Mechanism of increased clearance of glycated albumin by proximal tubule cells

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Wagner MC, Myslinski J, Pratap S, Flores B, Rhodes G, Campos-Bilderback SB, Sandoval R, Kumar S, Patel M, Ashish, and Bruce A. Molitoris BA. Mechanism of increased clearance of glycated albumin by proximal tubule cells. Am J Physiol Renal Physiol 310: F1089–F1102, 2016. First published February 17, 2016; doi:10.1152/ajprenal.00605.2015.—Serum albumin is the most abundant plasma protein and has a long half-life due to neonatal Fc receptor (FcRn)-mediated transcytosis by many cell types, including proximal tubule cells of the kidney. Albumin also interacts with, and is modified by, many small and large molecules. Therefore, the focus of the present study was to address the impact of specific known biological albumin modifications on albumin-FcRn binding and cellular handling. Binding at pH 6.0 and 7.4 was performed since FcRn binds albumin strongly at acidic pH and releases it after transcytosis at physiological pH. Equilibrium dissociation constants were measured using microscale thermophoresis. Since studies have shown that glycated albumin is excreted in the urine at a higher rate than unmodified albumin, we studied glucose and methylglyoxal modified albumins (21 days). All had reduced affinity to FcRn at pH 6.0, suggesting these albumins would not be returned to the circulation via the transcytotic pathway. To address why modified albumin has reduced affinity, we analyzed the structure of the modified albumins using small-angle X-ray scattering. This analysis showed significant structural changes occurring to albumin with glycation, particularly in the FcRn-binding region, which could explain the reduced affinity to FcRn. These results offer an explanation for enhanced proximal tubule-mediated sorting and clearance of abnormal albumins.

albuminuria; glycation; diabetes; cell biology and structure; proximal tubule; neonatal Fc receptor

ALBUMIN is the most abundant soluble protein in the body and the major protein in plasma (39). It has been extensively studied and has many important functions, including colloid osmotic pressure, as an antioxidant, transport of endogenous and exogenous compounds, and pH buffering capacity (44, 47). It is a protein of ~66 kDa, composed of three homologous domains that interact with a variety of ligands, fatty acids, drugs, proteins, and various small molecules, such as sugars. Some of these interactions alter its physical properties and are known to increase in certain disease states. In particular, glucosylated proteins derived from glucose and dicarbonyl α-oxaldehyde methylglyoxal (MGO) incubations are closely associated with hyperglycemia, poor control of diabetes, and increased susceptibility to diabetic complications, including nephropathy (8, 45, 48). These modifications are known to result in albuminuria; however, the mechanism(s) responsible for this is still being discovered.

The normal half-life of albumin is ~21 days in human and ~3 days in rats, and it is dependent on its interaction with the neonatal Fc receptor (FcRn) (27). FcRn is a heterodimeric integral membrane protein composed of an α-chain of the nonclassical major histocompatibility complex class I family and β2-microglobulin (69). It contains distinct and noninteractive binding sites for its two known ligands (IgG and albumin), and its affinity for each is increased >100-fold in an acidic environment (10). The FcRn-IgG interaction has been studied extensively, and multiple studies have supported increased ligand binding in endosomes that prevents ligand targeting to lysosomes followed by ligand transcytosis and subsequent extracellular release (18, 21, 37, 42). More recent studies have documented the presence of FcRn in the brush border of proximal tubule cells (20), direct visualization of albumin transcytosis in proximal tubule cells intravitaly (55), and molecular evidence for an active role of FcRn in proximal tubule albumin transcytosis (56, 68). Using fluorescently tagged albumin with intravitreal microscopy in Munich-Wistar-Frontier (MWF) rat kidneys has enabled real-time visualization and analysis of glomerular albumin filtration and proximal tubule reabsorption and trafficking (14, 55). Thereafter, endothelial transcytosis, mediated by FcRn, returns albumin to the circulation.

Characterizing the biochemical interaction between albumin and FcRn is essential to fully understand how albumin modifications impact urinary loss of albumin by affecting either binding and/or trafficking. Multiple studies using several animal models have shown that glycated albumin is handled differently compared with unmodified albumin (17, 29, 30). We recently showed in a diabetic model that reduced albumin uptake occurred by proximal tubules although no change in the of fluorophore-labeled albumin was measured (50). We also confirmed that the majority of albumin in urine is present as albumin-derived fragments of 300–500 Da (50). What is unknown is whether glycated albumin binding to albumin receptors is altered and could explain this observation. Microscale thermophoresis (MST) has proven to be a powerful method to address protein-protein interactions (60) and was used to evaluate albumin-FcRn interactions. In addition, previous studies have documented secondary structure changes in albumin when modified (24, 48, 61). To extend these findings to modified albumins of physiological importance, small-angle X-ray scattering (SAXS) was conducted to determine if there was a correlation between global shape changes and the observed altered binding. Both analyses were done in solution to mimic the possible changes occurring in vivo. In addition, we
evaluated the glomerular filtration of glycated albumin and proximal tubule uptake to provide a more complete picture of its kidney dynamics.

MATERIALS AND METHODS

Two-photon microscopy. Imaging was conducted using an Olympus FV1000 microscope adapted for two-photon microscopy with high-sensitivity gallium arsenide nondescanned 12-bit detectors with animal preparations, as previously described elsewhere (54, 55). Animals were anesthetized with pentobarbital sodium (50 mg/ml). A jugular venous line was used to introduce fluorescent albumin. As previously described, animal body temperature, saline bath temperature, and hydration was maintained by saline infusion. Glomerular sieving coefficients (GSCs) were determined using our previously published method (55). Briefly, z-stack images of the glomerulus before fluorescent rat serum albumin (RSA) infusion were collected to enable background fluorescence levels of the Bowman’s space and glomerular capillaries to be quantified. These values were subtracted from the same region after the fluorescent albumin infusion. Quantification of intensity values was performed using Metamorph (Molecular Devices) or ImageJ. Graphing and statistical analyses were performed using Microsoft Excel (Redmond, CA), KaleidaGraph (Synergy Software, Reading, PA), and GraphPad Prism 5 (La Jolla, CA).

Proteins. Soluble rat FcRn (srFcRn) and soluble human FcRn (shFcRn) were purified from Chinese hamster ovary cell lines generously provided by Dr. Pamela Bjorkman (Howard Hughes Medical Institute at California Institute of Technology) (16, 75). Cells were grown in custom -MEM (Lonza) plus 5% dialyzed FBS, penicillin, streptomycin, and methionine sulfoxime as previously described. srFcRn was purified from the culture supernatant using pH-dependent binding to the rat IgG agarose column as described previously (16). shFcRn was also purified from the culture supernatant using pH-dependent binding to the human IgG agarose column. Removal of bound BSA required further purification using an anion exchange column as previously described (75). Albumins were purchased from Sigma (bovine, mouse, and rat), Equitech-Bio (porcine, rabbit, and sheep), and Albumin BioScience (human). IgGs were purchased from Equitech-Bio (bovine, human, mouse, and rat) and Rockland Immunochemicals (rabbit). DQ red BSA was purchased from Life Technologies.

Modified albumin and FcRn. Rat and human albumin were glycated using glucose or methylglyoxal as previously described (24). Briefly, albumin was incubated at 37°C for 21 days in the presence of glucose (20, 200, and 500 mM) or methylglyoxal (1 and 5 mM). Conjugation of a fluorescent tag to albumin and FcRn was done using fluorescent dyes [NT-red and NT-blue from NanoTemper Technologies; Alexa 647, Alexa 568, Alexa 488, Texas red (TR), FITC, and TR-X from Life Technologies; CF594 was a free sample from Biotium] according to the manufacturers’ protocols. The dye-to-protein molar ratio was varied to achieve the conjugation desired, and free dye was separated by desalting and/or dialysis.

MST. MST was used to characterize binding affinity between purified FcRns and albumin (26, 60). Binding assays were performed with the Monolith NT.115 MST device using standard capillaries (NanoTemper Technologies, Munich, Germany). Measurements were performed at 25°C in 67 mM NaPO₄ buffer, 150 mM NaCl, and 0.05% Tween 20 at pH 6.0 or 7.4. The infrared laser power was between 20 and 60%, and 40–70% LED power was used. A laser on time of 30 s and a laser off time of 5 s were used. Data from a minimum of three replicate binding assays were analyzed using NanoTemper analysis and GraphPad Prism software. Final data normalization and curve fitting were performed with GraphPad Prism. It is important to note that this instrument requires a fluorescent tag be placed on either the receptor or ligand. Note that the weak binding measurements are approximate and the equilibrium dissociation constant (K₆) in this case is listed as >100 µM.

SAXS data acquisition. Lyophilized samples of FcRn, RSA, and its glycated versions [RSA modified with 20, 200, and 500 mM glucose (RSA, 20G, RSA, 200G, and RSA, 500G, respectively), were dissolved in 67 mM sodium phosphate buffer, 150 mM NaCl, 1 mM NaN₃, and 0.05% Tween 20. Sodium phosphate buffer was used for pH 6–8, whereas 10 mM sodium acetate buffer was used for pH 4 and 5. The purity of the samples was ascertained by single bands near the expected migration position in 12% SDS-PAGE relative to standard markers (Thermo Scientific), and their intact masses were obtained by matrix-assisted laser desorption/ionization-time of flight (TOF) mass spectrometry (AB-Sciex TOF/TOF 72092). Microdialysis was used to exchange buffer pH as required, and the last dialysis buffer was used as the matched buffer for SAXS experiments. Binding experiments were done at a 1:1 stochiometric ratio of FcRn and RSA. Before SAXS data collection, samples were centrifuged at 60,000 g using a Beckman TLA 120.2 for 1 h to remove any aggregates. Data were collected using a SAXSspace instrument with a sealed tube source (Anton Paar, Graz, Austria). All experiments were done using line collimation, and scattered X-rays were recorded on a one-dimensional CMOS Mythen detector (Dectris, Baden, Switzerland). In each case, 60-µl samples and their matched buffer were exposed for 1 h at 20°C in the same thermostated quartz capillary with a 1-mm diameter (63). The capillary was washed with water, 1 M NaOH, and isopropanol before and after each data collection. SAXSxtract software was used to convert scattering data as intensity points at each pixel and calibrate the position of the primary beam. Data were further processed using SAXSquant software to obtain intensity (I) as a function of Q [where Q = 4πsin(θ)/λ, where θ is the angle and λ is wavelength], i.e., I(Q), for all samples and buffers. Then, contribution of buffer was subtracted. In addition, the Porod constant (C) was calculated and subtracted from sample I(Q) to obtain an intensity profile, where the scattering of the particle decays as a function of Q⁻⁴. I(Q) profiles in reciprocal space were analyzed by generalized indirect Fourier transform software to J) desmear the data from line collimation and represent a true point collimation and 2) estimate the pairwise distribution function of interatomic vectors [P(r)] profiles of interatomic scattering vectors contributing to the obtained SAXS I(Q) profiles, as previously described (63).

SAXS data analysis and modeling. Desmeared I(Q) files were analyzed using Guinier analyses assuming globular- and rod-like scattering shapes of the scattering particles, which yielded the radius of gyration (Rg) and radius of cross-section (Rx), respectively. These analyses were done using the AUTORG program in the ATSAS 2.6 suite of programs (28). Kratyky plots [I(Q) × Q² vs. Q] of each data set were prepared to examine the nature of scattering protein in the solution. Having confirmed globular scattering profiles, the AUTOGNOM program (66) was used to perform indirect Fourier transformation of the scattering data to obtain P(r). During the transformation, the probability of finding a pairwise vector equal to 0 Å and the maximum linear dimension (Dmax) was considered to be zero. To visualize the predominant solution shape of FcRn, RSA, and its glycated versions, for each data set, 10 independent uniform density models were generated using Dammin software (65, 67). These models were compared with each other using the DAMCLUST program (40). As described below, the models with the best correlation between the experimental data and theoretical SAXS profile of the model were used for comparison with crystal structures and analysis. The CRYOSOL program was used to calculate the theoretical SAXS profile of the final residue level models and compare it with experimental data (64).

Isoelectric focusing. Novex pH 3–10 isoelectric focusing (IEF) protein gels and SERVA Liquid Mix IEF Marker pl 3.5–10.7 were purchased from Life Technologies. Three to five micrograms of each protein sample was used, and gels were run according to the manu-
facturer’s protocol, fixed with 12% TCA, and stained with Pierce GelCode Blue (Fisher Scientific).

Rat clearance experiments. Male Sprague-Dawley rats (180–220 g) were purchased from Harlan and acclimated for 4 days. Blood and urine were obtained to establish baseline values. Rats were anesthetized with isofluorane, and 1.5 mg of fluorescently tagged RSA were injected intraperitoneally or by tail vein. Blood was collected postinjection at 15 min, 2 h, and 24 h. Each tagged albumin was evaluated in four rats unless stated otherwise in the figures. Blood was collected in heparinized tubes, spun, and assayed for fluorescence using a Molecular Devices SpectraMax M5 plate reader. The 15-min collection time point was set to 100%, and the decrease in fluorescence followed at 2 and 24 h. All experiments followed National Institutes of Health Guide for the Care and use of Animals guidelines and were approved by the Animal Care and Use Committee of the Indiana University School of Medicine. All rats had normal blood and urine creatinine and protein values before infusion and at the end of the study.

RESULTS

Two-photon imaging shows no GSC increase and normal proximal tubule uptake of glycated albumin. To evaluate the glomerular filtration of glycated albumin and proximal tubule uptake, healthy MWF rats were infused with 1–3 mg of fluorophore conjugated glycated albumin (either glucose or methylglyoxal modified). The GSC was $0.014 \pm 0.003$ for 500 mM glucose-modified albumin and $0.014 \pm 0.010$ for 1 mM MGO-modified albumin. Note that these GSCs are consistent with our previously published values for normal albumin in these rats: $0.010 \pm 0.001$ (55). To evaluate albumin uptake, proximal tubules were examined after infusion. Figure 1A shows the uptake (~60 min after infusion) of MGO-modified albumin in proximal tubules, whereas Fig. 1B shows both low- and high-power views of glucose-modified albumin present in proximal tubules after infusion. Each image is presented in black and white as well as pseudocolor. Note that for both modified albumins, the uptake pattern was indistinguishable from our previous reports with unmodified albumin (50, 51, 55). These results are consistent with glycated albumin being filtered and taken up by proximal tubules normally, thus implicating a proximal tubule handling alteration.

MST characterization of rat FcRn binding to albumin and IgG from multiple species. Our overall focus was to define the mechanisms mediating albumin handling in the kidney. The MWF rat model enabled us to quantify both glomerular filtration and proximal tubule uptake of albumin using two-photon intravitral microscopy. This method also requires the attachment of a fluorescent probe to either the ligand, albumin, or receptor (FcRn) being studied. Since FcRn is a key albumin receptor responsible for albumin transcytosis, and thus the albumin salvage pathway in the proximal tubule, our initial experiments quantified $K_D$ using MST. Figure 2, A and B,

![Fig. 1. Proximal tubule uptake of glycated albumin is normal when infused into healthy rats. A: representative two-photon images of rats infused with methylglyoxal-modified albumin. B: representative two-photon images of rats infused with glucose-modified albumin. Each image is presented in both black and white as well as pseudocolor and shows significant proximal tubule (PL) uptake after infusion. G, glomerulus.](http://ajprenal.physiology.org/)

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Recent evidence has identified considerable species differences for FcRn binding to albumin and IgG. Graphs are presented as the fraction bound versus log of the concentration. $n = 3$. $K_D$ values of 10 and 1 $\mu$M were calculated for FcRn versus albumin and IgG, respectively, at pH 6.0.

Multiple animal studies have used albumin from different species interchangeably, but recent evidence has identified considerable species differences for FcRn binding to albumin and IgG (2, 3, 5, 10, 11, 15, 22, 34, 36, 38, 72, 75, 76). To extend these studies and define more completely the interactions of rFcRn, we compared albumins from several different species and IgGs against srFcRn using MST analysis. Figure 3A shows the binding curves for albumins at pH 6.0. srFcRn bound rabbit albumin with comparable affinity to rat albumin at pH 6.0, whereas bovine, human, mouse, porcine, and sheep albumin had $K_D$ values of >100 $\mu$M. Figure 3B shows the binding curves for IgGs at pH 6.0. srFcRn had a slightly stronger affinity to rabbit and human IgG than rat and bovine IgG, with mouse IgG having very weak binding. Table 1 shows our binding results and summarizes those of other laboratories who examined FcRn interactions with albumin and IgG. While not complete, these studies do indicate it is best to use species-specific ligand FcRn combinations since significant differences between species can be present.

**Glycation of albumin reduces affinity to FcRn.** Glycated albumin has been shown to be preferentially excreted in the urine (17, 29, 30). To evaluate whether decreased affinity for FcRn contributed to increased glycated albuminuria, we used MST to quantify $K_D$ values between modified albumins and FcRn. Albumin was glycated with three concentrations of glucose (20, 200, and 500 mM) for 21 days. Figure 4A shows the binding curves for glucose-modified rat albumin, and Fig. 4C shows human albumin at pH 6.0. For both rat and human FcRn, $K_D$ increased with the level of glycation, indicating that glycation of albumin does reduce its interaction with FcRn in a dose-dependent fashion. Methylglyoxal, which is also increased in diabetic patients, also led to a reduction in FcRn binding to albumin. Albumin was incubated with 1 or 5 mM methylglyoxal for 21 days. The MST binding curves show that both rat (Fig. 4B) and human (Fig. 4D) methylglyoxal-modified albumin had reduced binding to FcRn at pH 6.0. Table 2 shows the $K_D$ values for these modified albumins at pH 6.0 and 7.4. These data indicate that binding of albumin to FcRn is reduced by albumin modifications that occur clinically.

**SAXS data analysis from albumins with or without FcRn.** Intact molecular masses of RSA, RSA_20G, RSA_200G, and RSA_500G were observed to be 65.87, 67.46, 67.94, and 69.57 kDa, respectively. The graphs are presented as the fraction bound versus log of the concentration. $n = 3$. $K_D$ values of 10 and 1 $\mu$M were calculated for FcRn versus albumin and IgG, respectively, at pH 6.0.

![Graph](http://ajprenal.physiology.org/)

**Fig. 2.** Microscale thermophoresis (MST) confirming a pH-sensitive interaction between the neonatal Fc receptor (FcRn) and both albumin and IgG. MST was used to characterize the interaction between FcRn and rat serum albumin (RSA; A) and between FcRn and IgG at pH 6.0 and 7.4 (B). Equal amounts of fluorescently labeled (NT-647) rat (r)FcRn were titrated with unlabeled RSA or IgG. Graphs are presented as fraction bound versus log of the concentration; $n = 3$. $K_D$ values of 10 and 1 $\mu$M were calculated for FcRn versus albumin and IgG, respectively, at pH 6.0.

**Fig. 3.** MST documenting species differences in binding between rat FcRn and albumins and IgG molecules. MST was used to evaluate rabbit, human, bovine, and mouse albumin (A) and IgG (B) binding to FcRn at pH 6.0. Equal amounts of fluorescently labeled (NT-647) FcRn were titrated with unlabeled albumin or IgG. Graphs are presented as fraction bound versus log of the concentration. Rat albumin had a $K_D$ value of 11.8 $\mu$M, whereas all other albumins had $K_D$ values of >100 $\mu$M. In contrast, rabbit IgG ($K_D$: 0.19 $\mu$M) and human IgG ($K_D$: 6.63 $\mu$M) had a slightly stronger affinity with FcRn than rat IgG, whereas bovine IgG had a $K_D$ value of 1.5 $\mu$M and mouse IgG had a $K_D$ value of >100 $\mu$M. Interestingly, bovine IgG did not show pH-sensitive binding and retained a strong $K_D$ value of 1.2 $\mu$M at pH 7.4 ($n = 3$; see Table 1).
Assessment of rFcRn binding to albumins and IgGs using MST analysis

<table>
<thead>
<tr>
<th>Ligand</th>
<th>rFcRn pH 6 K&lt;sub&gt;D&lt;/sub&gt; μM</th>
<th>Human FcRn pH 6 K&lt;sub&gt;D&lt;/sub&gt; μM</th>
<th>Mouse FcRn pH 6 K&lt;sub&gt;D&lt;/sub&gt; μM</th>
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<tbody>
<tr>
<td>Rat</td>
<td>11.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>−1.1 (34)</td>
<td>SB (34)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>11.8&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>&gt;100&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.6 (22); 5.2 (38)</td>
<td>−86 (72)</td>
</tr>
<tr>
<td>Porcine</td>
<td>&gt;100&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.70 (2)</td>
<td>0.265 (10)</td>
</tr>
<tr>
<td>Mouse</td>
<td>&gt;100&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.8 (72)</td>
<td>9.3 (72); SB (34)</td>
</tr>
<tr>
<td>Bovine</td>
<td>&gt;100&lt;sup&gt;*&lt;/sup&gt;; WB (36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>&gt;100&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>Rhesus</td>
<td>WB (34)</td>
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IgGs

- Rat IgG2a: 0.014 (75); NB (75); SB (76)
- Rabbit: 0.19<sup>*</sup>; SB (76)
- Human: 0.63<sup>*</sup>; 0.70 (2); 0.265 (10)
- Human IgG1: 0.03 (75); 0.09 (75)
- Human IgG2: 0.50 (3); 2.35 (10); 1.2 (72)
- Rhesus: 0.019 (75); NB (75); 0.25 (75)
- Mouse IgG1: >100<sup>*</sup>; WB (76); 0.01 (72)
- Mouse IgG2: >100<sup>*</sup>; 0.75 (15)
- Rat and mouse IgGs: −0.05 (5)

Data shown are from the indicated references. *Data from the present study.

Assessment of FcRn binding to glucose- and methylglyoxal-modified albumin

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pH 6 K&lt;sub&gt;D&lt;/sub&gt; μM</th>
<th>pH 7.4 K&lt;sub&gt;D&lt;/sub&gt; μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat albumins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmodified</td>
<td>11.6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>20 mM Glucose</td>
<td>78</td>
<td>&gt;100</td>
</tr>
<tr>
<td>200 mM Glucose</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>500 mM Glucose</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>1 mM Methylglyoxal</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5 mM Methylglyoxal</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Human albumins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmodified</td>
<td>7.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>20 mM Glucose</td>
<td>36</td>
<td>ND</td>
</tr>
<tr>
<td>200 mM Glucose</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>500 mM Glucose</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>1 mM Methylglyoxal</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>5 mM Methylglyoxal</td>
<td>&gt;100</td>
<td>ND</td>
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</table>

ND, not determined.

kDa, respectively. This indicated that upon chemical modification, on average, 8.8 (close integer: 9), 11.5 (close integer: 11), and 20.5 (close integer: 20) molecules of sugar got attached to RSA, respectively. The I(Q) profiles, as plotted in log I(Q) versus log Q, indicated lack of aggregation or inter-particulate nature in the samples (Fig. 5A). The linear nature of the Guinier analysis of the data sets, presuming globular scattering shape, further confirmed the monodispersity of the samples (Fig. 5A, insets). The slope of the fit provided the scattering data profile is dependent on the molecular mass and relative abundance of each scattering species; thus, even in instances of partial binding, the SAXS data will be dominated by the complex.

**Global shapes of RSAs with or without rFcRn.** Solution shapes of the albumins were computed using the constraints offered in the SAXS data profiles. Shape thus restored for unmodified rFcRn matched well with the crystal structure of monomeric FcRn (Protein Database ID 4K2C). Automated alignment of inertial axes of the SAXS data-based model and crystal structure confirmed the similarity between the two results (Fig. 6). The crystal structure helped in realizing how the volumes of the three domains of albumin packed inside the overall envelope. Interestingly, shapes restored for RSA_20G, RSA_200G, and RSA_500G suggested that the domains of the proteins appeared to open up with increase in glycation. In the

Table 2. Assessment of FcRn binding to glucose- and methylglyoxal-modified albumin

- Rat albumins against rFcRn
  - Unmodified: 11.6, >100
  - 20 mM Glucose: 78, >100
  - 200 mM Glucose: >100, >100
  - 500 mM Glucose: >100, >100
  - 1 mM Methylglyoxal: >100, >100
  - 5 mM Methylglyoxal: >100, >100

- Human albumins against human FcRn
  - Unmodified: 7.5, >100
  - 20 mM Glucose: 36, ND
  - 200 mM Glucose: >100, ND
  - 500 mM Glucose: >100, ND
  - 1 mM Methylglyoxal: 42, ND
  - 5 mM Methylglyoxal: >100, ND

ND, not determined.
absence of the crystal structure of glycated albumin, these shapes could not be compared with high-resolution structures. The global shape of the 1:1 complex of RSA-rFcRn was compared with the complex of the same proteins by aligning the inertial axes in an automated manner (Fig. 7). The overlay confirmed that the two proteins remained tightly bound in solution, as seen in the crystal structure. Interestingly, the shapes of 1:1 complexes of rFcRn with glycated RSAs showed that the complexes seemed to extend out in one direction.

**Shape of rFcRn as a function of buffer pH.** SAXS data were collected for rFcRn in buffer with varying pH to determine if any shape changes occur as a function of pH. Guinier analysis suggested $R_g$ and $R_c$ values at pH 4, 5, 6, 7, and 8 to be 3.5, 3, 3.2, 3.1, and 3.5 nm and 2.1, 1.9, 1.8, 1.7, and 2 nm, respectively (Table 3). Based on these values, the estimated persistence length of rFcRn/β₂-microglobulin was estimated to be 9.7, 8.0, 9.2, 9.0, and 9.9 nm at pH 4, 5, 6, 7, and 8, respectively. Kratky analysis of the variable pH data sets supported globular profiles of molecules in solution. Furthermore, $P(r)$ analysis suggested $D_{\text{max}}$ values for pH 4, 5, 6, 7, and 8 to be 10.8, 10.4, 10.6, 10.8 and and $R_g$ values to be 3.5, 3.1, 3.3, 3.2 and 3.6 nm, respectively. SAXS data analysis indicated that the rFcRn/β₂-microglobulin complex appears to be most compact at pH 6 and open up on increasing or decreasing buffer pH. Additionally, molecular masses of the predominant species in the mixtures were estimated to be 81.79, 80.61, 84.24, 83.26, and 88.48 kDa, respectively.

Shapes of rFcRn/β₂-microglobin in different pHs were restored from their SAXS data profiles (Fig. 4, G–I). The dummy atom, a model computed for the rFcRn/β₂-microglobin complex, was compared with the crystal structure 3FRU, which showed that the solution shape matched the complex in crystalline state and is a dimer of the heteromeric complex (Fig. 8). The shape seemed to change little as the buffer was changed from pH 6 to 4, but at pH 8, the complex appeared to open up or the sample had partial aggregation or both. Probably, these changes compromised the ability of FcRn to bind albumins at physiological or high pHs.

**RSA charge is altered by glycation or tag addition.** IEF gels were used to determine the pI of modified albumins. Figure 9 shows a Coomassie blue-stained IEF gel (3–10) of unlabeled albumin and albumin labeled with multiple different fluorescent tags conjugated at different dye to albumin ratios. A commercially available TR-labeled BSA (DQ) is also shown along with the glycated RSA that was examined in earlier binding experiments. Note that Alexa 488, Alexa 568, and FITC all make RSA more anionic, whereas DQ BSA separated into a diverse set of protein bands. The shift to a more anionic albumin also occurs with glycation. Higher concentrations of glucose and MGO showed the greatest shift. Finally, TR, CF-594, and Alexa 647 caused little if any alteration in the pI of RSA when conjugated to RSA. These data indicate that both fluorescent tag conjugation and glycation of albumin can make
the pI of albumin more anionic, which may impact albumin interactions.

Altered binding of tagged or modified RSA to FcRn correlates with an increased clearance when examined in the rat. Table 4 shows RSA fluorophore conjugates, their dye-to-RSA ratio, and their respective $K_D$ values at pH 6.0 and 7.4. The conventional conjugation ratio is 4 dyes to 1 albumin molecule; note that most dyes at a $1:1$ ratio show reduced binding of RSA to FcRn, as measured with MST. Interestingly, TR sulfonyl chloride, when conjugated at 1:1, had normal binding at pH 6.0 but increased affinity at pH 7.4, whereas TR-X succinimidyl ester at 1:1 and 2:1 behaved like unlabeled RSA with strong binding at pH 6.0 and no binding at pH 7.4. Attachment of the tag at the only reduced thiol of albumin, using maleimide conjugates, also maintained physiological binding characteristics. The modification of albumin-FcRn binding by fluorescent probe conjugation may result from the fluorophore altering key amino acid interactions.

Fig. 5. Small-angle X-ray scattering (SAXS) data analysis from the samples of RSA (A–C), RSA-rFcRn (D–F), and rFcRn (G–I). A, D, and G: SAXS intensity I(Q) profiles of data sets and linear regions of Guinier approximations (insets). B, E, and H: Kratky profiles of the data sets. C, F, and I: pairwise distribution function of interatomic vector [P(r)] curves computed from indirect Fourier transformation of measured SAXS data. Top, unglycated RSA and its glycated versions [RSA modified with 20, 200, and 500 mM glucose (RSA_20G, RSA_200G, and RSA_500G, respectively)]. Middle, rFcRn and its complexes. Bottom, plots for rFcRn as a function of pH. I(Q), Kratky, and P(r) plots are arbitrarily translated vertically for clarity.
ance for all glycated RSAs, with the most rapid clearance taking place for RSA modified with 500 mM glucose. Since measurement of the different infused albumins is followed by their respective fluorescent tag, we always normalized 2- and 24-h time points to the early time point, <15 min, which is set at 100%. This controls for fluorescent variability and amount of albumin infused in each rat. Since GSC and proximal tubule uptake were normal for glycated albumins, the rapid blood clearance is consistent with the important role of the kidney in reabsorbing filtered physiological albumin for transcytosis and recycling into the circulation, whereas modified albumins with reduced FcRn binding has increased clearance due to lack of reclamation.

### Table 3. Structural parameters of rat FcRn, RSA, and its glycated versions and complexes as deduced from Guinier analysis and indirect Fourier transformation of small-angle X-ray scattering data

<table>
<thead>
<tr>
<th>Concentration, mg/ml</th>
<th>Guinier Analyses</th>
<th>Indirect Fourier Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R_g ), nm</td>
<td>( R_c ), nm</td>
</tr>
<tr>
<td><strong>Unliganded Protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSA</td>
<td>4.3</td>
<td>2.91</td>
</tr>
<tr>
<td>RSA_20G</td>
<td>4.1</td>
<td>3.48</td>
</tr>
<tr>
<td>RSA_200G</td>
<td>4.4</td>
<td>3.64</td>
</tr>
<tr>
<td>RSA_500G</td>
<td>4.1</td>
<td>4.06</td>
</tr>
<tr>
<td><strong>Protein mixture of rFcRn:RSA (1:1)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rFcRn_RSA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rFcRn_RSA20G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rFcRn_RSA_200G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rFcRn_RSA500G</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unmodified RSA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 4</td>
<td>4.3</td>
<td>3.49</td>
</tr>
<tr>
<td>pH 5</td>
<td>4.4</td>
<td>3.0</td>
</tr>
<tr>
<td>pH 6</td>
<td>4.3</td>
<td>3.22</td>
</tr>
<tr>
<td>pH 7</td>
<td>3.8</td>
<td>3.13</td>
</tr>
<tr>
<td>pH 8</td>
<td>3.6</td>
<td>3.56</td>
</tr>
</tbody>
</table>

RSA, rat serum albumin; RSA_20G, RSA_200G, and RSA_500G are RSA modified with 20, 200, and 500 mM glucose, respectively; \( R_g \), radius of gyration; \( R_c \), radius of cross-section; \( L \), persistant length; \( D_{\text{max}} \), maximum linear dimension.

Fig. 6. SAXS density models of RSA. **Top:** SAXS data-based uniform density model of unglycated RSA, its overlay with the crystal structure of ligand-free human serum albumin (Protein Database ID 4K2C), and an orthogonal view of the overlaid SAXS envelope. The red ellipses show the domains of albumin. **Middle:** average uniform density models of glycated versions of RSA. **Bottom:** schematic representation how domain opening and movement lead to shape changes upon increasing glycation.

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stain and fluorescence in each lane. Figure 11A shows blood proteins before and after infusion [early (<15 min), 2 h, and 24 h]. The Commissie blue stain in the control is representative of all Commissie blue-stained gels. The fluorescence shows a protein band with the expected molecular weight of albumin. The clearance is consistent with the fluorescent quantitation shown in Fig. 10, B and C. To determine whether urine albumin varies when albumins with different FcRn affinities are infused into rats, four rats were infused with control albumin (K_d for FcRn: 2.6 µM) and four rats were infused with modified albumin (K_d for FcRn: >100 µM; Fig. 11B). Little intact fluorescent albumin was detected in the urine from rats injected with control albumin. However, when nonbinding or modified albumins were injected, a clear fluorescent albumin band was observed in the urine. This is consistent with a previous report (50) showing that the majority of albumin in urine is degraded.
observed pH-sensitive binding to both albumin and IgG with the affinity has not been determined. Consequently, it is important for in vivo studies to understand how albumin probes interact with the endogenous receptor. At a minimum, one must be cautious of interpreting results when reabsorption requirements of glomerular filtration and proximal tubule processing of albumin was altered. Both cubulin/megalin receptor-mediated uptake and fluid phase uptake pathways could be participating in glycated albumin uptake, and any impact of glycated albumin on these pathways requires further investigation. However, to address whether the interaction of glycated albumin with FcRn is altered, both human and rat albumins were evaluated. Both glucose and MGO-modified albumins had significant decreased affinity for FcRn (Fig. 4, A–D). This would prevent these albumins from being transcytosed. Abnormal protein delivery to the proximal tubule has been shown to activate multiple phenotypic changes, including complement and inflammasome activation and autophagy.

**DISCUSSION**

The clinical importance of albumin and the role of the kidney in its long plasma half-life is well understood (14). Thus, understanding the reabsorption requirements of glomerular filtered albumin and how it is processed for either degradation or transcytosis by proximal tubule cells is an area of active investigation (7, 14, 35, 70). The present study focused on defining, in more detail, the interaction of rat FcRn with rat albumin. This is important since the kidney disease model best suited for intravital microscopy is the MWF rat model, which enables visualization of both glomerular filtration and proximal tubule uptake of filtered molecules.

**FcRn-albumin.** Since previous studies have documented affinity differences between FcRn and albumins from multiple species, our first step involved examining rat FcRn binding to albumins of different species. To establish that MST is a reproducible and valid method to look at this interaction, binding of rat FcRn to rat albumin and rat IgG was determined. Figure 2, A and B, show that, using this method, FcRn does exhibit pH-sensitive binding to both albumin and IgG with the observed $K_D$ values in agreement with previous studies. To address species differences, we determined $K_D$ values for seven different albumins and four IgGs (Fig. 3, A and B, and Table 1). Only rat and rabbit albumin displayed pH-sensitive binding with other albumins, showing weak binding at both pHs. Similarly, rabbit IgG was the only IgG that showed an affinity similar to rat IgG. Interestingly, bovine IgG had a high affinity at both pHs, whereas mouse IgG showed weak binding at both. Differences in the pH affinity if albumin are likely to alter its normal intracellular trafficking. Proteins that have increased affinity at both pHs would be expected to have shorter half-lives since the normal pH-sensitive steps would be inhibited and reclamation via transcytosis reduced. Consequently, it is important for in vivo studies to understand how albumin probes interact with the endogenous receptor. At a minimum, one must be cautious of interpreting results when the affinity has not been determined.

### Table 4. Assessment of fluorescent dye albumin conjugates on FcRn binding using MST analysis

<table>
<thead>
<tr>
<th>Fluorophore Conjugate (Ratio of Dye to Albumin)</th>
<th>pH 6 $K_D$, $\mu$M</th>
<th>pH 7.4 $K_D$, $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-594 maleimide* 1:3</td>
<td>1.5</td>
<td>NB</td>
</tr>
<tr>
<td>Texas red-X* 1:1</td>
<td>5.4</td>
<td>NB</td>
</tr>
<tr>
<td>Texas red* 4:1</td>
<td>$&gt;40$</td>
<td>NB</td>
</tr>
<tr>
<td>Alexa 488* 4.5:1</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>Fluorescein 6:4:1</td>
<td>69</td>
<td>1.0</td>
</tr>
<tr>
<td>Alexa 568 4:1</td>
<td>17</td>
<td>NB</td>
</tr>
<tr>
<td>Texas red-X 2:1</td>
<td>10</td>
<td>NB</td>
</tr>
<tr>
<td>Texas red-X 5:1</td>
<td>$&gt;100$</td>
<td>NB</td>
</tr>
<tr>
<td>Texas red 1:1</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Alexa 647 maleimide 1:1</td>
<td>11</td>
<td>NB</td>
</tr>
<tr>
<td>DQ BSA</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>No dye</td>
<td>11.6</td>
<td>$&gt;100$</td>
</tr>
</tbody>
</table>

*Data from present study.
taphagy (33, 77). This cascade of events initiated by increased delivery to and mishandling of proteins by the proximal tubule is known to result in kidney injury.

One possible explanation for the altered binding of modified albumin to FcRn was that glycation altered the normal structure of albumin (6, 45). Previous examination of glycated albumin using fluorescence (61), Fourier transformed infrared (12), circular dichroism and microviscometer (57), and assorted spectroscopic methods (24) all confirmed that glycated albumin had significantly decreased $\alpha$-helical and increased $\beta$-sheet structure compared with nonmodified albumin. To extend these findings as well as to examine the interaction of FcRn with albumin, SAXS analysis was conducted (59). Increasing concentrations of glucose increased the $R_g$ and $D_{max}$ values of albumin (Fig. 5 and 6 and Table 3). FcRn (Figs. 7 and 8 and Table 3) did not show significant structural changes between pH 6 and 7; however, changes were noticed at more basic and acidic pHs. When FcRn and the glycated albumins were incubated together to carry out binding experiments and SAXS was performed, both Guinier and Autognom analyses showed significant changes in shape, which increased with increasing glycation (Fig. 7 and Table 3). However, the exact organization of glycated albumins was unclear since the crystal structure is unavailable for glycated albumin and the data were fit using unglycated albumin. Our SAXS data-based models provide visual insights that with the increase in glycation, the domains of RSA progressively open away from each other.

Previous studies using amino acid analysis, mass spectrometry methods, and molecular modeling found preferred lysine residues for glycation (23, 32, 48). Most preferred sites are in

Fig. 10. Fluorophore conjugation of albumin can alter FcRn binding and the rate of vascular clearance. Rat albumin was conjugated to multiple fluorophores at different ratios, and binding to FcRn was evaluated with MST at pH 6.0 (A). An increased conjugation ratio of dye to protein resulted in decreased binding. Table 4 shows all dye-albumin conjugates examined and their affinity at pH 6.0 and 7.4. To address whether decreased binding to FcRn correlated with vascular clearance increase, we infused the four conjugates shown into rats and collected blood over a 24-h time period ($n = 3$). The 15-min collection time point was set to 100%, and the decrease in fluorescence was followed at 2 and 24 h. $B$: percent tagged albumin in blood at 2 and 24 h. Note that clearance was faster for albumins with decreased binding to FcRn. To address the clearance of glycated albumin, rats were infused with both methylglyoxal- and glucose-modified RSA. Clearance was again increased, most dramatically for the 500 mM glucose-modified albumin (C).

Fig. 11. Presence of control and modified albumin in blood and urine after infusion. Rat albumin was infused into rats, and blood and urine samples were collected and analyzed by SDS-PAGE. Gels were imaged for fluorescence and stained for total protein using Coomassie blue stain. $A$: blood samples quantitated in Fig. 10 showing the more rapid vascular clearance of the modified albumins. $B$: 24-h urine from 8 rats was analyzed. Rats 1–4 received modified albumin and rats 5–8 received control albumin. Note the increased presence of intact albumin in rats that received albumin with decreased FcRn binding.
albumin DII and DIII, with 33% of overall glycation occurring at Lys \(^{573}\) in albumin DIII. In addition, Lys \(^{573}\), which is unique to humans, is essential to interact with \(\beta_2\)-microglobulin by forming interfacial salt bridges with Glu \(^{41}\) (41). Previous studies have suggested that the attached light chain is essential for interactions with albumin, and glycation at Lys \(^{573}\) may reduce its interactions with the light chain of the neonatal receptor, which, in turn, reduces its affinity with serum albumin. Multiple sequence alignment showed the importance of conserved lysine residues in serum albums of different species in the context of glycation, which also provide the reason for the higher binding affinity of the human serum albumin K573P mutant. These results, along with our observations, suggest that the openings we observed in our models are due to glycation in albumin DII and DIII. However, a recent report (52) has also showed that both albumin DI and DIII are required for optimal pH-dependent binding to FcRn. Given these restrictions, it is still very clear that the glycation of albumin does alter its structure. This, combined with our binding data, supports that glycation through steric hindrance and/or modifications of key amino acids alter the normal FcRn-albumin binding site. Additional work is underway to determine how these modifications impact the interaction of albumin with cubilin, which may, in conjunction with megalin, be responsible for the significant albumin uptake in proximal tubule cells (1, 14). The pharmacokinetics of albumin and the impact of glycosylation have been previously reported, but this is the first report to address the mechanism (6). Given the multitude of modifications and associations that albumin undergoes, many correlate with disease processes, and deciphering how these “different” albumins bind to their receptors becomes critical for defining and eventually manipulating the uptake, transport, and recycling of albumin and its cargo.

**Two-photon imaging and modified albumin.** The advent of two-photon intravitreal microscopy has enabled multiple novel studies that have revealed and clarified existing and new cell dynamics in many tissues and organisms. We have used this technology to investigate kidney dynamics and, in particular, the glomerular filtration and proximal tubule reabsorption of albumin. One requirement of these studies is that tracer amounts of albumin are tagged with a fluorescent probe enabling real-time visualization of its movement. While this approach is commonplace in fluorescent studies, assessment of the impact of the tag on the molecule’s normal interactions is often not addressed. Therefore, we evaluated multiple fluorescent tags, at different conjugation ratios, and determined their effect on FcRn binding. Since charge has been shown to be a potential modifier of albumin’s interactions, we first examined some of the conjugates using IEF. Figure 9 (25, 62) shows that glycated and some fluorescent tagged rat albumins became slightly more anionic. TR, CF-594, and Alexa 647 fluorophores did not alter the pH of albumin, whereas DQ albumin was composed of multiple charged species. The placement and type of conjugation are clearly important, as seen with TR albumin, since the TR-X (contains spacer), even at a 2:1 conjugation, retained normal pH-dependent interactions with FcRn, whereas TR lacking the spacer showed abnormal pH-dependent binding even at a 1:1 conjugation (Fig. 10A and Table 4). Since there were clear differences in FcRn affinity, we predicted their serum clearance would correlate with binding differences. We found that those with normal pH-depen-

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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


