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

Jennifer Baraka-Vidot, Cynthia Planesse, Olivier Meilhac, Valeria Militello, Jean Van den Elsen, Emmanuel Bourdon, and Philippe Rondeau

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 pandemic 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. 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. Numerous studies showed that nonenzymatic glycation and AGE formation were associated with diabetic complications, such as retinopathy, nephropathy, neuropathy, and coronary artery disease.

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

Albumin exerts several physiological and pharmacological functions, including antioxidant properties, oncotic pressure regulation, pseudoenzymatic activities, and particularly binding and transport capacities for numerous endogenous and exogenous compounds, like drugs. 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. Thus, this plasma protein can act as a circulating depot for many drugs. Albumin affinity capacity plays a major role in absorption, distribution, metabolism, and excretion for drugs (ADME). 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. Sudlow site I is large and II. Sudlow site I is large and flexible and preferentially binds bulky heterocyclic compounds such as warfarin. By binding bulky heterocyclic compounds such as warfarin.
Glycation of HSA. The incubation of commercially available human serum albumin (HSA) and the esterase-like activity and a functional alteration caused by glycoxidation, we investigated elucidated. To clarify the relationship between structural and pharmacological properties of albumin remains to be investigated.14–19 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%),** bicinechonic acid, ketoprofen, d-(+)-glucose, Congo Red, m-aminophenolboronic 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 M stock solution. Glycated human serum albumin was purified by using the method developed by Johnson et al.15 with the nitroblue tetrazolium (NBT) reagent.

The 2,4,6-trinitrobenzensulfonic acid (TNBS) assay is a sensitive method for identifying the primary free amino groups in proteins.20 This method was described in detail in a previous study by our group.21 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 spectrophotometric assay based on recognition of protein-bound DNPH in carboxylated 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:

$$\text{carbonyl (mol)/protein (mol)} = \frac{\text{OD}_{370} \times \varepsilon_{370/\text{hydrazone}}}{\text{OD}_{276} - 0.43 \times \text{OD}_{370} \times \varepsilon_{370/\text{hydrazone}}}$$

In previous formulas, $\varepsilon_{370/\text{hydrazone}}$ equals 46824, the molar absorptivity of HSA, and $\varepsilon_{370/\text{hydrazone}}$ 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* glyated 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_{GP}.31 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.32 Discriminating analysis of glycated and nonglycated albumin was performed using MPBA polyacrylamide electrophoresis.33 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.

**Borane Affinity Chromatography.** The formation of Amadori products was also assessed by the percentage of Amadori products.
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.\textsuperscript{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 (2000g for 2 minutes), 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\textsubscript{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.

\textbf{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 \textmu L 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 mmol/L 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.

\textbf{Fluorescence AGE Determination.} The fluorescence emission intensity of the glycated product was obtained with 235 nm (pentosidine)\textsuperscript{34} and 380 nm (crossline + vepserlysine)\textsuperscript{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 + vepserlysine) was calculated using the following formula:

\[
\text{AGE}\% = 100 \left( \frac{I_{\text{max}G} - I_{\text{max}G0}}{I_{\text{max}G0}} \right)
\]

where AGE\% represents the relative percent of AGE, \(I_{\text{max}G}\) is maximal fluorescence intensity of glycated HSA, and \(I_{\text{max}G0}\) is the maximal fluorescence intensity of nonglycated HSA\textsubscript{0}.

\textbf{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.\textsuperscript{36,37} This assay based on the reduced binding affinity of human serum albumin for metal ions (cobalt, Co\textsuperscript{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.

\textbf{Affinity of Albumin for Ketoprofen.} This method is based on the quenching of albumin fluorescence induced by interaction with drugs.\textsuperscript{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.

\textbf{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,\textsuperscript{38,39} and the apparent hydrolysis rate constant (\(k_{\text{app}}\)) was calculated.

\textbf{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) were described in detail in several papers by our group.\textsuperscript{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 parameter.
Biochemistry

301 parameter (K_3 or k_{obs}), x_i is the measured value of predictor
302 parameter i, and C is the associated regression coefficient.

303 All samples were used to calibrate the PLS model, and the
304 performance of this model was assessed by the coefficient of
305 correlation (r²) and the root-mean-square error of calibration
306 (rmsec) between predicted and measured values of esterase
307 activity (k_{obs}) and binding affinity (log K_3) on a data set.

308 \[
309 \text{rmsec} = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n}}
310 \]

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

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

320 **RESULTS**

321 HSA was in vitro glycated by being incubated with increasing
322 glucose concentrations followed by a range of biochemical
323 characterizations. Functional assays were performed to
324 determine the impact of glucose-induced glycation on albumin
325 properties. The biochemical and structural parameters relating
326 to the in vitro glycated HSA models are summarized in Table 1.
327 Glycated HSA is termed HSAG\_X, where X is the concentration
328 of glucose incubated with albumin (5, 25, 50, 100, 200, or 500
329 mM).

330 **Structural Characterization of in Vitro Glycated HSA Samples.**
331 Biochemical characterization of albumin samples
332 revealed that glycation with increasing concentrations of
333 glucose caused a dose-dependent elevation in ketoamine levels
334 (up to 4-fold higher for HSAG\_500 compared to HSAG\_0 (p <
335 0.001)) and in glycated albumin proportion (+66.7% for
336 HSAG\_500 vs that for HSAG\_0). This increase in early glycation
337 products is accompanied by the formation of fluorescent AGE,
338 in a dose-dependent manner for pentosidine, vesperlysine, and
339 crossline products, as well. Indeed, glycation in the presence of
340 500 mM glucose induced the more intense formation of
341 pentosidine [34.5% (p < 0.001)] and vesperlysine [35.9% (p <
342 0.01)]. Two direct consequences are observed with the
343 glycation process with growing concentrations of glucose: the
344 increase in average molecular mass accompanied by aggregate
345 formation.

346 As reported in previous studies, glycation of albumin was
347 shown to generate thermodynamically more stable high-
348 molecular mass aggregates with high \( \beta \)-sheet structure content
349 leading to formation of amyloid-type structures.\(^{42-44}\) The
350 measurement of Congo red absorbance performed to detect \( \beta \-
351 fibrillar structure in albumin samples featured a significant
352 increase only for highly glycated models HSAG\_200 [15.4% (p <
353 0.05)] and HSAG\_500 [22.1% (p < 0.01)], attesting to the
354 presence of such aggregate structures. These results are in
355 accordance with those of the thioflavin T assay showing
356 enhanced fluorescence upon binding of the dye to amyloid
357 fibrillar structures in highly glycated albumins (data not
358 shown). In parallel, the significant increase in average molecular
359 mass was noticed for most glycated albumin samples. The

Table 1. Structural and Biochemical Parameters in Different HSA Samples.\(^b\)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescent AGE level (mol/mol)</th>
<th>ACB index</th>
<th>0.83 mol/mol ACB index vesperlysine</th>
<th>0.83 mol/mol ACB index pentosidine</th>
<th>0.83 mol/mol ACB index free amino groups/HSA</th>
<th>0.83 mol/mol ACB index carbonyl/HSA</th>
<th>0.83 mol/mol ACB index esterase activity (mol/mol)</th>
<th>Average molecular mass (MDa)</th>
<th>β-amyloid formation (% HSAG0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSAG_0</td>
<td>0.12 ± 0.015</td>
<td>0.099</td>
<td>0.83</td>
<td>0.099</td>
<td>0.099</td>
<td>0.099</td>
<td>0.099</td>
<td>0.099</td>
<td>0.099</td>
</tr>
<tr>
<td>HSAG_5</td>
<td>4.36 ± 0.21</td>
<td>4.36</td>
<td>4.36</td>
<td>4.36</td>
<td>4.36</td>
<td>4.36</td>
<td>4.36</td>
<td>4.36</td>
<td>4.36</td>
</tr>
<tr>
<td>HSAG_50</td>
<td>56.43 ± 1.23</td>
<td>56.43</td>
<td>56.43</td>
<td>56.43</td>
<td>56.43</td>
<td>56.43</td>
<td>56.43</td>
<td>56.43</td>
<td>56.43</td>
</tr>
<tr>
<td>HSAG_100</td>
<td>121.46 ± 3.45</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
</tr>
<tr>
<td>HSAG_200</td>
<td>121.46 ± 3.45</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
</tr>
<tr>
<td>HSAG_500</td>
<td>121.46 ± 3.45</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
<td>121.46</td>
</tr>
</tbody>
</table>

\(^b\)Glycation level determined by fluorescence spectroscopy (100% corresponding to complete glycation of HSA). Ketoneamine level obtained by the NBT assay. Unmodified primary amino group level as assessed by Ellman’s method. Carbonyl level as assessed by a spectrophotometric carbonyl assay. Unmodified primary amino group level as assessed by Ellman’s method. Carbonyl level as assessed by a spectrophotometric carbonyl assay. Albumin cobalt binding (ACB) index is calculated according to Pearson’s method. The Unscrambler (Camo ASA) was used to perform regression analysis. Statistical significance was 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.

The table above shows the various biochemical and structural parameters measured in different glycated HSA samples, including fluorescent AGE, ACB index, ketoneamine, free amino groups, carbonyl, esterase activity, and average molecular mass, along with the β-amyloid formation percentage compared to the starting HSA (HSAG\_0). The data are expressed as the means ± standard deviation (SD) from a minimum of three experiments. The Unscrambler (Camo ASA) was used to perform regression analysis. Statistical significance was 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.
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 a glycated HSA band (1) as a function of increasing glucose concentration, similarly, MPBA gel electrophoresis toward the anode as a function of glucose concentration. Similarly, MPBA gel electrophoresis of in vitro glycation HSA (Figure 1B) shows a clear increase in the level of retention and intensity of the glycation 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 vitro glycation of the protein prior to its purification from human serum.

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 (p < 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. 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. 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_τ)/F_τ for each albumin sample in Figure 17 where 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 × 10^7 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 HSAG500 (K_a = 4.98 × 10^7 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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glycation</th>
<th>Ketamine</th>
<th>Amine</th>
<th>Thiols</th>
<th>Carbonyl</th>
<th>ACB</th>
<th>MW</th>
<th>β-Amyloid</th>
<th>Fluo AGE</th>
<th>Esterase</th>
<th>Ket Affinity</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.737**</td>
<td>0.972***</td>
<td>0.956***</td>
<td>0.435</td>
<td>0.801**</td>
<td>0.901**</td>
<td>0.982**</td>
<td>0.782**</td>
<td>0.848**</td>
<td>0.723*</td>
<td>0.583*</td>
</tr>
<tr>
<td>Glycation</td>
<td>0.837***</td>
<td>0.788**</td>
<td>0.440</td>
<td>0.485</td>
<td>0.833**</td>
<td>0.824**</td>
<td>0.897**</td>
<td>0.941***</td>
<td>0.902**</td>
<td>0.868**</td>
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</tr>
<tr>
<td>Ketamine</td>
<td>0.920***</td>
<td>0.715*</td>
<td>0.138</td>
<td>0.785**</td>
<td>0.894**</td>
<td>0.993**</td>
<td>0.829**</td>
<td>0.890**</td>
<td>0.905**</td>
<td>0.754*</td>
<td>0.654*</td>
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<tr>
<td>Amine</td>
<td>0.060</td>
<td>0.243</td>
<td>0.102</td>
<td>0.050</td>
<td>0.930**</td>
<td>0.940**</td>
<td>0.857**</td>
<td>0.820**</td>
<td>0.807**</td>
<td>0.668*</td>
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<tr>
<td>Thiols</td>
<td>0.599</td>
<td>0.743*</td>
<td>0.715</td>
<td>0.050</td>
<td>0.263</td>
<td>0.102</td>
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<td>Carbonyl</td>
<td>0.929***</td>
<td>0.864**</td>
<td>0.899**</td>
<td>0.888**</td>
<td>0.881**</td>
<td>0.829**</td>
<td>0.902**</td>
<td>0.763*</td>
<td>0.646*</td>
<td>0.797**</td>
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<tr>
<td>ACB</td>
<td>0.930**</td>
<td>0.872**</td>
<td>0.899**</td>
<td>0.888**</td>
<td>0.881**</td>
<td>0.903**</td>
<td>0.902**</td>
<td>0.763*</td>
<td>0.646*</td>
<td>0.797**</td>
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</tr>
<tr>
<td>MW</td>
<td>0.833**</td>
<td>0.776**</td>
<td>0.888**</td>
<td>0.881**</td>
<td>0.881**</td>
<td>0.903**</td>
<td>0.902**</td>
<td>0.763*</td>
<td>0.646*</td>
<td>0.797**</td>
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<tr>
<td>Esterase</td>
<td>0.966***</td>
<td>0.966***</td>
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</table>

*Univariate correlation coefficients and values of significance between different structural and functional parameter values compared by peer were calculated according to Pearson’s method.
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$^{0}$) to 0.211 s$^{-1}$ (for HSA$^{500}$).

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 $\beta$-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 ($r^2 = 0.97$, rmse = 4.56).

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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$^{0}$): ***p < 0.001.
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. 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. 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. 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. 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. In this study, we first characterized numerous biochemical 
parameters of albumin (redox state, modified amino residues,

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 HbA1C (%) 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$_{G0}$): *$p < 0.05$; **$p < 0.01$.

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 HbA1C (%) 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): ***$p < 0.001$.
As expected and consistent with previous studies,\textsuperscript{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 $\beta$-structure aggregates associated with an increase in the albumin molecular mass due to the attachment of one or several glucose molecules to the protein.\textsuperscript{25} This conformational change of HSA into an intermolecular $\beta$-sheet structure suggests an impact of glycation on the tertiary structure of albumin and corresponds to results reported in previous studies.\textsuperscript{21,23,53} 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.\textsuperscript{55}

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.\textsuperscript{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.\textsuperscript{34} 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.\textsuperscript{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.\textsuperscript{36} 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.\textsuperscript{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,\textsuperscript{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 HbA1C [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.\textsuperscript{58} The HSA used in this study is not defatted and displayed an enzymatic activity ($0.273 \text{ s}^{-1}$) that is significantly higher than that of wild-type HSA ($0.085 \text{ s}^{-1}$), as determined by Watanabe et al.\textsuperscript{18} Similarly, the esterase activity of HSA for the hydrolysis of p-nitrophenol 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.\textsuperscript{61}

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.\textsuperscript{61} Interestingly, marked differences in enzymatic activity and binding properties were clearly noticed between commercial...
Indeed, the binding constant for ketoprofen of \textit{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.\textsuperscript{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 \textit{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.\textsuperscript{59}

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.\textsuperscript{60}

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\textsuperscript{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\textsuperscript{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.\textsuperscript{15} An enantiomeric form of ketoprofen was also associated with this hydrophobic pocket.\textsuperscript{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.\textsuperscript{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.\textsuperscript{59}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{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.\textsuperscript{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.}
\end{figure}
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.

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 misfolding 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 the species specificity of human serum albumin (HSA) is not altered by glycation.

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 produgs.

### ABBREVIATIONS

AGE, advanced glycation end product; HSA, human serum albumin; HSA_gly, 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); MPB, methacrylamido phenylboronic acid.

### REFERENCES


### AUTHOR INFORMATION

**Corresponding Author**

*DéTROI U1188, Université de la Réunion Plateforme,*

*CYROI 2, rue Maxime Riviere, BP 80005 97491 Sainte Clotilde, La Réunion, France. E-mail: rophil@univ-reunion.fr.*

**Telephone:** (+262) 262 93 86 48. **Fax:** (+262) 262 93 82 37.

**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.de.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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