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Glycation Alters Ligand Binding, Enzymatic, and Pharmacological Properties of Human Albumin

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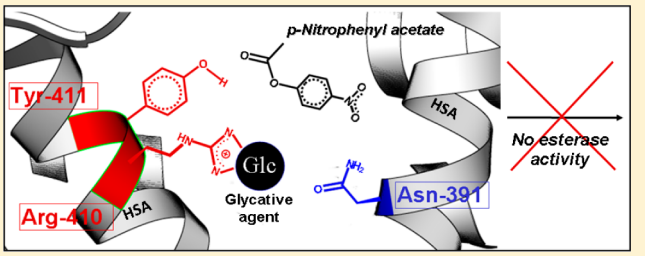
ABSTRACT: Albumin, the major circulating protein in blood plasma, can be subjected to an increased level of glycation in a diabetic context. Albumin exerts crucial pharmacological activities through its drug binding capacity, i.e., ketoprofen, and via its esterase-like activity, allowing the conversion of prodrugs into active drugs. In this study, the impact of the glucose-mediated glycation on the pharmacological and biochemical properties of human albumin was investigated. Aggregation product levels and the redox state were quantified to assess the impact of glycation-mediated changes on the structural properties of albumin. Glucose-mediated changes in ketoprofen binding properties and esterase-like activity were evaluated using fluorescence spectroscopy and *p*-nitrophenyl acetate hydrolysis assays, respectively. With the exception of oxidative parameters, significant dose-dependent alterations in biochemical and functional properties of *in vitro* glycated albumin were observed. We also found that the dose-dependent increase in levels of glycation and protein aggregation and average molecular mass changes correlated with a gradual decrease in the affinity of albumin for ketoprofen and its esterase-like property. In parallel, significant alterations in both pharmacological properties were also evidenced in albumin purified from diabetic patients. Partial least-squares regression analyses established a significant correlation between glycation-mediated changes in biochemical and pharmacological properties of albumin, highlighting the important role for glycation in the variability of the drug response in a diabetic situation.



Diabetes mellitus is now described as a pandemia affecting more than 300 million people worldwide. This disease is characterized by high blood glucose levels that result from defects in the body's ability to produce and/or use insulin. This chronic hyperglycemia can affect numerous proteins through a nonenzymatic process known as glycation or glycoxidation.[1] This condensation reaction between the aldehyde function of carbohydrates and amino groups of circulating proteins results in the formation of Schiff bases and subsequent Amadori products that can give rise to advanced glycation end products (AGEs) in the case of uncontrolled hyperglycemia.[2] Numerous studies showed that nonenzymatic glycation and AGE formation were associated with diabetic complications, such as retinopathy, nephropathy, neuropathy, and coronary artery disease.[3,4]

Albumin, the most abundant protein in blood plasma, is the most common protein affected by these glycoxidative alterations.[5,6] The high proportion of lysine and arginine residues of the albumin structure explains the potential target of this protein for glycation.[7]

Albumin exerts several physiological and pharmacological functions, including antioxidant properties,[8] oncotic pressure regulation, pseudoenzymatic activities, and particularly binding and transport capacities for numerous endogenous and exogenous compounds, like drugs.[9] Albumin can bind a remarkably wide range of therapeutic drugs. The albumin-bound form of the drug provides temporary storage as well as controlled release to the target receptor to prevent its rapid metabolism or toxicity.[10] Thus, this plasma protein can act as a circulating depot for many drugs.[11] Albumin affinity capacity plays a major role in absorption, distribution, metabolism, and excretion for drugs (ADME).[12] This key role is particularly important for drugs with a narrow therapeutic index, such as warfarin, which could be toxic in its free form.

The two major binding sites for drugs are called Sudlow sites I and II.[13] Sudlow site I is large and flexible and preferentially binds bulky heterocyclic compounds such as warfarin. By

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Biochemistry XXXX, XXX, XXX−XXX

contrast, site II, also named the indole-benzodiazepine site,[14] is small and less flexible and binds to aromatic carboxylic acid-containing drugs, such as ketoprofen, consisting of a hydrophobic center with a negatively charged carboxy group at one end of the molecule. The most prominent enzymatic, esterase-like, active sites of human serum albumin were shown to be closely related to its drug binding sites, because various drugs inhibited this activity.[15] Albumin exerts several types of hydrolytic activity, among which the esterase-like property is the most prominent. This activity was first discovered in 1951 and confirmed in 1991 by Honma et al.[16] The relation between the esterase activity of human serum albumin (HSA) and the mechanism of conversion of aspirin has been extensively studied for decades. Recently, it was shown that this activity is useful for converting prodrugs into active drugs.[17]

These two functions (drug binding capacity and esterase-like activity) play an important role in the pharmacological properties of drugs, especially because the interaction with HSA can be stereospecific.[18] Structural alterations caused by glycoxidation in a diabetic situation could alter these two properties, and recent studies from our group reported that *in vitro* and *in vivo* glycation of albumin indeed induced biochemical and structural modification affecting albumin antioxidant properties[19] and binding capacities.[20,21] However, the direct contribution of the glycation process to drug pharmacological properties of albumin remains to be elucidated. To clarify the relationship between structural and functional alterations caused by glycoxidation, we investigated the esterase-like activity and affinity capacity for ketoprofen of albumin in a diabetic context using *in vitro* and *in vivo* models of glycation. The *in vitro* models use glycated HSA resulting from the incubation of commercially available human serum albumin with increasing concentrations of glucose, and *in vivo* models consist of plasma-purified HSA from diabetic patients with different degrees of severity of the disease. In this paper, we use both models to establish the relationship between the degree of albumin glycation and its biochemical and functional properties by attempting a partial least regression method (PLS) across different *in vitro* models of glycated albumin.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Human serum albumin (96–99%), bicinchoninic acid, ketoprofen, D-(+)-glucose, Congo Red, *m*-aminophenylboronic acid-agarose, and *p*-nitrophenyl acetate were all purchased from Sigma-Aldrich (St. Louis, MO).

***In Vitro* Glycation of HSA.** Commercial human serum albumin was dissolved in phosphate-buffered saline (PBS) (pH 7.4) to yield a 0.6 mM stock solution. Glycated human serum albumin was prepared as previously described[22] by incubating filtered solutions of HSA prepared without and with glucose (5, 25, 50, 100, 200, and 500 mM) in PBS (pH 7.4) under sterile conditions and nitrogen gas in capped vials at 37 °C for 3 weeks. After being incubated, protein samples were dialyzed against PBS, sterile-filtered through a 0.2 μm Millipore filter, and stored at −80 °C. The final concentration of glycated proteins was determined by using the bicinchoninic acid assay (BCA).

Preparation of Glycated Human Serum Albumin from Diabetic Patients. Blood samples from diabetic patients (% $H_bA1c = 8.7 \pm 0.7$; $N = 15$) with different degrees of type 2 diabetes and nondiabetic subjects (% $H_bA1c = 5.5 \pm 0.3$; $N = 5$) were obtained by the Biochemistry laboratory of the Centre Hospitalier Universitaire (CHU, Saint-Denis, La Réunion). The procedures and the collection of human materials were approved by the local governmental French Ethical Committee and conformed to the standards set by the Declaration of Helsinki. All patients underwent an interview before the blood was taken to collect medical information. Albumin was purified from fresh human plasma by performing a dialysis against Tris-HCl followed by affinity chromatography using Cibacron Blue 3G linked to agarose (Amersham catalog no. 17-0948-01) as a ligand for albumin. A 1.5 M NaCl (pH 7.4) buffer was used for the desorption of bound albumin from Cibacron Blue-agarose following the elution of other plasma proteins with 50 mM Tris-HCl buffer (pH 7.4). Each fraction of eluate was examined by gel electrophoresis, and the most purified and concentrated extracts were pooled before the remaining immunoglobulins were removed with sepharose-protein A, followed by extensive dialysis against PBS and storage at −80 °C.

Biochemical Characterizations. Fructosamine and ketoamine derivatives were determined using the method developed by Johnson et al.[23] with the nitroblue tetrazolium (NBT) reagent.

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay is a sensitive method for identifying the primary free amino groups in proteins.[24] This method was described in detail in a previous study by our group.[25]

The thiol groups in modified albumin were measured by Ellman's assay using 5,5-dithiobis(2-nitrobenzoic acid) (DTNB),[26] which was described well in a previous study.[27]

Levels of carbonylation of proteins were determined by a spectrophotometric assay based on recognition of protein-bound DNPH in carbonylated proteins by using an anti-DNP antibody.[28] This method was described in detail in a previous study.[29] Carbonyl was expressed as moles of carbonyl per mole of protein and determined by the following formula:

$$\text{carbonyl (mol)/protein (mol)} = \frac{OD_{370} \times \varepsilon_{M276protein}}{(OD_{276} - 0.43 \times OD_{370}) \times \varepsilon_{M370hydrazone}}$$

In previous formulas, $\varepsilon_{M276protein}$ equals 46824, the molar absorptivity of HSA, and $\varepsilon_{M370hydrazone}$ equals 22000, the molar absorptivity of the hydrazone.[30]

The Congo Red probe is extensively used in the field of amyloid fibril analysis. For this study, an *in vitro* glycated albumin sample (2.5 μM) was incubated with a 100 μM Congo Red solution in PBS with 10% (v/v) ethanol. The absorbance at 530 nm was recorded, and results were expressed as a percentage of amyloid formation with regard to HSA_{G0}.

The global charge, size, and potentially shape modifications in glycated albumin samples were analyzed by native polyacrylamide gradient gels (from 5 to 15% acrylamide) and stained by Coomassie blue according to Laemmli's method.[31]

Discriminating analysis of glycated and nonglycated albumin was performed using MPBA polyacrylamide electrophoresis.[32] Methacrylamido phenylboronic acid (MPBA) was synthesized in the Department of Biology and Biochemistry of the University of Bath. MPBA-resolving acrylamide gels were prepared by adding 1% (w/v) MPBA to an 8% acrylamide solution. The 4% stacking acrylamide gel was prepared without boronic acid. Albumin samples (20 μg) were applied to the gel in denaturing sodium dodecyl sulfate (SDS) and reductive dithiothreitol (DTT) buffers.

Boronate Affinity Chromatography. The formation of Amadori products was also assessed by the percentage

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Biochemistry XXXX, XXX, XXX–XXX

retention of modified albumin on boronate affinity columns. This chromatography technique is based on the specific interaction between glycated protein and boronate anion immobilized within an agarose gel.[33] Here, the technique of batch separation was used. One milliliter of separating gel (*m*-aminophenylboronic acid-agarose) was transferred into 2 mL tubes equilibrated with 5 bed volumes of binding buffer [0.2 M ammonium acetate (pH 8.8)]. One milliliter of an albumin solution sample (diluted in PBS at 1 mg/mL) was applied to 1 mL of separating gel. After a first centrifugation (2000*g* for 2 min), the supernatant fraction was collected. The gel was washed five times with binding buffer, under the same conditions. When all nonmodified HSA, which was not retained on the gel, was entirely collected, "boronate-bound" albumin, corresponding to glycated HSA, was eluted using 5−6 bed volumes of elution buffer [0.15 M NaCl, 10 mM $MgCl_2$, and 0.2 M D-mannitol (pH 3.4)]. The absorbance at 278 nm was measured in all the fractions to monitor the presence of proteins and to determine the percentage of glycated albumin for each sample. The gel was regenerated successively with 3 bed volumes of 0.02 M NaOH, 0.05 M acetic acid, and binding buffer.

Mass Spectroscopy Analysis. Analysis of glycated albumin samples by mass spectrometry (MS) was performed using SELDI-TOF (surface-enhanced laser desorption ionization time of flight) MS technology (Bio-Rad). Ten micrograms of albumin samples was added to 100 μL of 100 mmol/L Tris-HCl (pH 8) (binding buffer) for incubation with Q10 ProteinChip arrays (Bio-Rad), an anionic exchanger surface. After incubation for 120 min while being gently shaken, the ProteinChip array was washed with binding buffer. Finally, arrays were washed with water and allowed to air-dry before the addition of the matrix, consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid matrix (Bio-Rad) in 100 μL of acetonitrile (100%) and 100 μL of trifluoroacetic acid (1%). The *m*/*z* values of proteins retained on the Q10 surface were determined from time-of-flight measurements using a ProteinChip Reader (PCS 4000, Bio-Rad). Data were collected by averaging 500 laser shots for each sample. The peak intensities were normalized by using the total ion current of all spectra.

Fluorescence AGE Determination. The fluorescence emission intensity of the glycated product was obtained with 335 nm (pentosidine)[34] and 380 nm (crossline + vesperlysine)[35] excitation wavelengths using a Horiba FluoroMax-4 spectrophotometer. The excitation and emission slits were equal to 5 and 10 nm, respectively. All protein samples were prepared at 1.5 mg/mL in 50 mM sodium phosphate buffer (pH 7.4). The relative percent of AGE formation (pentosidine and crossline + vesperlysine) was calculated using the following formula:

$$\mathrm{AGE\%} = 100\left(\frac{I_{\mathrm{maxG}} - I_{\mathrm{maxG0}}}{I_{\mathrm{maxG0}}}\right)$$

where AGE% represents the relative percent of AGE, I_{maxG} is maximal fluorescence intensity of glycated HSA, and I_{maxG0} is the maximal fluorescence intensity of nonglycated HSA_{G0}.

Albumin Cobalt Binding. The albumin cobalt binding (ACB) test reported by Bar-Or et al. was originally designed to detect ischemia-modified albumin (IMA) in patients with ischemia.[36,37] This assay based on the reduced binding affinity of human serum albumin for metal ions (cobalt, Co^{2+}) was used here for glycated albumin samples. Preparations for the Co(II) albumin binding protocol consist of the addition of 20 μL samples (0.15 mM) to 15 μL of a 0.2% cobalt chloride solution, followed by vigorous mixing and incubation at 37 °C for 15 min. Dithiothreitol (20 μL of a 1.5 g/L solution) was then added and mixed. After incubation for 2 min, 20 μL of a 0.9 M NaCl solution was added. The absorbance was read at 470 nm using a microplate reader. The blank was prepared similarly without DTT.

Affinity of Albumin for Ketoprofen. This method is based on the quenching of albumin fluorescence induced by its interaction with drugs.[10] The intrinsic fluorescence of human albumin is mainly attributed to the tryptophan residue (Trp-214). Different series of assay solutions were prepared by mixing 20 nmol of different HSA preparations with ketoprofen in variable amounts ranging from 0 to 160 nmol. Each solution was heated for 30 min at 37 °C and transferred into a quartz cell. The fluorescence spectra were recorded in the range of 250−500 nm under excitation at 270 nm. The excitation wavelength at 270 nm was chosen to prevent contributions of tyrosine to the tryptophan emission. The binding parameters (i.e., binding constant K_A and binding site number *n*) for ketoprofen were obtained from the equation given below:

$$\log\frac{F_0 - F_C}{F_C} = \log K_A + n\log[C]$$

where F_0 and F_C are the tryptophan fluorescence intensities in the absence and presence of a drug at concentration [C], respectively, and K_A is the constant for formation of the complex formed between the drug and albumin, expressed as liters per mole.

Esterase-like Activity. The reaction of *p*-nitrophenyl acetate with HSA was followed spectrophotometrically at 400 nm with an Infinite M200 pro spectrofluorometric analyzer (TECAN) by monitoring the absorbance of *p*-nitrophenol. The reaction mixtures contained 5 μM *p*-nitrophenyl acetate and 20 μM HSA in 67 mM sodium phosphate buffer (pH 7.4). Reactions were followed at 25 °C. Under these conditions, the pseudo-first-order rate constant analysis could be applied, as described in previous reports,[38,39] and the apparent hydrolysis rate constant (k_{obs}) was calculated.[18]

Partial Least-Squares Regression Approach. Partial least-squares regressions (PLS) were performed to establish the correlation between structural and functional data across different commercial glycated HSA samples. The principles of partial least-squares regression (PLS) determinations were described in detail in several papers by our group.[40,41] This method allows "principal component or PLS-factor" variable calculations from structural and biochemical data and functional data. The regression model equation obtained from the PLS algorithm after calculating these "PLS-factors" gives the regression coefficients that express the link between the variation in predictive parameters (structural and biochemical parameters) and the variation in response parameters (functional parameters). The predicted values are calculated on the basis of these regression coefficients by using the following equation:

$$\hat{y}_i = \sum_{i=1}^{m} C_i \times x_i$$

In this formula, *m* represents the number of predictor parameters (here 9), $\hat{y}_i$ is the predicted value of the response

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Biochemistry XXXX, XXX, XXX−XXX

parameter (K_A or k_{obs}), x_i is the measured value of predictor parameter i, and C_i is the associated regression coefficient.

All samples were used to calibrate the PLS model, and the performance of this model was assessed by the coefficient of correlation (r^2) and the root-mean-square error of calibration (rmsec) between predicted and measured values of esterase activity (k_{obs}) and binding affinity (log K_A) on a data set.

$$\text{rmsec} = \sqrt{\frac{\sum_{i=1}^{n} (\hat{y}_i - y_i)2}{n}}$$

where n is the number of data, y_i is the measured value of data point i, and $\hat{y}_i$ is the predicted value based on the model of calibration. The Unscrumbler (Camo ASA) was used to perform regression analysis.

Statistical Analysis. The data are expressed as the means ± the standard deviation (SD) from a minimum of three experiments. Statistical significance values were determined using one-way analysis of variance (followed by the Student's t test) for multiple comparisons; a p value of <0.05 was required for significance. Univariate correlation coefficients were calculated according to Pearson's method.

RESULTS

HSA was *in vitro* glycated by being incubated with increasing glucose concentrations followed by a range of biochemical characterizations. Functional assessments were performed to determine the impact of glucose-induced glycation on albumin properties. The biochemical and structural parameters relating to the *in vitro* glycated HSA models are summarized in Table 1. Glycated HSA is termed HSA_{Gx}, where x is the concentration of glucose incubated with albumin (5, 25, 50, 100, 200, or 500 mM).

Structural Characterization of *in Vitro* Glycated HSA Samples. Biochemical characterization of albumin samples revealed that glycation with increasing concentrations of glucose caused a dose-dependent elevation in ketoamine levels [up to 4-fold higher for HSA_{G500} compared to HSA_{G0} ($p < 0.001$)] and in glycated albumin proportion (+66.7% for HSA_{G500} vs that for HSA_{G0}). This increase in early glycation products is accompanied by the formation of fluorescent AGE, in a dose-dependent manner for pentosidine, vesperlysine, and crossline products, as well. Indeed, glycation in the presence of 500 mM glucose induced the more intense formation of pentosidine [34.5% ($p < 0.001$)] and vesperlysine [35.9% ($p < 0.01$)]. Two direct consequences are observed with the glycation process with growing concentrations of glucose: the increase in average molecular mass accompanied by aggregate formation.

As reported in previous studies, glycation of albumin was shown to generate thermodynamically more stable high-molecular mass aggregates with high β-sheet structure content leading to formation of amyloid-type structures.[42−44] The measurement of Congo red absorbance performed to detect β-fibrillar structure in albumin samples featured a significant increase only for highly glycated models HSA_{G200} [+15.4% ($p < 0.05$)] and HSA_{G500} [+22.1% ($p < 0.01$)], attesting to the presence of such aggregate structures. These results are in accordance with those of the thioflavin T assay showing enhanced fluorescence upon binding of the dye to amyloid fibrillar structures in highly glycated albumins (data not shown). In parallel, the significant increase in average molecular mass was noticed for most glycated albumin samples. The

Table 1. Structural and Biochemical Parameters in Different HSA Samples[a]

method	glycation level (%)	ketoamine/HSA (mol/mol)	free amino groups/HSA (mol/mol)	thiols/HSA (mol/mol)	carbonyl/HSA (mol/mol)	ACB index	fluorescent AGE (% HSA_{G0})		average molecular mass (kDa)	β-amyloid formation (% HSA_{G0})
							vesperlysine	pentosidine		
HSA_{G0}	0	4.77 ± 1.96	26.43 ± 1.15	0.122 ± 0.015	4.29 ± 0.83	0.404 ± 0.061	0	0	66.437 ± 0.036	0.0 ± 8.82
HSA_{G5}	7.49	4.61 ± 1.84	24.94 ± 1.86	0.119 ± 0.020	4.26 ± 0.71	0.407 ± 0.051	0.71 ± 0.95	6.06 ± 1.53	66.360 ± 0.096	2.58 ± 0.94
HSA_{G25}	15.79	5.60 ± 1.95	25.37 ± 1.97	0.126 ± 0.014	4.16 ± 0.57	0.441 ± 0.054	5.37 ± 1.72	11.93 ± 7.65	66.562 ± 0.068[d]	0.45 ± 1.25
HSA_{G50}	22.56	6.01 ± 1.64	23.43 ± 1.30[d]	0.162 ± 0.009	4.04 ± 0.03	0.460 ± 0.053	14.21 ± 5.89[d]	21.38 ± 2.49	66.631 ± 0.045[d]	10.58 ± 5.05
HSA_{G100}	46.5	8.20 ± 1.56[d]	21.40 ± 2.57[d]	0.102 ± 0.003	4.44 ± 0.87	0.467 ± 0.040	10.57 ± 3.36	15.27 ± 9.69	66.862 ± 0.069[c]	12.04 ± 4.03
HSA_{G200}	56.43	12.13 ± 1.82[c]	21.30 ± 0.63[c]	0.097 ± 0.004	4.32 ± 0.63	0.478 ± 0.041	25.78 ± 8.96[b]	27.38 ± 13.65[d]	67.242 ± 0.043[b]	15.38 ± 1.70[d]
HSA_{G500}	66.73	17.90 ± 3.84[b]	13.30 ± 0.77[b]	0.087 ± 0.003	4.97 ± 0.68	0.555 ± 0.052[b]	34.48 ± 5.11[b]	35.91 ± 10.96[c]	68.060 ± 0.013[b]	22.09 ± 3.46[b]

[a]Glycation level determined by boronate affinity chromatography (100% corresponding to complete glycation of HSA). Ketoamine level obtained with the NBT assay. Unmodified primary amino group content in proteins obtained by the TNBS assay. Free thiol group content as assessed by Ellman's method. Carbonyl level as assessed by a spectrophotometric carbonyl assay. Albumin cobalt binding (ACB) index. Percent increase in fluorescent AGE level obtained by the maximal fluorescence emission at an excitation wavelength of 335 nm (pentosidine) or 380 nm (vesperlysine). Average molecular mass obtained by ESI/MS. Percent increase in the level of β-amyloid aggregate formation probed with Congo Red reagent. All data expressed as means ± the standard deviation of three independent experiments. [b]Effect of glycation in native HSA (vs HSA_{G0}). $p < 0.001$ [c]Effect of glycation in native HSA (vs HSA_{G0}). $p < 0.01$. [d]Effect of glycation in native HSA (vs HSA_{G0}). $p < 0.05$.

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Biochemistry XXXX, XXX, XXX−XXX

molecular mass in HSA_{G25} was approximately increased by 127 Da, while glycation of HSA with 500 mM glucose led to an increase of approximately 1621 Da, corresponding to a condensation of ∼10 glucose units per molecule of albumin (one glucose unit is equivalent to a mass increase of 160 Da). As expected, the progressive glycation correlated with glucose concentration also showed a dose-dependent decrease in the number of free amino groups that react with the TNBS reagent. If native albumin (HSA_{G0}) displayed ∼26.4 free amine groups, this level dropped up to 13.3 for the most glycated HSA (HSA_{G500}), reflecting the direct involvement of lysine and arginine residues in the glycation reaction. As a direct consequence of the modification of these positively charged residues with glucose, a significant change in the isoelectric point of albumin can be observed with native polyacrylamide gel electrophoresis (PAGE) (Figure 1A), resulting in an enhanced electrophoretic migration toward the anode as a function of glucose concentration. Similarly, MPBA gel electrophoresis of *in vitro* glycated HSA (Figure 1B) shows a clear increase in the level of retention and intensity of the glycated HSA band (1) as a function of increasing glucose concentration as well as a reduction of native HSA levels (2). Incubation with 500 mM glucose (G500) results in an almost complete glycation of albumin with barely any unglycated protein that can be detected in the MPBA gel. The presence of a glycated HSA band in the G0 control sample could be the result of *in vivo* glycation of the protein prior to its purification from human serum.

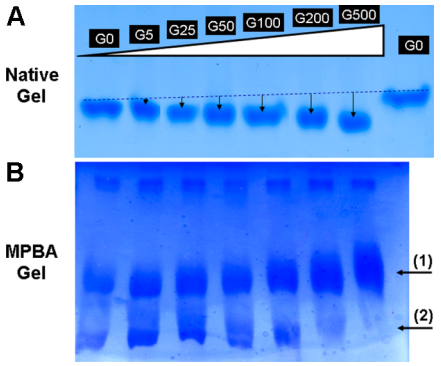

Figure 1. Characterization of glycated HSA by PAGE. (A) Electrophoretic migration profile in native PAGE (4 to 15% gradient polyacrylamide gel). (B) Separation of HSA samples using phenylboronate acrylamide gel electrophoresis (mPAGE) (12% polyacrylamide gel/0.5% MPBA). Arrows 1 and 2 indicate the localization of glycated and native forms of HSA, respectively.

Under our experimental conditions, glycation seemed to have only a mild impact on the oxidative state of albumin as evidenced by the significant but slight decrease in free thiol levels in albumin (from 0.122 ± 0.015 to 0.087 ± 0.003 mol/mol of HSA) upon glucose glycation. In addition, the increase in carbonyl levels (from 4.3 ± 0.83 to 4.9 ± 0.68 mol/mol of HSA) did not reach statistical significance.

Table 2 shows that the significant correlations (>0.78) established between main biochemical parameters (except for thiol and carbonyl levels) reflect the direct impact of the extent of glycation on major structural modifications of the protein.

Impact of Glycation on the Affinity of Albumin for Ketoprofen. To study the impact of glycation on the drug binding characteristics of albumin, we selected ketoprofen, a nonsteroidal anti-inflammatory drug with analgesic and antipyretic properties. Ketoprofen is a well-known site-selective probe for Sudlow site II, but it could also bind to site I of the protein. The nature of the binding site depends on the stereochemistry of the drug molecule that is used in a racemic form.[45] In addition, the albumin binding sites for ketoprofen are found to be located in the vicinity of major esterase activity sites. The choice of ketoprofen was also justified by the impaired drug binding capacities of glycated HSA for this drug evidenced in a previous study by our group.[20] The interaction between ketoprofen and glycated HSA *in vitro* models was investigated using a method based on fluorescence quenching. Ketoprofen acts as a quencher via its interaction with albumin and induces a reduction in tryptophan fluorescence (F) emission intensity as illustrated in Figure 2A. The binding constant (K_A), shown in Figure 2B, was calculated using the plots represented by $(F_0 - F_C)/F_C$ for each albumin sample in which binding site n is the slope and log K_a is the intercept (data not shown). The binding constant for ketoprofen (K_A = 4.75 × 10^8 L/mol) dropped considerably with the rate of glucose (c) condensed to the protein. This impairment of the affinity for ketoprofen was the highest for HSA_{G500} (K_A = 4.98 × 10^6 L/mol). In addition, the gradual loss of affinity of albumin for ketoprofen was confirmed by the reduction in the number of binding sites resulting from the glycation process [from 1.82 ± 0.05 to 1.41 ± 0.03 sites (data not shown)].

Table 2. Statistical Analysis of Biochemical and Functional Parameters[a]

	glycation	ketoamine	amine	thiols	carbonyl	ACB	MW	β-amyloid	Fluo AGE	esterase	Ket affinity
glucose	0.737*	0.972***	0.956***	0.435	0.801**	0.901**	0.982***	0.782**	0.848**	0.723*	0.583*
	glycation	0.837**	0.788**	0.440	0.485	0.833**	0.824**	0.897**	0.941***	0.902**	0.868**
		ketoamine	0.920***	0.138	0.715*	0.894**	0.993***	0.829**	0.905**	0.754*	0.654*
			amine	0.060	0.785**	0.930***	0.940***	0.857**	0.820**	0.807**	0.668*
				thiols	0.243	0.263	0.102	0.050	0.038	0.050	0.066
					carbonyl	0.599*	0.743*	0.477	0.424	0.388	0.263
						ACB	0.929***	0.864**	0.899**	0.888**	0.881**
							MW	0.829**	0.902**	0.763*	0.646*
								β-amyloid	0.903**	0.872**	0.797**
									Fluo AGE	0.833**	0.776**
										esterase	0.966***
											Ket affinity

[a]Univariate correlation coefficients and values of significance between different structural and functional parameter values compared by peer were calculated according to Pearson's method.

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Biochemistry XXXX, XXX, XXX−XXX

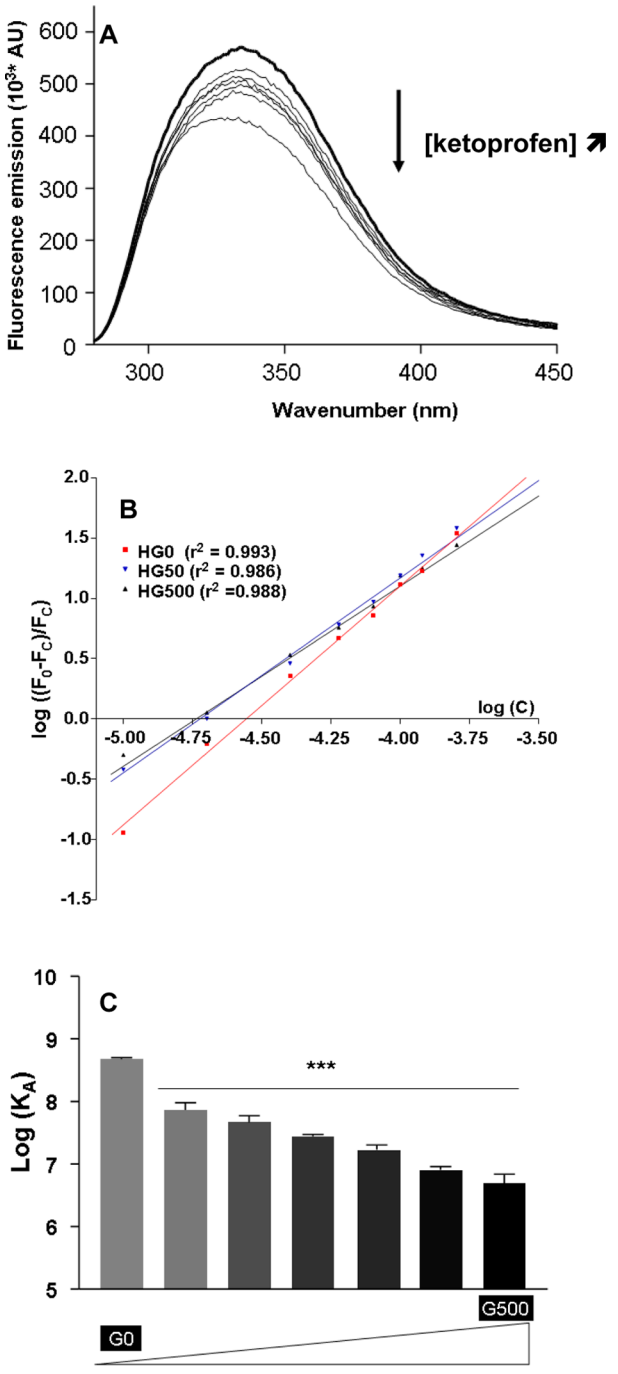

Figure 2. Binding of ketoprofen to *in vitro* glycated HSA samples obtained by fluorescence spectroscopy. (A) Effect of an increasing concentration of ketoprofen on the quenching fluorescence of albumin tryptophan. (B) Log plots of $(F_0 - F_C)/F_C$ vs log[C] for ketoprofen binding with HG0, HG50, and HG500. F_0 and F_C are the tryptophan fluorescence intensities of HSA in the absence and presence, respectively, of ketoprofen at different concentrations [C]. (C) Binding constant K_A for ketoprofen with albumin modified by increasing concentrations of glucose (5−500 mM) calculated as described in Experimental Procedures. Values are means ± the standard deviation for three experiments. Significance of differences compared with native albumin (vs HSA_{G0}): ***$p < 0.001$.

To translate these results to a clinical situation, the affinity for ketoprofen of albumins purified from plasma of nondiabetic and diabetic patients was also investigated. Figure 3A shows the log K_A values as a function of the HbA1c level of each patient. HbA1c corresponds to the glycated hemoglobin fraction and is considered one of the main clinical parameters used for monitoring chronic glycemic control. This parameter primarily reflects mean blood glucose levels over time and hyperglycemic severity. Though a moderate relationship was observed (r = 0.75) between albumin affinity and HbA1c levels, our results established that in a diabetic context, the affinity of albumin for ketoprofen ($K_A = 4.17 \times 10^4$ L/mol) is significantly impaired compared to the affinity of albumin of a healthy subject ($K_A = 1.82 \times 10^6$ L/mol).

Esterase-like Activity of Glycated HSA Samples. The effect of glycation of the enzymatic activity of glycated HSA was investigated by monitoring the hydrolytic conversion of *p*-nitrophenyl acetate to *p*-nitrophenyl. Preliminary experiments showed that human serum albumin had esterase-like activity significantly greater than that of bovine serum albumin (data not shown). Figure 4 shows initial rate constants (k_{obs}) for the hydrolysis of *p*-nitrophenyl acetate by HSA as a function of the extent of glycation. Similar to the affinity constant for ketoprofen, esterase-like rate constants decreased significantly in a dose-dependent manner with an increased level of glycation of the protein, as exemplified by the 22.5% drop in the initial rate constant from 0.273 s^{-1} (for HSA_{G0}) to 0.211 s^{-1} (for HSA_{G500}).

The *in vivo* glycated albumin samples purified from plasma of diabetic and nondiabetic patients showed a significant and inverse correlation between esterase-like activities of the protein and HbA1c that reflects the hyperglycemic severity. As shown in Figure 5A, a moderate correlation (r = 0.67) between both parameters was obtained. Comparison between nondiabetic and diabetic groups in Figure 5B further confirms these results.

Relationship between Structural and Functional Parameters: A Linear Regression Approach. To establish a potential link among albumin affinity for ketoprofen (log K_A), its esterase-like activity (k_{obs}), and the change in structural and biochemical parameters, we employed the partial least-squares regression method (PLS-2). The PLS-2 technique was performed on seven samples of human albumin incubated with increasing concentrations of glucose between 0 and 500 mM. All structural parameters for *in vitro* glycated albumin samples are independent predictor variables. Even if main biochemical and structural variables are strongly related to the glucose parameter, we could consider that their variations are independent. All structural parameters related to *in vitro* glycated albumin samples are included in this regression (ketoamine, free primary amine, thiol, ACB, carbonyl, fluorescent AGE, average molecular mass, and β-amyloid levels). Partial least-squares regression combined the features of principal component analysis and multiple regressions by compressing a large number of variables into a few latent variables (PLS factors) to find a linear regression model by projecting the predicted variables and the observable variables to a new space. Figure 6 illustrates the predicted values as a function of the measured values of log K_A (Figure 6A) and k_{obs} (Figure 6B). The regression models developed here produced log K_A and k_{obs} values very close to reference values. The PLS model related to albumin affinity for ketoprofen shows a very high correlation (r^2 = 0.96) with a very low root-mean-square error (0.159) indicating the good accuracy of this model of prediction of log K_A from structural parameters. The regression model generated for the prediction of esterase-like activity via k_{obs} values also shows satisfactory accuracy (r^2 = 0.97; rmsec = 4.56).

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Biochemistry XXXX, XXX, XXX−XXX

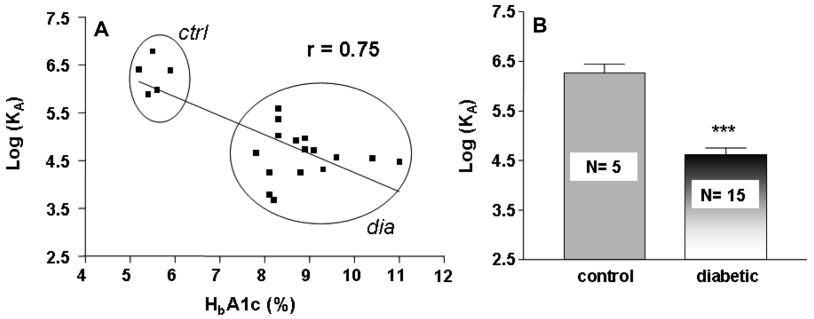

Figure 3. Binding of ketoprofen to *in vivo* purified HSA samples from plasmas obtained by fluorescence spectroscopy. Affinity of ketoprofen for *in vivo* glycated albumin purified from plasma of nondiabetic subjects (*ctrl*; $N = 5$) and diabetic patients (*dia*; $N = 15$) investigated with 20 nmol of albumin samples. (A) Plots of binding constant K_A values vs H_bA1C (%) for 20 purified HSAs. (B) Comparison of binding constant K_A average values between control ($N = 5$) and diabetic ($N = 15$) groups. Significance of the difference between both groups (vs control): ***$p < 0.001$.

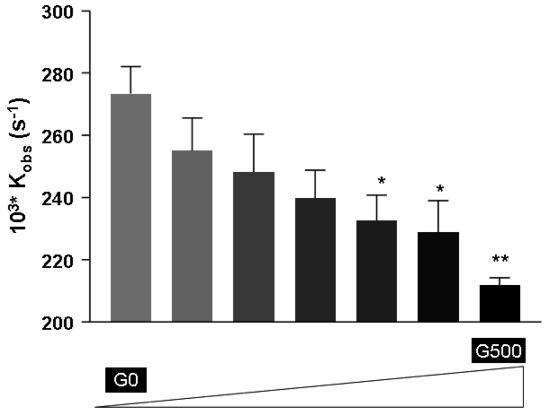

Figure 4. Esterase-like activity of *in vitro* glycated HSA samples. Hydrolysis rate constants (k_{obs}) were determined after reacting 5 μM *p*-nitrophenyl acetate with 20 μM native or glycated HSA in 67 mM sodium phosphate buffer (pH 7.4), followed at 25 °C. Shown are mean values ± the standard deviation for three experiments. Significance of differences compared with native albumin (vs HSA_{G0}): *$p < 0.05$; **$p < 0.01$.

Both PLS models show that the two functional properties of *in vitro* glycated HSA (affinity and esterase activities) could be correctly estimated from structural parameters.

DISCUSSION

Chronic diabetic complications (nephropathy, atherosclerosis, etc.) have been shown to be closely linked to protein glycoxidation in cellular physiopathology.[3,46] Numerous *in vitro* studies report on the impact of glycoxidation on the main functional properties of albumin, the major target protein of glycoxidation in the circulatory system.[19−21] These observations suggest that the severity of the diabetes-associated complications could be intricately linked to albumin glycation extent. However, none of these studies have been able to establish whether there is a quantitative relationship between the degree of glycation and the extent of biochemical and functional modifications of *in vitro* and *in vivo* glycated HSA. In this study, we sought to establish this link by focusing on two important functions of HSA involved in the pharmacokinetics of therapeutics: drug binding and esterase-like properties.

With respect to the main aspects of the pharmacokinetics of drugs (absorption, distribution, metabolism, and excretion), HSA plays a major role in the distribution of drugs through the plasma. Alterations in the binding affinity of albumin could have serious consequences for the pharmacokinetic and pharmacodynamic properties of a wide variety of drugs. For example, an impaired drug binding capacity of HSA could lead to increased levels of the pharmacologically active fraction of a drug in the circulatory system and thereby contribute to its side effects.[47] In addition to albumin's crucial pharmacological role in binding and transporting therapeutic drugs, it also exhibits a range of hydrolase-type activities, among which the esterase property is the most prominent.[48] Esterase enzymes are known to be involved in the conversion of many prodrug esters to active drugs such as aspirin, ketoprofen glucoronide, and nicotinic acid.[49−51]

In this study, we first characterized numerous biochemical parameters of albumin (redox state, modified amino residues,

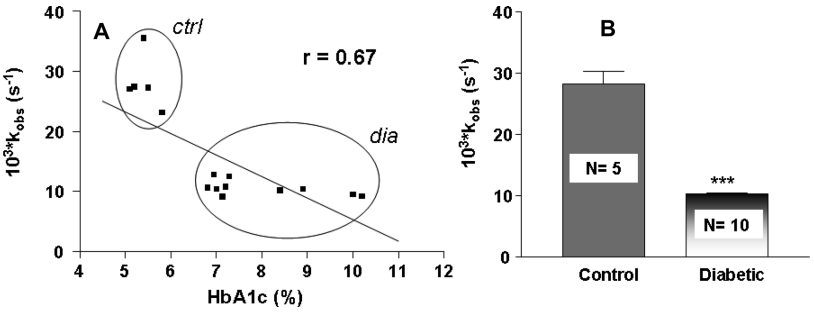

Figure 5. Esterase-like activity of *in vivo* purified HSA samples from plasmas. Hydrolysis rate constants (k_{obs}) of *in vivo* glycated albumin purified from plasma of nondiabetic subjects (*ctrl*; $N = 5$) and diabetic patients (*dia*; $N = 10$). (A) Plots of k_{obs} vs H_bA1C (%) for 15 purified HSAs. (B) Comparison of k_{obs} average values between control ($N = 5$) and diabetic ($N = 10$) groups. Significance of difference between both groups (vs control): ***$p < 0.001$.

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Biochemistry XXXX, XXX, XXX−XXX

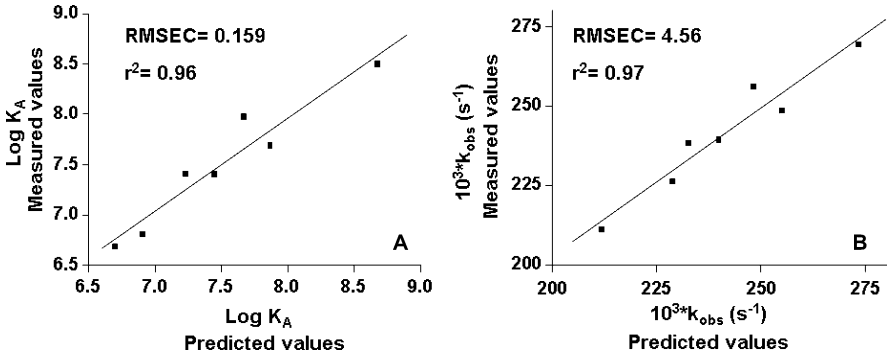

Figure 6. PLS regression model performance linking structural and biochemical parameters to ketoprofen binding capacity and esterase-like activity. Scatter plots describing the measured and predicted values for (A) binding constant K_A for ketoprofen and (B) hydrolysis rate constant k_{obs}. The relationship between structural and functional data was determined by using the PLS algorithm. r^2 is the coefficient of determination between model predictions and measured values, and rmsec is the root-mean-square error of data prediction.

aggregation, glycation product formation, and molecular mass) as a function of the dose of the glycation agent. We compared *in vitro* models of glycated albumin mimicking normoglycemia with a physiological concentration of glucose (5 mM) and HSA glycated with pathological glucose concentrations, reflecting the conditions found in diabetes (25 mM) and suprapathological conditions (50−500 mM) that are often used in numerous studies related to diabetic pathology.[42,52]

As expected and consistent with previous studies,[19,21] we found that *in vitro* glycation of albumin with glucose contributed to the formation of intermediary (ketoamine) and advanced (fluorescent AGE) glycation products. Structural characterization of glycated HSA indicated that glycation promoted the formation of β-structure aggregates associated with an increase in the albumin molecular mass due to the attachment of one or several glucose molecules to the protein. This conformational change of HSA into an intermolecular β-sheet structure suggests an impact of glycation on the tertiary structure of albumin and corresponds to results reported in previous studies.[21,25,53] The decrease in the level of free amino groups with glycation indicates the involvement of numerous exposed positively charged residues such as lysine and arginine that are neutralized by glucose. Our data show that all these glycation-induced structural parameters occur in a gradual manner and are strongly correlated with glucose concentration.

By contrast, the oxidative parameters of HSA (thiol and carbonyl levels) appear to be less impacted by incubation with glucose. The short incubation time could explain the lower levels of oxidation. However, previous studies have shown significant biochemical alterations in glycated HSA without marked changes in oxidation.[21,25]

The affinity of albumin for ketoprofen was evaluated by using tryptophan fluorescence quenching caused by molecular rearrangement or a change in the microenvironment close to Trp-214, resulting from drug-induced unfolding. This fluorescence quenching can occur via two mechanisms: static and dynamic.[54] Numerous studies reported that the mechanism of quenching of HSA by ketoprofen is employed in static mode, corresponding to ground state formation of a nonfluorescent complex between the fluorophore and the quencher.[10,55] Although the main binding site of ketoprofen is located in Sudlow site II of albumin, ketoprofen would be closer to Trp-214 than a ligand in site I.[56] Then, ketoprofen may act as a quencher because of the proximity of site II to the albumin fluorophore (approximately 10−15 Å). Using this spectrophotometric method, we were able to accurately investigate the binding characteristics of interaction between ketoprofen and the protein by calculating the number of binding sites and the affinity constant (K_A). The results suggest that the glycated albumin affinities were considerably altered as a function of the concentration of glucose used in the glycation process. The reduction in the binding constant was associated with a decrease in the number of binding sites. We have already reported, in a previous study, such impairment of albumin capacity for ketoprofen and also for warfarin, an anticoagulant drug, in a glycative context.[20]

Although it has been reported on several occasions that oxidation contributes to biochemical changes in HSA, we saw no significant impact of glycation on the oxidative state of albumin,[57] indicating that the impaired binding properties observed here result rather from the glycation process. This finding is confirmed by the results obtained with *in vivo* glycated HSA purified from diabetic patients, showing a strong correlation between altered drug binding capacity and the levels of H_bA1C [used as a measure of *in vivo* glycation in the blood over a long period (around 3 months)]. This monitoring parameter was already found not to be correlated with the oxidative state of plasmatic albumin from diabetic patients determined via the free thiol level parameter (unpublished data).

In addition to the evaluation of the affinity properties of albumin for ketoprofen, we investigated the enzymatic activity of the glycated albumin with respect to the hydrolysis of *p*-nitrophenyl acetate. As reported by a number of authors, the esterase-like hydrolytic activity of HSA depends on the source of the protein and also on its content in fatty acids, which inhibit this activity.[58] The HSA used in this study is not defatted and displayed an enzymatic activity (0.273 s^{-1}) that is significantly higher than that of wild-type HSA (0.085 s^{-1}), as determined by Watanabe et al.[18] Similarly, the esterase activity of HSA for the hydrolysis of *p*-nitrophenyl acetate was partially impaired by the *in vitro* and also the *in vivo* glycation process. A comparison of the two sets of *in vivo* data showed that esterase activity in human albumin of healthy subjects was ∼3 times higher than that in HSA purified from diabetic patients.

To the best of our knowledge, no results about binding properties and esterase-like activities were reported for purified albumin from plasma of diabetic patients or healthy subjects. Interestingly, marked differences in enzymatic activity and binding properties were clearly noticed between commercial

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Biochemistry XXXX, XXX, XXX−XXX

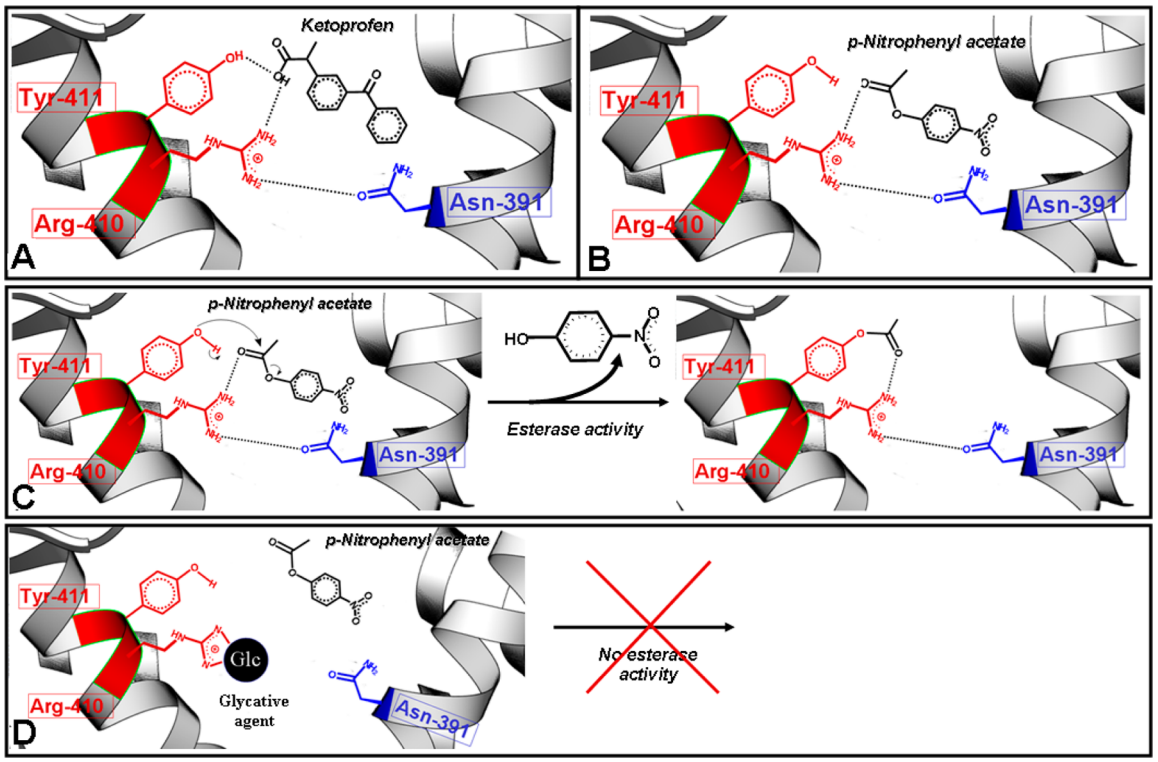

Figure 7. Molecular modeling of ketoprofen interaction and esterase active site in Sudlow site II (domain IIIA) in the presence or absence of glycation adducts according to Ahmed et al.[61] (A) Model of ketoprofen interaction in binding site II. (B) Model of *p*-nitrophenyl acetate in the main esterase active site. (C) Catalytic mechanism of esterase activity of HSA with *p*-nitrophenyl acetate in the absence of glycation. (D) In the presence of a glycative agent on Arg-410.

albumin (*in vitro*) and purified albumins from plasmas (*in vivo*). Indeed, the binding constant for ketoprofen of *in vitro* HSA was found to be 100 times higher than for HSA from nondiabetic subjects. Similarly, there was an 8-fold increase in esterase activity for HG0 compared with that of nondiabetic albumin. As shown in another study, our experimental conditions for the purification of albumin from plasma seem to impact significantly certain intrinsic albumin properties such as esterase and affinity properties.[59]

Finally, our study showed that partial least-squares regression was an effective method for predicting functional parameters such as esterase activity or binding capacity from biochemical parameters of *in vitro* models of glycated albumin. Despite the use of very few samples, this PLS model featured good predictive performance. Such a regression method could be applied, in the future, for plasmatic samples obtained from a large panel of diabetic patients displaying variable severities of their diabetes and their complications.

Impaired esterase and affinity properties of albumin following the glycation process with increasing concentrations of glucose could be explained by two mechanisms: direct chemical modification of some sensitive residues at the active sites of the protein and/or by a conformational alteration around these sites.

As far as the three-dimensional structure of HSA is concerned, drug binding and esterase activity sites are located very close to each other. The primary reactive center of esterase activity is consists of the catalytic triad of Arg-410, Tyr-411, and Asn-391[18,60] and is located in Sudlow site II (domain IIIA), which also acts as the binding site for ketoprofen and several benzodiazepines. Molecular docking experiments performed by Ahmed et al. showed that the chemical functions of Arg-410 and Tyr-411 strongly stabilized the binding of ketoprofen to HSA through hydrogen bonds[61] (Figure 7A). Similarly, the catalytic triad of Tyr-411, Arg-410, and Asn-391 could positively influence HSA esterase activity through a stabilization of the *p*-nitrophenyl acetate substrate by similar hydrogen bonding with Arg-410 (Figure 7B). These interactions with the active site of the protein could stimulate the conversion of *p*-nitrophenyl acetate by esterase activity into *p*-nitrophenol (Figure 7C).

A second esterase site was reported to be in the vicinity of the warfarin binding site (Sudlow site I) located in a large hydrophobic cavity of domain IIA and to imply the sole tryptophan residue (Trp-214) in albumin.[15] An enantiomeric form of ketoprofen was also associated with this hydrophobic pocket.[45] In addition to Arg-411 in Sudlow site II, it was clearly established that several positively charged residues such as lysine and arginine are located in these hydrophobic cavities contributing potentially their conformation and thereafter enhanced the binding of ketoprofen or ligand, including *p*-nitrophenyl acetate, via hydrogen bonds.[62]

We showed that incubation of albumin with increasing concentrations of glucose affects an increasing number of amine residues (up to 13 residues) and leads to an incremental enhancement of the average molecular mass of the protein due to the incorporation of increasing glycation adducts (up to more than 10 units). If numerous lysine residues (including Lys-525 and Lys-199) were identified as preferential targets for glycation, many arginine residues such as Arg-218 and Arg-410 in sites I and II, respectively, could be affected by glycation. For example, Arg-410 is known to be the most reactive glycation site together with Lys-525.[61]

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Biochemistry XXXX, XXX, XXX−XXX

First, albumin esterase activity and affinity for ketoprofen could be prevented by glycation at some arginine or lysine residues, which are not necessarily directly implicated in the active site of the protein but contributing to the hydrophobic pocket conformation. Previous studies have clearly established that the regions mostly affected by the glycation-induced changes in the three-dimensional conformation of HSA were located around tryptophan residue Trp-214 and involved the partial unfolding of the hydrophobic pockets of albumin where Sudlow sites I and II are located.[25]

Second, the ligand stabilization within the active site through hydrogen bonding could be directly hampered by the glycation of the Arg-410 residue. The presence of a glycation adduct at this residue level would prevent hydrogen bonds from forming between ketoprofen or *p*-nitrophenyl acetate and the protein and consequently minimize the esterase catalytic reaction (Figure 7D).

As a consequence, protein modification at specific amino residues highly involved in the active sites combined with protein misfolding has a direct impact on the intrinsic biological functionality of albumin, including its binding and enzymatic capacities.

Two important findings emerge from this work. (1) The reduced binding capacity of glycated albumin for ketoprofen was observed for both *in vitro* and *in vivo* glycated HSA and is strongly correlated with the extent of glycation. This functional impairment of albumin was also associated with an alteration of its esterase-like capacity. (2) The linear relationship established between glucose and the biochemical and functional parameters of albumin in a glycative context demonstrated that pharmacological properties of albumin are strongly correlated with the glycation process alone.

In summary, alterations in the structure and function of glycated HSA are strongly linked to glucose concentration, indicating that the severity of hyperglycemia in diabetes can lead to significant disturbances in drug metabolism. This could be of utmost importance for drugs with a narrow therapeutic index or for the more recent use of prodrugs.

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Author Contributions

J.B.-V. researched data, contributed discussion, and wrote and reviewed the manuscript. C.P. researched data and reviewed the manuscript. O.M. contributed discussion and reviewed the manuscript. V.M. contributed discussion and reviewed the manuscript. J.V.d.E. reviewed the manuscript. E.B. contributed discussion and reviewed the manuscript. P.R. researched data and wrote, reviewed, and edited the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AGE, advanced glycation end product; HSA, human serum albumin; HSA_{Gx}, commercial human serum albumin incubated with *x* mM glucose; HbA1C, glycated hemoglobin level; PBS, phosphate-buffered saline; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); MPBA, methacrylamido phenylboronic acid.

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Biochemistry XXXX, XXX, XXX−XXX

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