Review

The glycation of albumin: Structural and functional impacts

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A B S T R A C T

Oxidative stress and protein modifications are frequently observed in numerous disease states. Glucose constitutes a vital nutrient necessary to cellular oxygen metabolism. However, hyperglycemia-associated damage is an important factor in diabetes disorders.

Albumin, the major circulating protein in blood, can undergo increased glycation in diabetes. From recent studies, it has become evident that protein glycation has important implications for protein activity, unfolding, and degradation, as well as for cell functioning.

After giving a brief overview of the key role of albumin in overall antioxidant defense, this review examines its role as a target of glycation reactions. A synthesis of state of the art methods for measuring glycation on the structure of albumin and its various activities, especially its antioxidant and binding capacities. The biological impact of glycated albumin on cell physiology is also discussed, specifically the role of the protein as a biological marker of diabetes.

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1. Introduction

During their lifetime, proteins are exposed to a multitude of altering factors, including enzymatic and non-enzymatic mechanisms. Oxidation and glycation are among the major non-enzymatic mechanisms. Such alterations can affect our tissues and are accumulated throughout our lifetime with an undeniable effect on metabolism. These molecular and cellular alterations, though initially harmless, can become damaging and pathogenic when sufficiently abundant. Among the various targets that are sensitive to alteration, it has been suggested that blood plasma is sustainably and continually exposed to numerous metabolites, which could, in particular, induce an oxidative stress. For instance, in the case of hyperglycemia, reducing sugar could induce a non-enzymatic glycation of several proteins in the circulatory system, with albumin being the most important of these.

Albumin is known to have a set of quite diverse functions. After a brief introduction to the properties of albumin, this article reviews the different aspects of albumin glycation by focusing on the impact of this process on the structural, biological and physiological modification of the protein. The effects of in vitro or in vivo glycation on the binding properties of albumin, in particular, are detailed. Finally, and with regard to its clinical and biological relevance, the importance of glycated albumin as a biological marker of hyperglycemia and diabetes is discussed.

2. Albumin, a multifunctional protein

With a normal concentration of between 35 and 50 g/l, serum albumin represents the most abundant protein in plasma and exerts a wide variety of physiological and pharmacological functions. In particular, human albumin constitutes some 50% of the protein present in the plasma of normal healthy individuals [1]. The flexibility of albumin’s structure is due to its organization into three domains, I, II and III, each subdivided into two subdomains, A and B [2]. 17 intramolecular disulfide bonds ensure rigidity within each subdomain of the protein, but allow significant modifications in the shape and size of albumin in response to pH changes or other biophysical influences [3]. Many activities exerted by the protein can be attributed to its different levels of structure. For instance, albumin contributes to the maintenance of oncotic pressure due to its low molecular weight (67 kDa), compared with other plasmatic globulins [4], and
also because of its weak isoelectric point, which gives to the protein a global negative charge at physiological pH [5].

In addition, albumin’s tertiary dimensional structure allows it to bind and transport quite diverse small size metabolites such as metal ions, fatty acids, bilirubin and drugs [6,7]. Albumin is notably involved in the pharmacokinetics of many therapeutic drugs that can be bound to the protein. Indeed, conjugated with this long half-life protein, the therapeutic peptides of cytokines have improved their pharmacokinetic profile [8]. Affinity for these metabolites depends on the tertiary structure of the binding sites, which are distributed all over the molecule. The three most prominent binding sites for the main drugs and ligands have been identified [2]. The major sites for drugs are site I and site II located on subdomains II A and II A, respectively [2,9], whereas aminoterminus binding sites have a high affinity for metal ions (Co2+, Cu2+ and Ni2+). Both hydrophobic pockets (sites I and II) have been found to bind specifically aromatic and heterocyclic ligands [2,10]. Because of its large size and its adaptability, site I tends to bind bulky endogenus substances, including bilirubin and porphyrins [11]. Indeed, flexibility of the albumin structure allows the protein to accommodate to molecules of many different structures [12]. For instance, human serum albumin (HSA) can adopt numerous conformations depending on the ligand bound to the protein. By contrast, site II, which is smaller and less flexible, induces more stereospecific binding. For instance, paracetamol, a commonly used analgesic drug, binds to residues located in the subdomain III A [13]. Some other metabolites have been found to bind within other hydrophobic pockets such as fatty acids, whose seven binding sites are localized in subdomains IB, II A, and II B [14]. In addition, a few residues, including cysteine, lysine, serine and arginine, have been described as being able to covalently bind many drugs [15]. When protein modification induced by physiological or pathological changes occurs, an alteration of the native conformation and efficiency of these binding sites can be expected [16].

Among different endogenous substances accumulating in plasma, uremic toxins can bind to albumin via the Sudlow’s site I and II [17,18]. Uremic toxins are proteins and low molecular mass peptides, which were involved in chronic renal failure [17,18]. Even if AGEs were considered as a novel class of uremic toxins [19], there is no evidence on the potential binding of circulating AGEs by albumin.

In addition to its determinant role in molecule binding and transport, albumin is known to exert determinant antioxidant activities in plasma, a body compartment exposed to continuous oxidative stress [20,21]. There are several important antioxidants in human plasma including ceruloplasmin, albumin, and uric acid [22]. The predominant contribution assigned to albumin as antioxidant in plasma is mostly due to the fact that several residues may be available to work as antioxidant [20,23]. The main sites in albumin have been previously described for their involvement in this antioxidant activity [23]. Of these sites, Cys-34, the only free cysteine residue not involved in disulfide bond formation constitutes a powerful antioxidant. About 70–80% of albumin Cys-34 in a healthy person is in reduced form (free sulphhydryl group) [16]. Indeed, in reduced form, Cys-34 represents an important redox regulator in extracellular compartments, and is able to scavenge hydroxyl radicals [24,25]. The majority of antioxidant functions that have been ascribed to albumin, depend on the exposure of Cys-34 in the native conformation of the protein [26]. In addition, Cys-34 is able to bind covalently drugs including cisplatin, α-penicillamine and N-acetyl-cysteine [27]. In this context, the redox state of the protein, especially Cys-34, becomes important for covalent binding.

Other main binding sites could be mentioned for their involvement in the antioxidative properties of albumin. For instance, the more exposed methionine residues are particularly susceptible to oxidation, and could serve as an ROS (reactive oxygen species) scavenging system to protect proteins from extensive alteration [28,29]. Similarly, bound to albumin via three main sites (Lys-351, Lys-475 and Arg-117), long chain fatty acids may be protected from oxidant-mediated damage [30]. An initial study in this area has suggested that, at the very least, albumin may protect other proteins from glycation in the initial stages of diabetes [31]. Histidine, lysine and cystein residues in proteins may be the target of the electrophilic lipid oxidation product 4-hydroxy-2-nonenal (HNE) by Michael-type addition [32]. Noteworthy enough, Cys 34 and Lys199 in albumin were shown to be the most reactive HNE-adduction sites [33].

3. How does albumin glycation occur?

Because of its long half-life time, compared to other proteins (about 21 days), and its high concentration, serum albumin is a plasmatic protein highly sensitive to glycation. However, other circulatory proteins, including hemoglobin, insulin and immunoglobulins are also affected by glycation. In addition, the glycation process affects long half-life proteins of the extracellular matrix such as collagen, laminin and fibronectin [34,35]. This process, also known as the Maillard reaction, is a slow non-enzymatic reaction that initially involves attachment of glucose or derivatives with free amine groups of albumin to form reversibly a Schiff base product, leading to the formation of stable fructosamine residue (ketamine) following Amadori rearrangement [36,37]. This is the early glycation process: Schiff’s base and fructosamines have been called early glycation adducts [38]. The Amadori products could subsequently cyclize, forming pyranose or furanose carbohydrate adducts [39]. Further modifications in these early stage glycation products, such as rearrangement, oxidation, polymerization and cleavage give rise to irreversible conjugates, called advanced glycation end products (AGE) [40]. Fig. 1 provides a summary of such AGE identified in several previous studies [38,41–44]. The glycation process is often associated with the phenomenon of oxidation, called “glycoxidation”, which occurs when oxidative reactions affect the early stage glycation products [45].

Other sugars as glucose can also modify albumin such as galactose and fructose [46]. In galactosemia and fructosemia where those sugars are shown to be increased, enhanced hemoglobin and lens protein glycation and increased oxidative stress were measured [47–49]. No information is available concerning albumin modification in these metabolic diseases. Only an in vitro study has shown that galactose could induce a faster and more intensive glycation in human albumin than glucose [50]. Glycation process can also occur with highly reactive derivatives such as α-oxoaldehydes (glyoxal, methylglyoxal or 3-deoxyglucosone). α-oxoaldehydes, which are important precursors of AGE products, can be formed endogenously by degradation of glucose and early glycation products, as well [51]. Among α-oxoaldehydes, methylglyoxal can also be formed as a side-product of different metabolic pathways. MGO can derive from glucose and AGE formation but also through an enzymatic elimination of phosphate from glycerone phosphate or glyceraldehyde 3 phosphate [52], or from 3-aminoaceton in the threonine catalysis [53], through lipid peroxidation [54] and also from the degradation of DNA [55]. With saccharide derivatives, the glycation efficiency depends on the nature and the anomerization of the carbohydrate involved in the process. Indeed, the open chain form of a given monosaccharide, more electrophilic than the acyclic form, leads to the formation of more reactive carbohydrate [56]. In comparison with glucose, ribose induces a faster glycation process with albumin because of its greater fraction of acyclic form [57]. The percentage of modified residues depends on the reactivity of the saccharide derivatives involved in glycation process and increase with the time of incubation [58].
In vivo, the proportion of glycated albumin in healthy persons is in the range of between 1% and 10% [2, 59], and in the case of diabetes mellitus, the proportion may increase two- to threefold [60]. Glycated albumin levels, determined in different in vivo studies for different pathologies associated to diabetes mellitus, are given in Table 1 [61–66]. This glycated albumin rate can even reach more than 90% for severe diabetic patients with poor diabetic control [67]. Noteworthy, in the later study, AGEs were measured using fluorescence detection but majority of AGEs do not emit fluorescence [68]. Hence the level of AGEs in a diabetic patient given in this study remains insecure.

4. Methods for determining albumin glycation

Various methods, well described by Desch, have been used to characterize and quantify glycated proteins, including albumin glycated in vitro or in vivo; these methods are summarized in Table 2 [69]. The thiobarbituric acid assay (TBA) is a common colorimetric method showing the amount of ketoamine bound to albumin. This assay is based on the release, by hydrolysis, of adducted glucose to albumin as 5-hydroxymethylfurfural (5-HMF), which can react with thiobarbituric acid [70, 71]. Another colorimetric assay, using nitroblue tetrazolium (NBT) reagent, is a suitable method for measuring albumin fructosamine, which is useful as an index of diabetic control [72, 73]. The assay is based on a reduction reaction on NBT, with ketoamine giving rise to Formazan, which can be photometrically quantified. However both previous methods present some major drawbacks and suffer from a high degree of unspecificity [74]. Concerning the TBA method, HMF formed in the reaction is heat labile and free glucose could interfere in the test [75]. The NBT method is vulnerable to interferences with thiol group, uric acid or lipemia [76, 77]. A third colorimetric procedure, based on the formation of phenylhydrazone adduct, resulting from the reaction of fructosamine with phenylhydrazine can be used for Amadori product quantification [78, 79]. However, this last method was not developed for use in the clinical laboratory [80]. The following assays for fructosamine provide improved specificity and reliability compared to conventional NBT or TBA based methods described previously. Indeed, Glycated albumin can also be assayed more easily and rapidly by an enzymatic method using albumin-specific proteinase, ketoamine oxidase and bromocresolpurple reagent (BCP) for albumin measurement [81]. In comparison with colorimetric methods, this method enables a more accurate analysis of glycated albumin without any interference. Chromatography is an alternative technique for determining concentrations of glycated albumin, such as high-performance liquid chromatography using consecutively exchange chromatography to separate albumin, and boronate affinity chromatography to separate glycated and non-glycated albumin [82]. Similarly to the enzymatic assay, this method is not influenced by albumin concentration. Another sensitive method for quantifying the fructose-lysine linkages present in glycated albumin and performed by high-performance liquid chromatography (HPLC)
can be used routinely in the clinical laboratory [83]. In this method, fructosamine products are subjected to hydrolysis and dehydration, giving rise to the formation of furosine, which can be quantified [84]. In this method, furosine procedure-HPLC [83]. In this method, fructosamine products are subjected to hydrolysis and dehydration, giving rise to the formation of furosine, which can be quantified [84].

Table 1

<table>
<thead>
<tr>
<th>Type of diabetes</th>
<th>n</th>
<th>Glycerated albumin level %</th>
<th>References</th>
</tr>
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<td>Type 1 diabetes DM</td>
<td>93</td>
<td>15.8 ± 5.6</td>
<td>[66]</td>
</tr>
<tr>
<td>Type 2 diabetes DM</td>
<td>75</td>
<td>23.1 ± 4.4</td>
<td>[66]</td>
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<tr>
<td>Gestational diabetes GDM</td>
<td>22</td>
<td>14.5 ± 2.7</td>
<td>[64]</td>
</tr>
<tr>
<td>Diabetes in children</td>
<td>26</td>
<td>39.1 ± 9.1</td>
<td>[63]</td>
</tr>
</tbody>
</table>

5. Impact of glycation on albumin structure

The main studies dealing with the structural, biological, and physiological characterizations of glycated albumin have been performed using an in vitro or in vivo model of glycation derived from both human albumin and bovine albumin.

Compared sequences of human and bovine albumin and found to be of striking homology (round 80% [32]). Furthermore, the differences observed are mainly of a structurally conservative nature, e.g. hydrophobic amino acids are replaced by other hydrophobic amino acids and not by polar ones.

Because of its striking sequence homology with the human form (80%), bovine serum albumin (BSA) appears to be a common albumin used in such studies. Indeed, the differences are mainly due to hydrophobic amino acid replacements by other hydrophobic amino acids with no consequence on tertiary and secondary structures [89]. The glycation and glycoxidation processes of albumin induce several structural modifications, including an increase in the molecular weight of the protein, associated with the extent of glycation [90,91]. The increase in molecular weight with glycation corresponds to one several glucose units attached to the amino residues of the protein. Non-enzymatic glycosylation of albumin in vivo occurs at multiple sites corresponding to arginine, lysine and also cysteine residues.

Numerous studies have identified the main sites that can be modified by glycation for serum albumin in vivo. Because of their high nucleophile properties, lysine, arginine and cysteine are the sole residues prone to glycation. About 29 sites have been described, including 18 lysine residues, as shown in Table 2. In several studies, lysine-525 is depicted as the predominant site of the non-enzymatic glycosylation of human serum albumin in vivo [59,92,93]. Non-enzymatic addition of glucose at this residue accounts for 30% of the overall glycation [93]. An important decrease in the affinity of in vivo and in vitro glycated albumin for different ligands suggests that this principal glycation site may play a key role in these bindings. Indeed, affinity both for long chain fatty acids and for bilirubin has been shown to be drastically altered when albumin is modified [59]. Lys-525 appears to be the most reactive glycation site, despite its low surface exposure and accessibility in native conformation [94]. Along with Lys-525, three other lysine residues in positions 199, 281, and 489, have been clearly established, but with a lower contribution to the overall glycation than Lys-525 [67,93,95,96]. For instance, Lys-525 appears to be the most reactive glycation site, despite its low surface exposure and accessibility in native conformation [94]. Along with Lys-525, three other lysine residues in positions 199, 281, and 489, have been clearly established, but with a lower contribution to the overall glycation than Lys-525 [67,93,95,96]. For instance, Lys-199 and Lys-525, the main glycation sites are located in the vicinity of known drug binding sites in HSA [96].

The location of these glycated sites could be explained by the presence in their close vicinity of basic amino acid residues, such as lysine or histidine. For instance, the principal glycated site, Lys-525, lies in a Lys–Lys sequence, while Lys-489 is located near a histidine residue [93]. These positively charged amino acid residues at physiological pH may afford local acid-based catalysis for Amadori rearrangement [97,98]. Lys-199 and Lys-281 are close to disulfide bridges, which place a positively charged amino group, located in a remote part of the sequence, close to these sites. A higher accessibility of some amine residues depends on the tertiary structure conformation of albumin.
Evidence for the assignment of other lysine sites, such as Lys-12, Lys-233, Lys-317, or Lys-351, is less certain [93,99]. In addition, lysine residue located at the aminoterminal part of the albumin molecule is a putative glycation site because of the contribution and involvement of the close DAHK sequence for Amadori rearrangements [100,101]. This hypothesis is reinforced by the fact that in vivo copper-albumin complex cannot be glycated. The extent of glycation in vivo depends on the glycemic status of the subject and also the length of the half-life of the protein [100]. A recent study established a strong relationship between glycated albumin levels and the number of glycation sites in the plasmatic protein [67]. Indeed, in that study, ten major glycation sites were detected in a diabetic patient with poor glycemic control (glycated albumin 94%), an intensive insulin therapy decreased glycated albumin level (19.9%) and a decreased number of glycation sites (3).

In addition, several specific lysine residues have been identified as preferential sites for glycation in HSA. Moreover, the types of early and advanced glycation adducts that involve these lysine residues have been characterized [96]. Numerous residues (Lys-12, Lys-51, Lys-199, Lys-205, Lys-439 and Lys-538) have been found to be modified through the formation of fructose-lysine, while the modification of Lys-159 and Lys-286 has been found to be involved in the formation of pyrraline and N-carboxymethyl-lysine (CML), respectively. Finally, Lys-378 has been shown to be modified by N-carboxyethyl-lysine (CEL). In addition, some lysine residues of bovine serum albumin glycated in vitro with glucose have been shown to be involved in the formation of versperlysine products [43]. Other studies revealed lysine-524 (equivalent to Lys-525 of HSA) as the major glycation site in BSA [102,103]. Other residues, such as Lys-275 (equivalent to Lys-276 of HSA), Lys-232 (equivalent to Lys-233 of HSA) and Lys-396 constituted amino acids susceptible to be glycated [46,102]. A very recent work reported similar privileged sites of glycation and galactation in human albumin [104]. Lys-12, Lys-233, and Lys-525 have been also characterized as the principal sites of galactation in HSA incubated with a high level of α-galactose.

Though less abundant in the amino acid sequence of albumin than lysine residues (23/24 for 59 lysine residues), arginine residues can also be involved in glycative modification (Fig. 2b). Of the six arginine residues identified in glycated albumin in vivo, Arg-410 appears to be the predominant site of glycation by methylglyoxal [105]. It has been reported that methylglyoxal-mediated glycation mainly involves arginine residues, and that it leads to the formation of hydroimidazolone (MG-H1, 3DG-H1 and G-H1) (Fig. 1) [41]. In that particular study, the tryptic peptide mapping of modified human serum albumin, in vitro and in vivo, by methylglyoxal, indicated the major modification at Arg-410, which is located in drug binding site II. Minor arginine sites involved in glycation, such as Arg-114, Arg-160, Arg-186, Arg-218 and Arg-428, have also been identified [96,106]. N-carboxymethyl-arginine (CMA) constitutes an acidolable AGE implying arginine residues. CMA represents the major glycated product in collagen and has been detected in small amounts in human serum albumin obtained from diabetic patients [107,108].

Finally, the thiol group of cysteine residues is a well known powerful nucleophile, which can also be glycated in vitro by methylglyoxal to give rise to advanced glycation end-products such as S-carboxymethyl-cysteine (CMC) [109]. In vivo, CMC and also S-carboxymethyl-cysteine (CEC), identified and quantified in plasma proteins from diabetic patients, suggest the involvement of Cys-34 in the formation of such products [110]. In vitro, despite the very low levels of thiol groups in both BSA and HSA (less than one sulfhydryl group compared with 83 amino and guanidino groups), Cys-34 can be highly reactive with methylglyoxal (up to 80%) [111]. In addition, the involvement of Cys-34 in glycation with this glucose derivative contributes to the formation of protein cross-links.

As seen in Fig. 1, AGE adducts exhibit a wide range of chemical structures and thereby different biological effects [41,112,113]. In a recent study, Thornalley et al. have described the quantitative screening of a comprehensive range of AGE localized in cellular and extracellular proteins and also in body fluids [114]. Noteworthy enough, they reported that among different AGE products found in blood plasma, hydroimidazolone (MG-H1, 3DG-H1 and G-H1) constitutes the more abundant (15.54, 5.922 and 0.962 μM respectively) with CML (1.109 μM) in comparison with Pentosidine or MOLD (0.474 μM and 0.042 μM, respectively). Besides, they observed an accumulation of these AGEs in plasma protein of human subjects with renal failure with an increase of about 60 to 80% for hydroimidazolone and until more than one fold for pentosidine (+180%), CML (+400%) and MOLD (+600%).

Glycoxidation of albumin in both in vitro and in vivo models is associated with important structural modifications. In particular, tertiary structure conformation, probed by tryptophan fluorescence, is significantly affected by glycation. The bovine form of serum albumin contains two tryptophan residues, Trp-134 and
Trp-214, located in hydrophobic pockets near domain I and in domain II, respectively. For the human form, the sole tryptophan, Trp-214, is located in domain II. Several studies have reported the quenching of tryptophan fluorescence in modified albumin upon glycation, reflecting a local unfolding around these residues [60,115,116]. This structural modification has been shown to be accompanied by a partial opening of hydrophobic pockets for albumin (BSA and HSA) glycated in vitro for 21 days with glucose [116,117]. Indeed, the stronger affinity of these modified proteins for the anilinonaphthalene-8-sulfonate (ANS) probe is attributed to a higher exposure of hydrophobic sites to the solvent [118]. Conversely, a longer incubation (more than 21 days) of albumin with glucose tends to inhibit penetration of ANS into the hydrophobic domains of the protein [117,119]. The decrease in hydrophobicity of the long-term modified albumin is explained by the formation of the molten globule-like state and amyloid nanofibril after 3 weeks and 20 weeks of incubation with glucose, respectively [58,117]. Alterations of tertiary structure with the glycation process generally do not significantly impact the secondary structure of the protein [116,120]. However, a prolonged incubation with carbohydrate (glucose, fructose or ribose) induces a transition in albumin from a helical to a β-sheet structure, which is the basis of amyloid formation [58]. In this last study, Sattarahmady et al. reported three different structural organizations for HSA incubated for a long time with glucose: large branched chains of globular aggregates with 20–40 nm average diameters, bundles of unbranched fibrillar aggregates with 140 nm average lengths and fine amorphous aggregates. The nature of carbohydrate attached to the protein has strong impacts on the appearances of amyloid structures. Indeed, incubation of HSA with ribose leads to formation of several forms of aggregates, such as long straight amyloid fibrils with 15–20 nm average diameter and fibrous sheet-like structures (140 nm), drastically different to those obtained for HSA incubated with glucose [58]. Glycation of albumin has been shown to be able to generate thermodynamically more stable high molecular weight aggregates with a high β-sheet structure content compared with its non-glycated form [121]. This conversion of albumin into a β-structure is well supported by the fact that both glycated ligands derived from albumin and amyloid ligands can bind to the same multiligand receptors, called t-PA, which are responsible for the regulation of fibrinolysis in blood [122]. By contrast, aggregate formation, induced by glycation, is not necessarily associated with secondary structure modification. Short-term incubation (7 days) with D-ribose induces albumin to undergo rapid misfolding and to form globular amyloid-like aggregations without any change in α-helix/β-sheet proportions [123].

In addition, glycation of albumin results in an overall stabilization in both its tertiary and secondary structure. Indeed, glycation of human albumin alters the unfolding of the protein in the presence of a chemical denaturant reagent [120]. This increase in the protein stability attributed to glycation could have an impact on life time and enhance the residence time of glycated albumin in the circulatory system. In addition, alteration in the folding pathway for albumin modified by glycation can explain the involvement of this process in reducing albumin aggregation during heating treatment [116,124]. Fig. 3 is an illustration of the role played by glycation in limiting albumin aggregation (unpublished results). These previous studies indicated that there is an inhibition in aggregate formation with increasing concentration of glucose used for albumin glycation.

MOLD or pyrraline (Fig. 1). Several fluorescence studies performed on glycated albumin have reported that glycophore formation is associated with an apparent reduction in tryptophan during glycation [59]. An later study showed that glycophore fluorescence is not dependent on the protein’s folded conformation [39]. Finally, the isolectric point of albumin is impaired by the glycation process. Indeed, the decrease in cationic charges in BSA after glycation, shown by native electrophoresis, can be attributed to the involvement of positively charged residues (arginine and lysine) in condensation with carbohydrate [125].

6. Structural consequences on the properties of albumin

Glycation-induced modifications have a determinant impact on albumin functional properties, which can be related to alteration in its conformation. In addition, glycation is accompanied by oxidative modifications, which occur during aging of the albumin molecule. Most of the available studies have shown an increase in oxidation during glycation of albumin. This oxidative state has been revealed both by an increase in the carboxylated protein level and by a decrease in the reduced state of Cys-34 [125–127]. Therefore, the antioxidative capacities of albumin are impaired as the free sulphydryl group of Cys-34 [125–128]. However, in the case of in vitro modified bovine serum albumin, it has been shown that glycation of the protein tends to strengthen its antiradical properties, while the redox status of Cys-34 remained unaffected [60]. These conflicting results suggest that the scavenging properties of albumin depend not only on the redox status of Cys-34, but also on the structural conformation of the protein. By contrast, in vitro glycation of the human form of albumin...
has been shown to impair drastically its antiradical activities [90].

*In vitro* modified albumin exhibits different behavior, which can be explained by the heterogeneous products formed by glycation, depending on the nature of the albumin used (bovine or human), the nature and concentration of the carbohydrates involved (glucose, methylglyoxal... ) and the conditions of incubation applied (3 weeks or more).

Moreover, these glycation modifications that induce conformational changes in the protein structure can also change the binding properties of albumin, though they sometimes occur at distant residues from the binding sites (Table 3). Major studies on the binding capacity of glycated albumin have been performed using a great variety of techniques including high-performance liquid chromatography, circular dichroism, nuclear magnetic resonance and fluorescence spectroscopy. According to Shaklai et al. (1984), glycation affects both the tertiary conformation of albumin and its binding functions.

In addition, Oettl et al. (2007) have contributed to this area of study, through a detailed review on the impact of oxidation on albumin binding affinity. Their review discussed contradictory data regarding the affinity of modified albumin for some ligands. For instance, in one study, the binding of trypthophan, which is a site II ligand, was found not to be affected by glycation in albumin [129], whereas, in another study, a lower affinity for this ligand was reported for albumin glycated with low concentrations of glucose [130]. Similarly, the affinity of warfarin for the site I binding site has been shown not to be affected in the case of human albumin glycated with glucose [131], while a decrease in binding occurring with early stage glycated albumin has been attributed to conformational changes or to steric hindrance of the protein [132,133]. In contrast, an excessive glycation (60 days) of albumin with a large amount of glucose (9 mol glucose/mol albumin) has been shown to enhance warfarin binding to recombinant albumin [134].

Contradictory results are also observed for copper binding capacity in aminoterminus binding sites. Indeed, for Argirova et al., early glycated albumin has a lower capacity to bind copper ions in strong complexes, with the consequence of alteration in the redox abilities of protein-copper complexes [135]. In contrast, in another study using BSA extensively glycated with glucose (500 mM), an increase in the binding capacity for copper was found in comparison with native albumin, with a maintenance of the redox activity of copper bound to glycated albumin [136]. In this last study, glycated albumin was found to be able to bind up to 3 times as much copper as native albumin. These last reports highlight the importance of having an accurate knowledge of how the glycation of albumin is performed and the methods used for the measurement of ligand binding. For instance, a decrease in the binding affinities of glycated HSA toward several fatty acids was observed by a colorimetric determination of non-esterified fatty acids (NEFA), whereas with an ANS fluorimetric method no significant change was evidenced [137]. In addition, a better knowledge of the mode of purification of the albumin (defatted or not) used for *in vitro* glycation is necessary in order to interpret binding studies accurately. Indeed, the presence of free fatty acids bound to the protein contributes to a reduction in the affinity of glycated human serum albumin for dansylsarcosine [138].

Most ligands have an affinity for modified albumin when the protein is glycated. This is true for bilirubin, whose binding capacity has been shown to reduce to about 50% in comparison with untreated albumin [59]. Similarly, monoacetyldiaminophenyl sulphone, a ligand with the location of its binding site similar to bilirubin, appears also to present a decreased affinity [139]. Binding of the site I ligands, dansylsarcosine, dansylpropion and ibuprofen (three major ligands) have been found to be less bound to modified albumin [131,140]. In parallel, the binding of some ligands of Sadow’s site I or II, including diazepam, naproxen and dansylamine has been shown to be similar in both modified and nonmodified albumin [131,132,139]. Of these ligands with an affinity for albumin not affected by glycation, captopril appears to be an interesting therapeutic drug for preventing serious long-term diabetes-related complications. Indeed, this angiotensin-converting enzyme inhibitor has the ability to reduce *in vitro* glycation of albumin and has similar binding affinities toward both native and glycated albumin. Through its unchanged pharmacokinetic parameters, captopril could provide an essential protection for diabetic patients for maintaining the structural and functional properties of the albumin molecule [141].

Regarding fatty acids, which exhibit a strong affinity for albumin via several lysine and arginine sites, non-enzymatic glycation alters slightly oleate, laurate, caproate and linoleate binding [137], whereas palmitate uptake from albumin has been shown not to be influenced by glycation [142]. Finally, because of conformational changes resulting from this process, glycation could also lead to the appearance of new binding sites for specific ligands. Indeed, native bovine albumin exhibits two main binding sites for lipoic acid, an effective antioxidant, whereas methylglyoxal-modified protein shows three sequential binding sites with a reduction in affinity for the main one [143].

Clinical implication of albumin glycation is that the binding of HSA to some drugs may be altered at various stages of diabetes [84]. Among drugs whose affinity for albumin binding are impaired in diabetic patients, feature diazepam, sulfisoxazole, phenytoin, cyclosporine and valproic acid [144]. However these alterations are not always associated with an increase in the albumin glycation rate. For some of these drugs (sulfisoxazole, phenytoin), the

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**Table 3**

Effects of glycation on albumin binding properties.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Modification of binding affinity</th>
<th>Reference</th>
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<td>Warfarin</td>
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<td>[131]</td>
<td>Diazepam</td>
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<td>Ibuprofen</td>
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<td>Danaslyamide</td>
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<td>Dansylsarcosine</td>
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<td>Phenylbutazone</td>
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<td>Salicylate</td>
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<td>Copper</td>
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<td>Iron</td>
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reduced drug binding is directly a consequence of albumin alteration by glycation [145,146]. In contrary, the decreased plasma binding for diazepam is rather caused by enhanced free fatty acid levels than by glycation [146,147]. Similarly, the lower binding of valproic acid in T1DM patients involved glucose-independent modifications in albumin molecule.

7. Biological impact on cell physiology

The structural and functional alteration of glycated proteins, including albumin, have important clinical implications. Elevated levels of glycated albumin induce irreversible damage associated with the metabolic disorders observed in diabetes mellitus, such as retinopathy, nephropathy, neuropathy and coronary artery disease [39,148].

The deleterious effects of glycated albumin and the resulting advanced glycation end-products have been highlighted in many in vivo and in vitro studies. For example, most of the available studies conducted by Cohen have shown the physiopathological connection between glycated albumin and diabetic renal deficiency and microangiopathy [149–151]. This involvement of Amadori products is confirmed by the fact that glycated albumin is preferentially transported across the renal glomerular capillaries and is taken up by mesangial and epithelial cells, with a consequent increase in oxidative products strongly involved in nephropathy development [152]. The role of glycated albumin in diabetes-related vascular complications has also been established in the case of diabetic retinopathy [153]. In addition, there is increasing evidence of the role played by advanced glycation end products derived from albumin in the promotion of cardiovascular diseases. Indeed, among its multitude properties, albumin also has an anticoagulant and antithrombotic effects through its ability to limit platelet aggregation, with the consequence of preventing cardiovascular disease progression. Several in vitro studies have pointed out the implication of glycated albumin in the up-regulation of platelet activation and aggregation [154,155]. Moreover, the hyperaggregating effects of glycated albumin have also been demonstrated in erythrocytes [156].

In addition to its implication in the pathogenesis of diabetic micro and macrovascular complications, glycation of albumin can also affect glucose metabolism in both skeletal muscle and adipocyte cells [157,158]. For instance, in mouse adipocyte cell lines, albumin-derived AGE has been shown to trigger the generation of intracellular ROS leading to an inhibition of glucose uptake [159]. The attenuation of adipocyte insulin sensitivity in the presence of glycated proteins indicates their role in the development of obesity-related insulin resistance. The causal factor of insulin resistance is oxidative stress and intracellular ROS generation. Furthermore, it is well established that advanced glycated proteins, including albumin, contribute to oxidative modification of intracellular proteins in adipocyte cells [160,161].

One mechanism through which glycated albumin affects cellular biology and consequently induces the development of these different metabolism complications, is through interaction with specific cellular receptors, the best known being the receptor for AGE, called RAGE. Interactions between cells and glycated albumin modulate signal transduction though ROS species formation (Fig. 3) [40,162]. Cellular oxidative stress triggered by AGE-derived albumin results in the activation of a cascade of intracellular signals involving p21ras and Mitogen Activated Protein Kinase (MAP-kinase) pathways enhancing phosphorylation of extracellular signal regulated kinase (ERK) [40] and culminating in the activation of the NF-kB transcription factor [163,164]. NF-kB induces firstly, the expression of various genes involved in inflammation processes, such as the cytokines Chemoattractant Peptide (MCP-1) and IL-6 in vascular smooth muscle cells [165,166]; secondly, mRNA expression of tumor necrosis factor (TNF-a) in various monocyte cell models including THP-1 and RAW 264.7 [167,168]; thirdly, extracellular secretion of TNF-a [169].

Interaction of AGE-derived albumin with RAGE also up-regulates the expression of adhesion molecules, including the intercellular adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule (VCAM-1), which are involved in atherosclerotic lesion formation [170,171]. In addition to RAGE, several other receptors of glycated albumin, which are less selective for AGE, have been characterized in macrophage cell lines, such as Galectin-3 [172] and scavenger receptors type I and II (SRB1 and SRBII) [173]. For instance, albumin–AGE is taken by mouse or human adipocyte cells via CD-36 receptor, which belongs to SRBII family [174], triggering a down-regulation of leptin expression via the ROS system in mice 3T3-L1 cell lines [175]. Noteworthy, only proteins highly-modified by AGES were shown to bind SRB1 and II of macrophages and to be eliminated from circulation [113]. Since physiological AGE-modified proteins have not yet been shown to bind to scavenger receptors, the physiological importance of AGE and scavenger receptor interaction remains unclear [113].

As described above, most studies have focused on the role of AGE in diabetes-associated vascular complications. It was only recently that the role of Amadori-glycated proteins has come under consideration [176]. Early glycation products (Amadori adducts) were shown to be also important contributors to the development of diabetic complications such as microangiopathy [149,177], Fructosylsine, which is the main Amadori adduct in extracellular proteins [178], may trigger cellular events of many cell types (macrophages, monocytes...) through ligand binding, described recently as fructosylsine receptors [179–181]. Two binding proteins of about 100 and 200 kDa, which were described as specifically interacting with Amadori-modified glycated albumin appeared to not have any homologies with AGE receptors [182]. Besides, the partial amino-acid sequencing of the 100 kDa protein showed homologies with the glycosylated nuclear protein nucleolin, while the 200 kDa binding protein was characterized as glycosylated cellular myosin heavy chain [182,183]. More recently, the nucleolar protein nucleophosmin was also localized in monocyte-like cell membrane and characterized as a component of the 200 kDa protein specifically engaged in binding of fructosylsine moieties of glycated albumin [184]. Calnexin, a transmembrane protein, was also reported to be one of the mesangial cell receptors for Amadori-glycated albumin [185].

Similarly to AGE, Amadori-modified albumin could also upregulate expression of different cellular signaling through the activation of NFkB and AP-1 via their specific receptors [40,166]. A recent study on the glycated HSA signaling pathway performed in endothelial cell, revealed an activation of NADPH oxidase activity through a non RAGE pathway, leading to E-selectin upregulation via Jun N-terminal kinase (JNK) pathway [186] (Fig. 4). Like most modified proteins, AGE-derived albumin can exhibit antigenic properties, which can elicit an autoimmune response [187,188]. The presence of autoantibodies directed against AGE-derived albumin in serum from diabetic patients provides evidence that advanced glycation of proteins, including albumin, causes their immunogenicity [189–191]. If the immunogenicity involving Amadori products is limited, terminal products trigger an intense immune response with a significant production of AGE antibodies [192]. This anti-AGE autoantibody binds to AGE modified circulating protein to form immune complexes (ICs), which are likely to be involved in the onset and progression of late complications of diabetes and particularly in the atherosclerosis process [190].

Some in vitro results indicating glycated albumin as a potent trigger for molecular mediators contributing to diabetes...
complications are supported by the results of *in vivo* studies indicating that inhibition of albumin glycation or neutralization of its biological effects can improve cell biology and functionalities and consequently prevent the progress of diabetes complications. A significant delay in the progression of diabetic nephropathy has been demonstrated in diabetic db/db mice treated with an injection of monoclonal antibodies directed against glycated albumin (A717), reducing its deleterious effects [150,193]. Similarly, inhibition of albumin glycation, with EXO-226 by impeding condensation between glucose and lysine residues, or with 23CPPA, has been shown to improve diabetes-associated abnormalities including nephropathy in the db/db mouse and in streptozotocin-diabetic rats respectively [151,194]. Diabetic vascular disease can also be attenuated by interaction blockade between AGE-derived albumin and RAGE. Intravenous injection in diabetic rat models of soluble RAGE (sRAGE), which prevents ligand/RAGE interaction or specific RAGE antibody, could favorably impact microvascular leakage and atherosclerotic lesions [195,196]. These *in vivo* findings support the concept of interaction between AGE and the cellular receptor RAGE as playing an essential role in main diabetic vascular diseases.

Regarding to these numerous studies, non-enzymatic glycation appears to play a major role in the development of diabetic complications. Following the discovery of fructosamine-3-kinase (F3NK), Swergold et al. have suggested a new theory on the etiology of diabetic complications: the non enzymatic glycation/enzymatic deglycation hypothesis [197]. They reported that intracellular nonenzymatic glycation was controlled by an enzymatic deglycation process involving F3NK which phosphorylates fructosyllysine to fructosyllysine-3-phosphate ([F3LP]) [198]. Because of its intrinsic instability, F3LP spontaneously decomposes to lysine, 3-deoxyglucosone, thereby regenerating an unmodified protein and decreasing glycated albumin level. The evidence of enzymatic deglycation activity in *vivo*, raised the question of whether enzymatic deglycation is truly an important defense mechanism or merely an epiphenomenon [199].

However, most of the available *in vitro* studies on the biological effects of glycated albumin on cellular physiology were performed with *in vitro* AGE models, which do not reflect the physiopathology of a hyperglycemic situation. In order to obtain significant cellular responses, glycated albumin models have been obtained by incubating albumin with an excessive amount of glucose (up to 1 M) beyond the physiological (5 mM) and pathological (25 mM) concentrations, which can be encountered in a normal subject or diabetic patients, respectively. For instance, Stolzing et al. focused on the ability of microglial cells to degrade extracellular AGE modified albumin prepared with 1 M of glucose [127]. In addition, the majority of the literature in this area reports the use of bovine serum albumin instead of the human form in an *in vitro* model of glycation. A recent comparative study reported the differential biological responses of monocyte cell lines treated with in vitro glycation models derived from both BSA and HSA [90]. In that particular study, glycated BSA appeared to enhance proteolytic activities in THP-1 cells, whereas glycated HSA induced an inhibition of these proteosomal activities.

Many studies dealing on cell activation by RAGE have employed albumin highly modified by AGES (30–40 modified groups per protein). Proteins modified so highly are rarely found in tissues and body fluids in vivo [114]. The role of endotoxin binding to RAGE in cell activation cannot be ignored; contamination of AGE modified albumin with endotoxin may have compromised many studies of AGES-protein/RAGE receptor involvement in proinflammatory responses. RAGE binds member of the S 100/calgranulin group of proteins: S100A12 (EN-RAGE) and S100b [200]. The protein expression of S100/calgranulin indeed increases in clinical diabetes [201] and other inflammatory disorders [113]. S100b induced increased expression of genes linked to inflammatory responses in endothelial cells, whereas endotoxin-free AGE-modified protein did not [202].

Regarding previous in vitro studies dealing with the biological effects of AGE in diabetic physiopathology, one essential question arises:

As albumin represents the most abundant protein in plasma, what is the contribution of advanced glycation end products derived from this protein in the case of biological abnormality leading to diabetes-related diseases?

### 8. Glycated albumin, an underestimated marker of diabetes?

Protein glycation is increased in diabetic patients compared to non-diabetic subjects. These glycated proteins are strongly involved in the development and progression of chronic diabetic complications. The two main clinical parameters used for chronic glycemic control are the glycated hemoglobin (HbA1C) level and the blood glucose measurement (SMBG) [203]. If measurement of glucose levels in patients with hemolytic anemia [204], or those glycated albumin levels are underestimated in relation to blood glycated hemoglobin. Most of these report that glycated hemoglobin levels are underestimated in relation to blood glucose levels in patients with hemolytic anemia [204], or those submitted to hemodialysis [61], and underestimated in patients with iron deficiency [205].

Indeed, in contrast to glucose levels, HbA1C values are profoundly affected by a shortening of the erythrocyte lifespan [206,207]. In the case of numerous chronic diseases associated with diabetes (hemolytic or renal anemia, liver cirrhosis...), HbA1C gives incorrect values and is known to be unsuitable as a glycemic control marker [208,209]. Because of its shorter half-life (21 days) compared with hemoglobin, glycated albumin can constitute a better and shorter-term glycemic control state in several diabetes-associated pathologies compared to glycated hemoglobin. Indeed, the glycated albumin level is not affected by abnormal hemoglobin metabolism encountered in some cases of type 2 diabetes [210,211]. Moreover, the rate of non-enzymatic glycation of

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**Fig. 4.** Cellular signaling pathway of AGE-derived albumin via RAGE receptor. Most cellular receptors are: SRB, scavenger receptor class B; OST-48, oligosaccharyltransferase-48; RAGE, receptor for AGE.
this protein *in vivo* is approximately 9 times that of human hemoglobin [92]. Finally, albumin glycation reaction occurs ten times more quickly than hemoglobin glycation [93]. As glycemic fluctuation can affect plasmatic protein more easily than hemoglobin, the glycated albumin level appears to be a good marker for evaluating glycemic excursion in type 1 and 2 diabetes [66].

Numerous studies have reported glycated albumin as an alternative marker for glycemic control. First of all, it has been shown that glycated albumin is strongly involved in the development of major diabetes complications, including nephropathy [212], retinopathy [213] and Alzheimer’s disease [214]. In addition, in contrast to HbA1C, glycated albumin level is associated with peripheral vascular calcification [215] and arterial stiffening [216], which are two of the most common diabetic complications. Recent clinical studies have suggested glycated albumin as the ideal marker of glycemic control in numerous physiopathological states, including hemodialysis patients [217], gastrectomized subjects [218], or for gestational diabetes [205]. All these data support the utility of glycated albumin in the detection of short-term changes in glycemic control.

However, in some pathologies that affect albumin metabolism, such as thyroid dysfunction, nephrotic syndrome or liver cirrhosis, glycated albumin level is not a suitable indicator for glucose excursion [219,220]. Furthermore, the smoking status of diabetic patients has been identified as a significant negative explanatory variable, which affects the correlation between glycated albumin and plasma glucose levels [221]. Similarly, other studies have demonstrated that glycated albumin could be influenced by other physiological or pathological conditions, such as body mass index (BMI) [222] or the age of diabetic patients [223].

### 9. Conclusions

Albumin not only represents the most abundant protein in plasma, it also constitutes the most studied protein. Even so, when discussing the importance of the protein, most underestimate the impact of protein modification, arguing the high quantity of this “sponge”, which binds all kinds of molecules in the circulation. The quantity of albumin certainly constitutes a determinant health/nutritional indicator: the odds of death increase by about 50% for each 2.5 g/l decrement in the initial albumin level [224]. The enhanced glycation status of albumin under hyperglycemic conditions leads to the occurrence of a protein that has reduced beneficial activities and that exerts new detrimental actions. In other words, following glycation, albumin disappears as a friend and emerges as a foe. Koch’s postulate that a biological compound could constitute a causative agent in a disease seems to be fulfilled by glycated albumin.

Glycated albumin appears to be an underestimated potential index to fill the gap between self-monitoring of blood glucose and hemoglobin A1C testing in diabetes management. Additional studies, especially *in vivo*, are highly warranted in order to achieve a better understanding of the impact of albumin glycation in pathological progression.

### Acknowledgments

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