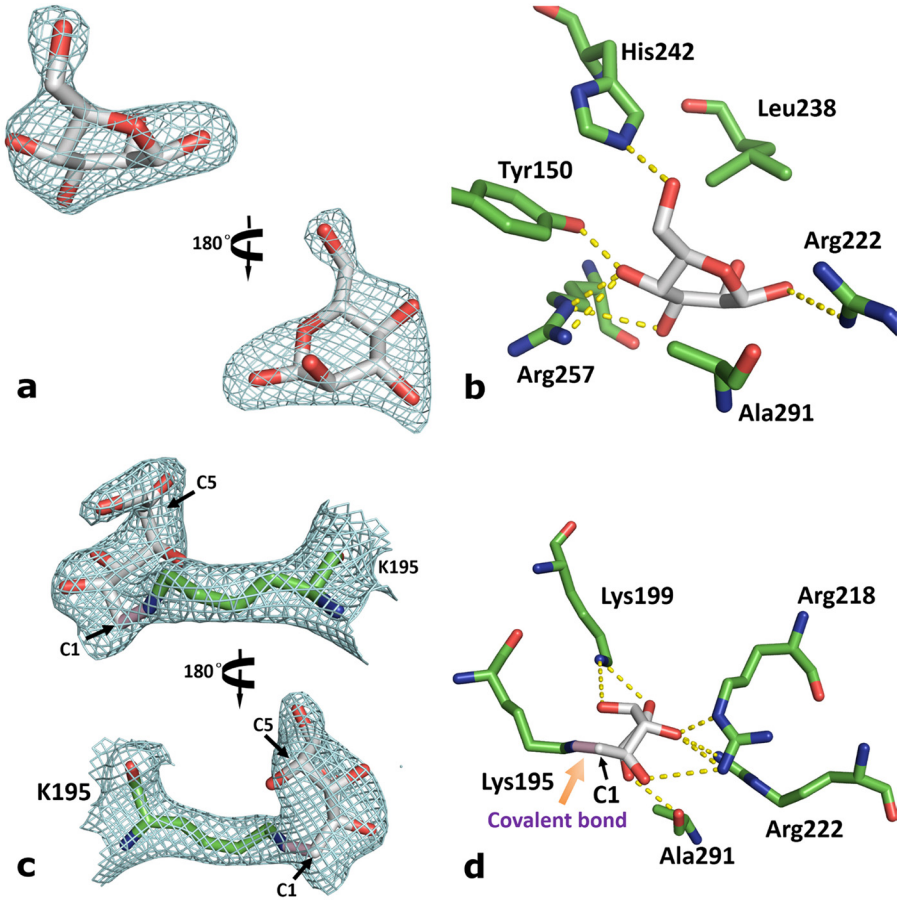

FIGURE 2. **Two glucose molecule bind at Sudlow site I of HSA.** *a*, the pyranose form of glucose is surrounded by several polar residues of HSA, which are shown as stick representation with atoms color-coded by atom type: glucose carbon in *white*; HSA carbon in *green*; oxygen in *red*; and nitrogen in *blue*. Hydrogen bonds are shown in *dotted lines*. *b*, an electron density map (*cyan* mesh net representation, σ-weighted $2F_{obs} - F_{calc}$ omit map, contoured at 1.0σ) shows that a glucose in pyranose cyclic form was found at the bottom (Fig. 1*c*) of Sudlow site I. *c*, an electron density map shows that a second glucose was in linear conformation and covalently linked to the Lys-195 residue of HSA. The large, elongated electron density was not big enough to fit to a glucose at pyranose form (ring form) and thus was assigned to a linear glucose with confidence. *d*, the linear glucose is anchored to Sudlow site I by polar residues of HSA.

The linear glucose is anchored by the positively charged residues of rHSA located at Sudlow site I (Lys-199, Arg-218, Arg-222, and Ala-291) through a hydrogen-bonding network, which stabilizes its current conformation (Fig. 2*d*). Thus, the structure of the rHSA·glucose complex shows that the pyranose ring of glucose was opened in the presence of rHSA.

Ring Opening of Fructose without Glycation of Human Serum Albumin—The structure of rHSA·glucose has limited resolution. To further confirm the results of glucose ring opening in the presence of rHSA, we crystallized another monosaccharide, fructose, in the presence of recombinant HSA at a molar ratio of 15:1. The resultant rHSA·fructose crystal structure, at a resolution of 2.56 Å, revealed the presence of two fructose molecules based on the electron density maps. Again, both fructose molecules were located in Sudlow site I. One fructose molecule was in a location similar to the pyranose form of glucose in the rHSA·glucose structure (Fig. 3, *a* and *b*). This fructose makes a number of hydrogen bonds with three HSA residues (Tyr-150, Arg-222, and Arg-257) (Fig. 3*b*). Also, based on the electron density map, another fructose molecule was found to adopt a linear conformation (Fig. 3*c*).

This linear fructose is located at the position of the linear glucose in the rHSA·glucose structure. Similarly, we found that this linear fructose makes hydrogen bonds to the positively charged HSA residues (Lys-195, Lys-199, Arg-218, and Arg-222) (Fig. 3*d*). However, we did not see the direct link between fructose and rHSA residue Lys-195. The electron density of the linear fructose and Lys-195 was clearly absent, strongly suggesting the absence of a direct covalent link. This is quite different from the rHSA·glucose structure.

DISCUSSION

Comparison of Our Results with Previous Studies—Some proteins in healthy persons are found to be glycated. For serum albumin, the proportion of glycated albumin in healthy persons is in the range of 1–10% (5, 8), which increases by 2–3-fold in the case of patients with diabetes mellitus (9). Glycation occurs on amine groups on the protein surface, commonly on lysine residues, but also on other residues (15, 17, 30).

We observed directly by x-ray crystallography the glycation of the residue Lys-195 upon incubation of rHSA with glucose at 22 °C for 3–4 weeks. We did not measure the glycation of the rHSA·monosaccharide complex in our crystals by other methods, *e.g.* mass spectrometry, because this observation of glycation of rHSA at position Lys-195 was supported by many other studies. Lapolla *et al.* (16) found by mass spectrometry that

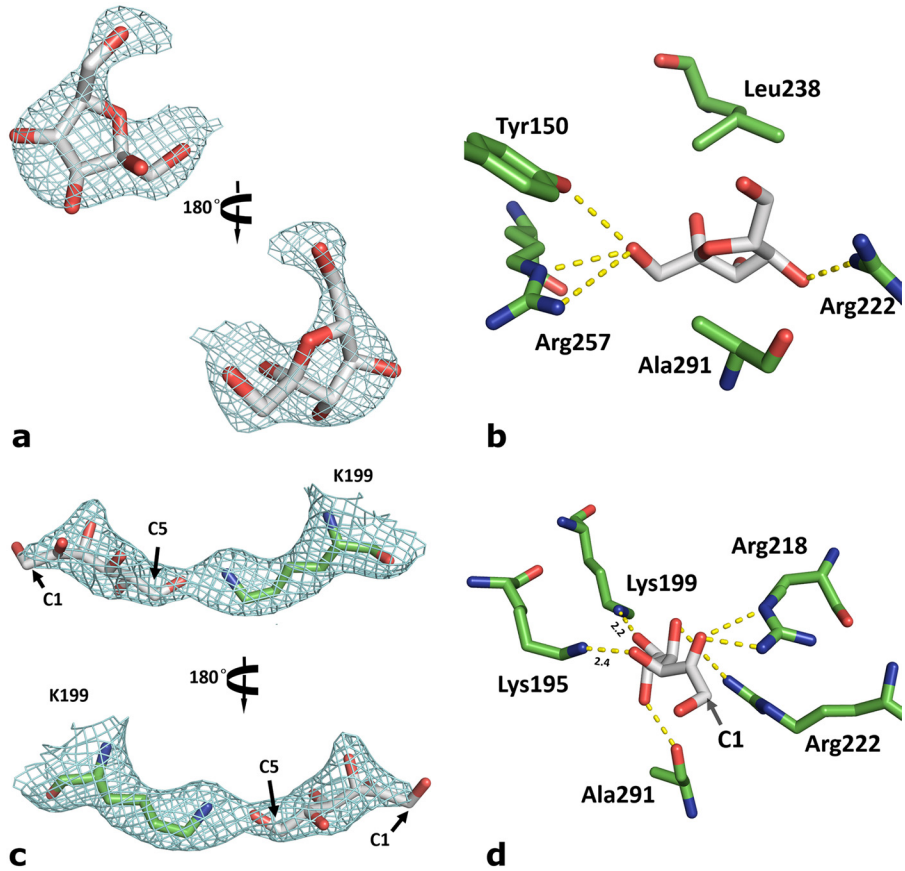

FIGURE 3. **Two fructose molecules bind at Sudlow site I.** *a*, one of the fructose molecules was in cyclic conformation as shown by its electron density map. The polar residues of HSA are shown as stick representation with atoms color-coded by atom type: glucose carbon in *white*; HSA carbon in *green*; oxygen in *red*; and nitrogen in *blue*. Hydrogen bonds are shown in *dotted lines*. *b*, this fructose was found at the bottom of Sudlow site I, in a location similar to the first glucose in the rHSA·glucose structure. *c*, the electron density map shows that the second fructose is in linear conformation. *d*, the linear glucose does not have a covalent bond to the Lys-195 and Lys-199 residues but is stabilized by a network of hydrogen bonds to a position near the entrance of Sudlow site I.

residues 181–200 and residues 198–218 of rHSA were glycated when glucose was allowed to react with HSA for 1–2 weeks. The native HSA was cleaved by trypsin at residues Lys-181, Lys-195, and Arg-197. The Lys-195 trypsin cleavage site was lost upon glucose glycation (16), further supporting the glycation at Lys-195. Wa *et al.* (17) used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to study HSA that was minimally glycated (from Sigma-Aldrich) prepared *in vitro*. They found that peptide 191–205 of HSA was glycated and suggested that the glycation site is Lys-199 and not Lys-195 (36). Recently, Stefanowicz *et al.* (37) prepared glycated HSA by heating an HSA-glucose mixture in an air oven at 80 °C for 25 min and used mass spectrometry combined with isotopic labeling to demonstrate that both Lys-190 and Lys-195 were glycated in peptide 187–197. For serum albumin purified from healthy donors, it was found by ion trap-time-of-flight mass spectrometry that the peptide 187–197 was glycated and that Lys-195 or Lys-190 was the glycation site in this peptide (38). In other studies, Sattarahmady and co-workers (39) used molecular dynamics simulation to investigate the interaction of HSA with the open form of glucose and concluded that residue Lys-195 was the primary site for interaction with glucose. Residue Trp-214 of HSA is located near residue Lys-195 in the Sudlow site, and its intrinsic fluorescence is sensitive to its environment. Binding of small molecules to HSA at Sudlow site 1 usually quenches the tryptophan fluorescence (8, 40, 41). Mendez *et al.* (42) showed that that the tryptophan fluorescence signal of HSA was reduced 25–30% upon glycation when compared with nonglycated albumin, also consistent with the glycation of residue Lys-195.

Other glycation sites on HSA were identified previously in a number of independent studies (15–18, 30). Lys-525 was often observed to be glycated by glucose in a number of *in vivo* studies (15, 16, 18). Other lysine residues (Lys-12, Lys-51, Lys-93, Lys-159, Lys-205, Lys-233, Lys-276, Lys-286, Lys-378, Lys-414, Lys-439, Lys-538, Lys-545) and some arginine residues were identified to be glycated by different groups using mass spectrometry *in vitro* (16–18). We did not observe the glycation of Lys-525 in our crystal structure of the rHSA·glucose complex, but we cannot rule out such a possibility. The glycation of HSA at these sites may require higher temperature (our glycation reaction was carried out at 22 °C for 3–4 weeks), or perhaps the glycation was not visible by x-ray crystallography due to the potential high flexibility of glycated surface residues. The linearized monosaccharides that we observed in our rHSA·monosaccharide crystals are located in a pocket (Sudlow site I). The restrained movement in the pocket is likely the factor allowing us to see the linearized monosaccharide directly.

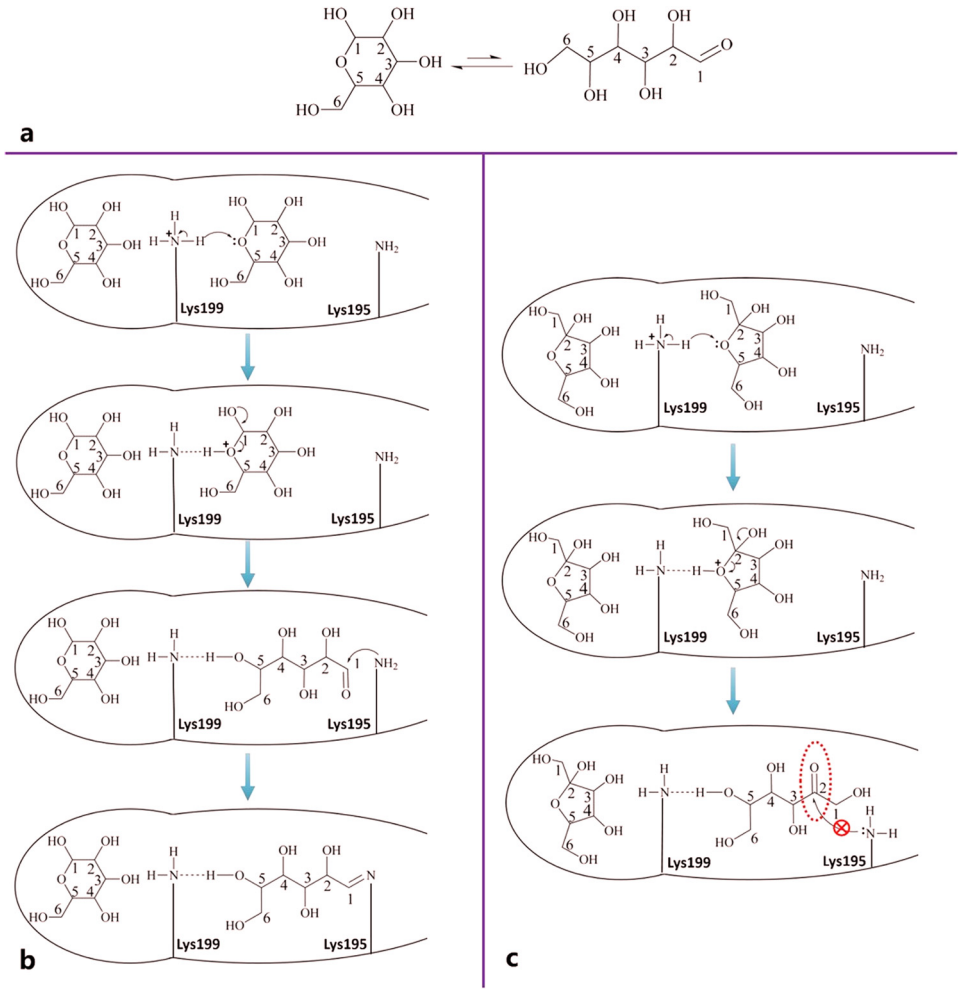

FIGURE 4. **Ring-opening mechanism of glucose and fructose by HSA.** *a*, glucose is in an equilibrium between the ring-closed forms and the open-chain aldehyde form in solution. *b*, glucose 1 enters Sudlow site I and is trapped at the bottom of Sudlow site I in pyranose form (*left*). The second glucose was attacked by the catalytic residue, Lys-199, at its O5 atom, leading to the break of the C1–O5 bond. The newly formed C1 aldehyde springs over to near the Lys-195 residue and forms a covalent bond to the Lys-195 residue. *c*, the ring-opening process for fructose by HSA is similar to glucose, except that no covalent bond is formed between the Lys-195 residue and the linear fructose. This is likely due to lower reactivity of the ketone.

Molecular Mechanism of Glucose and Fructose Ring-opening Reaction in Sudlow Site I Pocket of HSA—Glucose combines a delicate balance between chemical stability and chemical reactivity for metabolism and synthesis, and exists in several structural conformations: two major forms of pyranose (six-member rings with anomeric carbon at position 1), one minor form of furanose (five-member ring), and an open aldehyde form (43). Only about 0.004% of glucose exists in the open linear form (44). However, the linear aldehyde form is the essential intermediate for the interconversion among these forms (Fig. 4*a*) (45). The mechanism of the ring-opening reaction is of great interest to understand the stability of glucose and to understand the specificity of glycation of proteins by glucose (45).

Based on our rHSA·glucose and rHSA·fructose structures, we propose a molecular mechanism for the monosaccharide ring-opening reaction in Sudlow site I of HSA. The residue Lys-199 at Sudlow site I of HSA seems to be the key residue catalyzing the ring-opening reaction of glucose. The Lys-199 residue of HSA has an unusual low pK_a of 7.47 when compared with the normal lysine residues, *e.g.* the pK_a of 10.76 for Lys-195 (17). This low pK_a of the Lys-199 residue is most likely due to the buried and hydrophobic environment of the residue, and it makes Lys-199 acidic and a strong proton donor. There are many evidences showing the chemical reactivity of Lys-199 (46–49)). For example, we previously observed the hydrolysis of aspirin to acetylsalicylic acid, which is also catalyzed by the Lys-199 residue of rHSA (33).

Sudlow site I of HSA is a large binding site (Fig. 1) with its opening defined by three helices (the long h10 of domain I and the h3 and h6 of domain II). Charged residues, Lys-195, Arg-218, and Glu-292, are located at the opening of Sudlow site I, whereas the catalytic residue Lys-199 is located at the middle of site I and defines one wall of the binding site. The SAL subsite (33) is located at the bottom of site I and is hydrophobic in nature. The other two subsites (the indomethacin subsite (34) and a 3′-azido-3′-deoxythymidine subsite (22)) are near the entrance of Sudlow site I and separated by Lys-195 when viewing perpendicular to the long h10 of domain I. Our current rHSA·glucose/fructose structures suggest that the first monosaccharide diffusing into Sudlow site I passes the Lys-195 and Lys-199 residues and ends up being trapped at the SAL subsite and held between the side chains of Leu-238 and Ala-291 (Figs.

2*a* and 4*a*). This first monosaccharide is kept some distance away from residues Lys-195 and Lys-199 and maintains its cyclic conformation. The second glucose entering Sudlow site I is then near the catalytic Lys-199 residue (2.5 Å as observed in the rHSA·glucose structure). Lys-199 protonates the ring oxygen atom (O5) of the second glucose, leading to the break of C1–O5 bond of the glucose. Upon the ring opening of glucose, the C1 terminus of the glucose springs away from Lys-199 and is trapped by the residue Lys-195, forming a covalent bond through a nucleophilic addition reaction between NH_2 of Lys-195 and the C1 aldehyde (Fig. 4*a*). For fructose, no glycation of Lys-195 was observed (Figs. 3 and 4*b*). The absence of HSA glycation by fructose is likely due to two reasons: 1) the decreased reactivity of the ketone from linearized fructose and 2) the inaccessibility of the ketone from Lys-195 (a distance of 5.1 Å between the NH_2 of Lys-195 and the carbonyl of fructose).

This mechanism shows that although Lys-199 serves as a catalytic residue for the ring opening, Lys-195 provides an acceptor for glucose. Such a mechanism lends support to the previous proposal highlighting the importance of dibasic amino acids in facilitating the glycation reaction (15). Our current mechanism of protonation of the O5 atom is consistent with the ring opening of another sugar, D-xylose, by its isomerase (50). Kovalevsky *et al.* (50) showed that this reaction was driven by a basic amino acid (histidine or lysine), which provided a proton to O5 of D-xylose as a key step for the ring-opening reaction.

Physiological Consequence of Glycation of HSA at Lys-195—Sudlow site I of HSA is the largest cavity of HSA, contains several positively charged residues, and is an important binding site for drugs or other endogenous compounds. Lys-195 is located at the entrance region of this site. The glycated Lys-195 forms hydrogen bonds to another residue (Arg-218) located at the entrance of Sudlow site I, which block the access to this important drug binding site (Fig. 1). Thus, the glycation of Lys-195 by glucose may have important consequences for the structure and functions of HSA. Indeed, the glycation of albumin results in an overall stabilization in both its tertiary and its secondary structure toward chemical denaturation (42). The cationic charges of albumin decreased after glycation as shown by native electrophoresis (13). These observations can be explained by the "seal-off" of the Sudlow site I. Sudlow site I is the main binding site for aspirin based on crystallographic studies by us (33) and others (51). Day *et al.* (52) observed that the affinity of aspirin binding was reduced considerably in glycated HSA, consistent with our observation of the seal-off of Sudlow site I.

Effect of Fatty Acid on HSA Glycation—Our structural studies also suggested that Lys-195 glycation is inhibited by the presence of fatty acid. In this study, we used defatted rHSA without any fatty acid. In defatted rHSA, residue Glu-450 of rHSA domain III forms salt bridges with another residue (Arg-348) of domain III. In the presence of fatty acid in HSA, a fatty acid will occupy fatty acid site 3, which disrupts Glu-450–Arg-348 hydrogen bonding (7) and perturbs the conformation of a neighboring residue (Asp-451). The Asp-451 residue moves away from domain III and forms salt bridges with Lys-195 of domain I (Fig. 5). These salt bridge interactions between oppositely charged residues can be quite strong and can result in major conformational changes of HSA, and at the same time, can shield Lys-195 from modification by glucose (21). We reason that the uncommon observation of Lys-195 glycation is likely due, in part, to the common presence of fatty acid in most preparations of HSA.

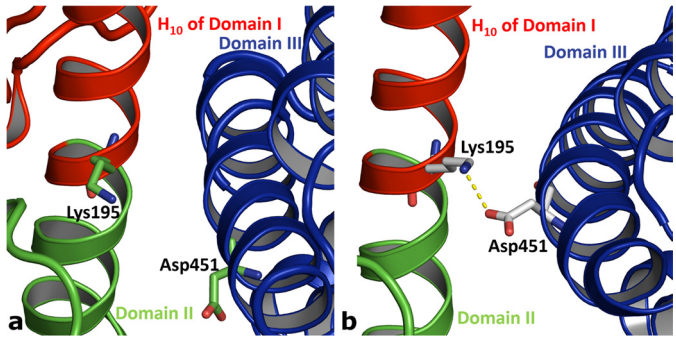

FIGURE 5. **The orientation of Lys-195 is affected by the fatty acid binding to HSA.** *a*, in ligand-free HSA, residue Lys-195 does not have direct interaction with residue Asp-451 of domain III. *b*, in the presence of fatty acid, the Asp-451 of HSA undergoes a major movement due to the binding of fatty acid at domain III and forms a hydrogen bond to residue Lys-195. This hydrogen bond may prevent residue Lys-195 from modification by glucose.

Conclusion—Glycation of proteins by sugars has been studied for a long time. We employed HSA as a model protein to study the structure of the glycation product. We determined the crystal structures of the rHSA·glucose and rHSA·fructose complexes, identified a catalytic center that converts ring sugar into linear, and showed that Lys-195 and Lys-199 play collaborative chemical roles involving glucose ring opening and covalent binding.

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