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CHARACTERIZATION OF GLYCATION ADDUCTS ON HUMAN SERUM ALBUMIN BY MATRIX ASSISTED LASER DESORPTION/ IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

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Abstract

Background—Non-enzymatic glycation of human serum albumin (HSA) is associated with the long-term complications of diabetes. We examined the structure and location of modifications on minimally glycated HSA and considered their possible impact on the binding of drugs to this protein.

Methods—Minimally glycated and normal HSA (used as a control) were digested with trypsin, Glu-C or Lys-C, followed by fractionation of the resulting peptides and their analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) to determine the structures and locations of glycation adducts.

Results—Several specific lysine and arginine residues were identified as modification sites in minimally glycated HSA. Residues K12, K51, K199, K205, K439 and K538 were found to be modified through the formation of fructosyl-lysine, while the modification of K159 and K286 involved the formation of pyrraline, N_{ε}-carboxymethyl-lysine respectively. Lysine K378 was found to give N_{ε}-carboxyethyl-lysine in some forms of glycated HSA but fructosyl-lysine in other forms. Residues R160 and R472 produced a modification based on N_{ε}-(5-hydro-4-imidazolon-2-yl) ornithine. Lysine R222 was modified to produce argpyrimidine, N_{ε}-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine or tetrahydropyrimidine.

Conclusions—With the exception of K12, K199, K378, K439 and K525, all of the observed sites of modification for minimally glycated HSA were new to this current study. The fact that many of these glycation-related modifications are located at or near known drug binding sites on HSA explains why some differences have been previously noted in the binding of certain drugs to normal vs glycated HSA.

Keywords

Non-enzymatic glycation; human serum albumin; diabetes; matrix-assisted laser desorption/ ionization mass spectrometry

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1. Introduction

Human serum albumin (HSA) is the most abundant protein in serum. This protein has a molecular weight of 66.5 kDa and contains 585 amino acid residues that are arranged in a single polypeptide chain stabilized by 17 internal disulfide bonds [1]. HSA has a variety of functions in the body, including its ability to act as a buffering agent and its role in the control of colloidal osmotic pressure in blood. HSA also sequesters exogenous toxins and plays a role in binding and transporting fatty acids, hormones (e.g., L-thyroxine and steroids) and numerous drugs. HSA contains 2 major binding sites for such solutes. These sites are known as Sudlow sites I and II (i.e., the warfarin-azapropazone and indole-benzodiazepine sites) [2,3] and are located in the IIA and IIIA subdomains of HSA [4].

It is known during diabetes that HSA and other proteins in blood (e.g., hemoglobin and transferrin) can have modifications to their structure due to the addition of glucose [5,6]. This process, known as glycation, is a slow non-enzymatic reaction that initially involves the reaction of glucose with free amine groups on HSA to form a reversible Schiff base linkage, giving a glycosylamine residue [6–8]. The first step of this process is reversible; however, the glycosylamine can later undergo an Amadori rearrangement to form a stable fructosamine residue (i.e., an early stage glycation product). Further rearrangement and cleavage of this fructosamine can lead to other modifications, known as advanced glycation end products (AGEs). Figure 1 provides a summary of such products, as identified in previous work with HSA and other glycated proteins [6,9–20].

These modifications are of interest since past studies have suggested that the glycation of HSA can lead to alterations in the binding of this protein to drugs such as phenytoin, salicylate, ibuprofen, phenylbutazone and warfarin [8,21–24]. Furthermore, the effect of glycation appears to vary between different binding regions on HSA [23,24]. Determining the locations and types of modifications that occur on glycated HSA would help give a better understanding of these effects. This study examined such modifications by using *in vitro* glycated HSA, which has proposed to be a good model for glycated HSA in serum and plasma [18,19]. The extent to which HSA can be glycated *in vitro* can range from minimal levels (<10 mol modification sites per mol protein) to high levels (30–40 mol modification sites per mol protein) [9,10], with previous studies indicating that minimally glycated HSA is the better model for individuals with well-controlled diabetics [11,17].

In this study the modifications produced in minimally glycated HSA were examined by using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS). This method began by separately digesting glycated HSA and normal HSA with several proteolytic enzymes. The resulting peptides were then fractionated and analyzed by MALDI-TOF MS. Differences in the mass spectra for peptides found in the glycated and normal HSA samples were used to identify regions on HSA that are involved in the formation of early and advanced glycation endproducts (Fig. 2). Using this approach, it was possible to not only determine which sites on minimally glycated HSA were being altered but to also determine which modifications were taking place at these sites. This information was then used to provide clues as to how glycated HSA may differ from normal HSA in its binding to drugs and other small solutes.

2. Materials and Methods

2.1 Materials

The minimally glycated HSA was from Sigma-Aldrich (St. Louis, MO), with the same batch being used throughout this study (~95% protein and 99% pure HSA glycated *in vitro*, giving an assayed value of 8.0 mol modified sites per mol protein). The trypsin (sequencing grade), endoproteinase Lys-C (Lys-C, sequencing grade), and endoproteinase Glu-C (Glu-C,

sequencing grade) were also from Sigma-Aldrich. The guanidine hydrochloride (>99% pure), D/L-dithiothreitol (DTT, >99% pure), iodoacetamide (99% pure) and formic acid (96% pure) were also from Sigma-Aldrich, as well as α-cyano-4-hydroxycinnamic acid (CHCA, >99% pure), 2,5-dihydroxybenzoic acid (DHB, 98% pure), *des*-Arg-bradykinin (99% pure), angiotensin I (97% pure, acetate salt), and Glu-fibrinopeptide B (97% pure). The Slide-A-Lyzer dialysis cassettes (7 kDa MW cutoff, 0.5–3 ml capacity) were from Pierce (Rockford, IL). All aqueous solutions were prepared using water from a Nanopure water system (Barnstead, Dubuque, IA) and filtered using 0.22 μm nylon filters from Fisher (Pittsburgh, PA).

2.2 Apparatus

Small volume samples and solutions were measured and applied using a 0.5–10 μl digital pipette from Fisher Scientific (Pittsburgh, PA). ZipTip$_{\mu\text{-C18}}$ pipette tips (5.0 μg capacity) were from Millipore (Billerica, MA). The overhead transparency film for sample/matrix mixing was from C-line Products (Des Plaines, IL). The MALDI-TOF MS experiments were carried out with a Voyager 6184 system (Applied Biosystems, Foster City CA) operated in a positive-ion delayed extraction reflection mode. The instrument settings for this analysis were as follows: accelerating voltage, 20 kV; grid voltage, 76% of the accelerating voltage; guide wire voltage, 0.008% of the accelerating voltage; delay time, 100 ns.

2.3 Sample Pretreatment & Digestion

The glycated and normal HSA samples were denatured, reduced and alkylated before digestion according to a previous described procedure [25]. First, 3 mg of glycated or non-glycated HSA was denatured by dissolving it in 1 ml of a denaturing solution containing 6 mol/l guanidine hydrochloride in pH 8.5, 100 mmol/l ammonium bicarbonate buffer, followed by shaking of this mixture for 1 min. Next, 15 μl 1.0 mol/l DTT dissolved in pH 8.2, 100 mmol/l ammonium bicarbonate buffer was added to this solution. The new mixture was incubated at 37°C for 30 min to break the disulfide bonds in the protein sample. Finally, a 36 μl portion of 1.0 mol/l iodoacetamide in 1.0 mol/l sodium hydroxide was then added to alkylate the free cysteine groups generated in the glycated or non-glycated HSA. This solution was incubated in the dark for 30 min at room temperature. A 150 μl portion of the 1.0 mol/l DTT reagent was then added to remove any remaining iodoacetamide in the sample.

After pretreatment, a 400 μl portion of the glycated or non-glycated HSA solution (i.e., about 1 mg protein) was placed into a 0.1–0.5 ml dialysis cassette to remove excess chemicals before digestion with trypsin. The sample was dialyzed twice for 4 h at room temperature against 2 500 ml portions of water, followed by dialysis against 500 ml of pH 7.8, 100 mmol/l ammonium bicarbonate buffer for an additional 4 h at room temperature. A 1 μg/μl solution of trypsin was prepared in pH 7.8, 100 mmol/l ammonium bicarbonate buffer. A 50 μl aliquot of the dialyzed HSA sample (containing about 75 μg HSA) was combined with 2.5 μl of the trypsin solution (giving a substrate-to-enzyme ratio of 30:1), with this mixture being incubated at 37 °C for 18 h.

Another 400 μl of the pretreated HSA solution (to be used for digestion with Glu-C) was placed into a 0.1–0.5 ml dialysis cassette. This sample was dialyzed twice against fresh portions of 500 ml water for 4 h at room temperature, followed by dialysis against 500 ml of pH 7.8, 100 mmol/l ammonium bicarbonate buffer for another 4 h. A 1 μg/μl solution of Glu-C was prepared in pH 7.8, 100 mmol/l ammonium bicarbonate buffer. A 50 μl aliquot of the dialyzed HSA solution (containing about 75 μg HSA) was combined with 7.5 μl of the Glu-C solution (giving a substrate-to-enzyme ratio of 10:1) and incubated at 37°C for 8 h. An additional 3.8 μl of the Glu-C solution (giving a new substrate-to-enzyme ratio of 20:1) was later added to the sample and incubated for another 18 h at 37°C.

Samples to be digested with Lys-C were prepared by taking a 400 μl aliquot of a pretreated glycated or non-glycated HSA solution (containing about 1 mg HSA) and placing this into a 0.1–0.5 ml dialysis cassette. This sample was then dialyzed twice against fresh portions of 500 ml water for 4 h at room temperature, followed by dialysis against 500 ml of pH 9.0, 100 mmol/l Tris-HCl buffer for another 4 h. Lys-C was prepared as a 1 μg/μl solution in pH 9.0, 100 mmol/l Tris-HCl buffer. A 50 μl aliquot of the dialyzed HSA (containing about 75 μg protein) was combined with 2.5 μl of the Lys-C solution (giving a substrate-to-enzyme ratio of 30:1) and incubated at 37 °C for 18 h.

After digestion (with either trypsin, Glu-C or Lys-C), 50 μL of the resulting digest for glycated or non-glycated HSA was combined with 5 μl of concentrated formic acid or 5 μl of 1% trifluoroacetic acid (TFA) to adjust the pH to <4. Each of these samples was then divided into 10μl aliquots and stored at −20°C prior to further use.

The $ZipTip_{\mu\text{-}C18}$ pipette tips were treated with 10 μl of a 50% (v/v) solution of acetonitrile and water, followed by a wash with 10 μl of 0.1% TFA. A 10 μl aliquot of the desired digest of glycated or non-glycated HSA digest was loaded onto the $ZipTip_{\mu\text{-}C18}$ pipette tip by performing 15–20 aspiration-dispensing cycles with this aliquot. Salts in the HSA sample were washed away by twice applying 10 μl of a 5% (v/v) mixture of methanol with water containing 0.1% TFA. Peptides retained by the pipette tip were eluted with a series of 1 μl washes with solvents containing 0.1% TFA in water plus 5%, 10%, 20%, 30%, or 50% (v/v) acetonitrile. The use of this step elution scheme with the ZipTips was found to quite reproducible, as indicated by the fact that almost all of the non-glycated peptides identified in the different elutes of glycated HSA digests were also identified in the same elutes of non-glycated HSA digests (see reference [25] and Supplemental Information). An alternative approach to this fractionation method would be to use microbore HPLC coupled with a fraction collection on a MALDI plate.

2.4 Mass Spectrometric Analysis

The matrix used for the MALDI-TOF MS analysis of glycated and normal HSA digests was a mixture of CHCA and DHB [25,26]. This matrix was obtained by first preparing a 20 μg/μl solution of CHCA in a 70:30 (v/v) mixture of acetonitrile and 5% formic acid; a DHB solution was prepared in a similar manner but was instead placed into a 70:30 (v/v) mixture of acetonitrile and 0.1% TFA. These CHCA and DHB solutions were combined in a 1:1 (v/v) ratio. A 0.5 μl aliquot of this matrix and 0.5 μL of a digested sample of glycated or normal HSA (containing about 15 pmol protein) were placed on a transparency film and mixed together with a pipette tip. The final mixture was aspirated and applied by pipette onto a spot on a MALDI plate.

A stock solution of several standard peptides was prepared by combining 18.4 μλ of 1 μg/μl des-Arg-bradykinin, 33.6 μl of 1 μg/μl angiotensin I, and 408 μl of 0.1 μg/μl Glu-fibrinopeptide B with 7540 μl of a 50:50 (v/v) mixture of acetonitrile and water. This stock solution was divided into several aliquots and frozen at −80°C until use. A 4 μl portion of this stock solution was mixed with 96 μl of the MALDI matrix, giving a final concentration for each standard peptide in this mixture of approximately 1.0–1.3 pmol/μl. A 1 μl portion of this standard mixture was spotted on each well of every other row on the MALDI plates used in this study. The spotted MALDI plate was allowed to air dry for 15–20 min before analysis. The mass scale on the mass spectrum was adjusted based on the calibration data obtained with the given standard peptide mixture.

Each sample spot on the MALDI plate was examined using a final mass spectrum that represented the sum of 250 laser shots over a mass range of 500–3500 Da. All peaks containing ions with a single charge and having a signal-to-noise ratio greater than five were considered

for further analysis. The mass accuracy was estimated to be better than 50 ppm under these conditions.

2.5 Analysis of Peptides in Digests of Glycated & Normal HAS

Non-glycated peptides in the digests of glycated or normal HSA were identified by comparing their observed masses in the mass spectra with those masses that were predicted by PeptideMass using the primary sequence of HSA within ≤50 ppm [25,27]. Modified peptides were initially identified by using those peaks that appeared only in mass spectra for glycated HSA under a given set of digestion and analysis conditions.

The identification of potential modified regions on glycated HSA relied on the relative mass shift (ΔM) caused by the formation of glycation adducts (Fig. 3). First, a "Theoretical Digestion Table" was generated from the primary sequence of HSA by using PERL script algorithm digest.pl [15] along with the following parameters: 1) all cysteines were assumed to be treated with iodoacetamide; 2) the oxidation of methionines was allowed, and 3) the maximum number of missed cleavage sites allowed was three. Next, the expected difference in mass (ΔM) was subtracted from the masses of all peaks seen in only the digests for glycated HSA; these results were then saved as a text file. This latter file was then compared with peptide masses listed in the "Theoretical Digestion Table" for a digest of HSA by using PERL script alogorithm searchSIM.pl. All mass values in this text file that could be matched within 50 ppm to a predicted mass were considered further as potential regions for modification in glycated HSA. This level of mass accuracy was found to be sufficient for assigning the structures of these modifications based on the observed masses by MALDI-TOF MS, as has noted in previous work [28,29].

The types of modifications that occur in minimally glycated HSA were initially determined based on the observed change in the mass-to-charge ratios for peptides from these regions vs the mass-charge ratios for non-glycated peptides (note: this approach works well for a preparation of a single protein, as used in this study; however, for more complex mixtures it may be necessary to use multiple stages of mass spectrometry to first isolate individual peptide ions and then fragment these ions for their identification and assignment of modifications). The modification sites on peptides that contained more than one possible residue for such changes were identified by comparing the pK_a values and fractional solvent accessible surface areas of the lysines and arginines residues in these peptides. The pK_a values were calculated using PROPKA [30]. The fractional solvent accessible surface areas were calculated by using VADAR, along with a solvent probe radius of 1.4 Å [31]. The regions determined to be at or near possible modification sites were then mapped onto the 3-dimensional structure of HSA by using Protein Explorer 2.45 Beta [32].

3. Results and Discussion

3.1 General Considerations in Method Development

The potential modification sites on HSA that could, in theory, be involved in glycation or AGE formation include 59 lysines, 23 arginines and the *N*-terminal amine. These sites are distributed in a relatively uniform pattern throughout both the primary sequence and tertiary structure of HSA, as shown in Figure 4. To examine as many of these possible modification sites as possible by MALDI-TOF MS, the samples examined in this work were treated using multiple enzyme digestion (based on trypsin, Lys-C and Glu-C) and ZipTip fractionation. When using this approach to study normal HSA, a sequence coverage of 97.4% has been reported [25]; the same conditions were used in this current study to examine both glycated and normal HSA.

When only the non-modified peptides in the digests of glycated HSA were considered during this study, the sequence coverage of the glycated HSA was 97.8% under the conditions used in Ref. [25]. When both modified and non-modified were considered, the overall sequence coverage for glycated HSA reached 98.8% (see Fig. 4). This sequence coverage included 58 out of 59 lysine residues in HSA (98.3%) and all of the arginine residues in this protein. Of all the sites on HSA that could potentially be involved in glycation or AGE formation, only residue K4 and *N*-terminus were not included in this sequence coverage. The most likely reason why these residues were not detected is that the relatively small peptide containing these sites and formed from residues 1–4 (DAHK, 469.24 Da) was obscured or suppressed by the matrix or other low mass peaks that occurred in the mass spectra for normal HSA and glycated HSA.

The sequence coverage obtained in this study was found to be sufficient for examining the locations of all possible non-crosslinking modifications that have been previously reported for minimally glycated HSA that have been previously prepared *in vitro*, as based on chromatographic studies [9,33]. As indicated in Figure 1, these modifications include both early and advanced glycation adducts involving lysine and arginine residues.

3.2 Tryptic Digests of Glycated HAS

A total of 10 modified peptides were identified in tryptic digests of glycated HSA, representing 23.2% of the primary sequence of HSA (see the Supplemental Information for a list of these and all other peptides that were detected in this study). A total of 53 non-modified peptides were also identified in tryptic digests of glycated HSA, covering 82.4% of the sequence for this protein. When these results for modified and non-modified peptides were combined, the overall sequence coverage for the glycated HSA was found to be 91.3% based on only its tryptic digest.

Among the ten modified peptides that were identified in the tryptic digests of glycated HSA, all but one peptide could be assigned to a given sequence of HSA within a mass tolerance of 50 ppm. This particular peptide had a mass of 1820.00, giving it a fit with either residues 191-205 (with a mass increase of 144.04) or 337-351 (with a mass increase of 39.99) within a mass tolerance of 50 ppm. However, it gave the best match to residues 191-205 when using a mass tolerance of 20 ppm.

An example of one modified peptide noted in the digest of glycated HSA had a mass of 971.50, as seen in Figures 5(b). This peptide was not detected in the digests of normal HSA under the same digestion and analysis conditions, as indicated in Figure 5(a). The mass of this peptides indicated that it originated from the 535–541 section of HSA and represented a fructosyl-lysine modification, giving a mass increase of 162.05 Da vs the theoretical mass of the non-glycated peptide from the same region in normal HSA. Although the mass for many of the modified peptides seen in such spectra could be linked to a particular type of modification, those glycated peptides with a mass increase of 144.04 Da (e.g., modified peptides originating from the 191-205, 219-240 and 349-372 regions of HSA) could have been due to the formation of a fructo-lysine with the subsequent loss of one water (FL-1 H_2O), the formation N_ε-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl)ornithine (3-DG-H1) or the creation of tetrahydropyrimidine (THP) at an arginine residue. In some cases it was possible to discriminate between these possibilities since there were only lysine or arginine residues in the given section of HSA. There was also one glycated peptide in the tryptic digest that had two modifications. This latter peptide originated from the 196-209 region of HSA and involved the addition of glucose to both K199 and K205, resulting in a total mass increase of 324.11 Da.

A comparison of non-modified peptides found in tryptic digests of normal HSA [25] and those noted in this study for minimally glycated HSA gave similar results for most of these peptides. However, there were five peptides that were only observed in the tryptic digests of normal

HSA. These five peptides had masses of 658.33, 875.50, 1305.61, 2545.17 and 2599.28 Da, representing residues 94-98, 219-225, 277-286, 501-521 and 390-410, respectively. One reason these peaks may not have appeared in the tryptic digests of glycated HSA is due to their complete shift to a different mass as a result of modification. For example, a peak noted with a mass of 875.51 Da in the tryptic digests of normal HSA shifted to a mass of 955.54 Da in glycated HSA as a result of the addition of argpyrimidine on R222. Another possible cause of this effect, as will be seen later in the work with Lys-C, is that sometimes the glycated HSA and normal HSA gave different digestion patterns for trypsin due to modifications that occurred at or near the terminus of a given peptide sequence. A similar effect has been noted in a previous study of highly glycated HSA [14].

3.3 Glu-C Digests of Glycated HAS

Digests prepared with the enzyme Glu-C were also used with MALD-TOF MS to examine the modified and non-modified peptides in minimally glycated HSA. A total of 11 modified and 30 non-modified peptides were identified in these digests, representing 20.7% and 71.1% of the primary sequence of HSA, respectively. When the results were combined, the overall sequence coverage for glycated HSA in the Glu-C digest was 77.8%.

Of the 11 modified peptides that were identified for glycated HSA in the Glu-C digest, 10 were found to have unambiguous locations in the sequence of HSA when using a mass tolerance of 50 ppm. The only peptide that did not have a specific assigned site within a mass tolerance of 50 ppm was a peptide with a mass of 2118.07. This mass gave a fit with that predicted for either residues 384-400 after a N_{ε}-carboxymethyl-lysine modification or with residues 426-442 after a fructosyl-lysine modification. However, the best match (within a mass tolerance of 10 ppm) for this peptide was to residues 426-442 after a fructosyl-lysine modification.

Figure 5(d) showed an example of one modified peptide detected in the Glu-C digest of glycated HSA. This peptide had a mass of 1130.56 and was not detected in the digests of normal HSA under the same digestion and analysis conditions, as shown in Figure 5(c). It was identified that this peptide comes from the 286-294 section of HSA and represents a N_{ε}-carboxymethyl-lysine modification at K286, giving a mass increase of 58.01 Da vs the theoretical mass of the non-glycated peptide from the same region in normal HSA.

A comparison of non-glycated peptides in the Glu-C digests of normal HSA [25]and glycated HSA revealed that three peptides were present only in the Glu-C digests of non-glycated HSA. These peptides had masses of 599.35, 1432.72 and 1798.83 Da, representing residues 281-285, 322-333 and 506-520. Modifications at or near these regions are again thought to be responsible for the lack of appearance of these peaks in the mass spectra for glycated HSA. For example, the absence of a peak with at mass of 599.35 Da (originating from residues 281-285) in the glycated HSA implies that either K281 or K286 was modified. This was supported by the appearance of a modified peptide originating from 286-294 in the Glu-C digests of glycated HSA that resulted from the CML modification of residue K286.

3.4 Lys-C Digests of Glycated HAS

A total of 2 modified peptides and 44 non-modified peptides were identified in the Lys-C digests of glycated HSA, representing 4.4% and 75.7% of the primary sequence of HSA. When both the modified and non-modified peptides were considered together, the overall sequence coverage for glycated HSA in the Lys-C digest was 75.7%.

A comparison of non-glycated peptides in the Lys-C digests of normal HSA [25] and glycated HSA revealed that three peptides were present in only the digests of normal HSA. These peptides had masses of 544.34, 1498.54 and 3119.56 Da, representing residues 196-199, 52-64

and 390-414 of HSA. As was noted for the tryptic and Glu-C digests, modifications at or near these regions is thought to be responsible for the lack of any peaks from these peptides in the mass spectra for glycated HSA. As an example, the lack of a peak with a mass of 1498.54 Da implied that either K51 or K64 was being modified in glycated HSA. This was supported by the fact that one modified peptide originating from sequence 49-57 was found in the Glu-C digests of glycated HSA, representing the formation of a fructosyl-lysine residue at K51.

3.5 Characterization of Modification on Glycated HAS

The results for the various digests were combined to determine the specific residues in minimally glycated HSA that were being modified. Some of these modification sites could be clearly identified because only a single possible site of modification (e.g., a single lysine or arginine) was present in the modified peptide's sequence. This group of peptides included modification sites that involved seven lysine and three arginine residues, as summarized in Table 1. Each of these residues had 1 specific type of modification except K378, which was modified to give a N_{ε}-carboxyethyl-lysine (CEL) in some forms of minimally glycated HSA but underwent a fructosyl-lysine modification in others.

There were also some modified peptides where 2 or more potential modification sites were available. This second group is summarized in Table 2. For these peptides, further analysis was performed to determine the most likely residues that were involved in the modification process. This analysis included estimation and comparison of the pK_a values of all lysines or arginines in these peptides, as well as a comparison of the calculated fractional accessible surface areas for these lysine and arginine residues. During this analysis, K199, R222, K439 and K538 were all identified as likely modification sites because they had either relatively low pK_a values (K199 and R222) or large fractional accessible surface areas (K439 and K538) vs other lysines or arginines in the same regions of glycated HSA. For example, K199 has a predicted pK_a of 7.47 vs a range of 6.23–11.11 and average of 10.08 for all lysines in HSA; similarly, R222 has a predicted pK_a of 7.56 vs a range of 7.56–16.02 and average of 12.34 for all arginines in HSA. This feature is important to consider since lysines or arginines with low pK_a values would have a larger fraction of their residues in a non-protonated state, as is required for the nuclophilic substitution and/or nucleophilic addition during glycation for AGE formation [34,35]. Examples of residues in Table 2 with good accessibility to the surrounding solvent are K439 and K538, which have ASA values of 0.94–0.96 vs a range of 0.01–0.96 and average of 0.52 for all lysines on HSA; this high level of accessibility should give these residues a higher chance of interacting with glucose or related intermediates involved in AGE formation.

For some peptides in Table 2, none of the potential modification sites had either a low pK_a or high ASA values. In these cases other factors such as structural flexibility or a favorable microenvironment may be required for a given lysine or arginine to take part in glycation. As an example, K525 was found both in this study and in previous *in vivo* [36] or *in vitro* [14] studies to be a potential site of glycation even though it does not have a particularly low pK_a or high level of accessibility to solvent.

Previous chromatographic studies of modifications that occur in minimally-glycated HSA have noted mainly FL and CML residues, plus minor amounts of modifications involving N_{ε}-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1), argpyrimidine, 3-DG-H1, THP and N_{ε}-(5-hydro-5-methyl-4-imidazolon-2-y1)ornithine (MG-H1) [9]. In this earlier work, the average concentrations of FL and CML modifications were determined to be 1.21 and 1.42 mol/mol HSA, respectively, but the locations of these modifications were not reported. In this current study, it was found that FL modifications could occur on HSA at K12, K51, K199, K205, K378, K439 and/or K538 (Tables 1 and 2). Other possible sites of FL modifications included K136 or K137, K159 or K162, K212 or K225 and K351 or K359. The only site found to have any detectable amount of CML modification was at K286. It was further found that the sites on

minimally glycated HSA that gave detectable amounts of G-H1 and argpyrimidine modifications were R160 or R472 and R222, respectively. Since both THP and 3-DG-H1 modified peptides have a mass shift of 144.04, these particular modifications could not be discriminated based only on the mass spectra used in this study. However, it was found that the only site on HSA with have any measurable amount of modification by THP or 3-DG-H1 was again R222. This is not surprising considering the fact that R222 has the lowest predicted pK_a value (7.56) of all arginine residues on HSA. No sites on minimally glycated HSA that were modified by MG-H1 were noted in this current study. This may be either due to the low intensity of the MG-H1 modified peptides or their suppression on the mass spectrometry by the matrix or other peptide peaks produced in this work. In addition, pyralline was found on K159 in this study, as well as on K378 or K389 and K573 or K574; this particular modification has not been noted in previous work with glycated HSA and is unique to this current study.

Of the various modification sites listed in Tables 1 and 2, 4 (K12, K199, K439 and K525) have been identified in a previous studies of *in vivo* glycated sites of HSA [36–38]. In addition, modification at K378 has been reported based on MALDI-TOF analysis of highly glycated HSA that has been produced *in vitro* [14]. The remaining modification sites (involving at least five lysines and three arginines) for minimally glycated HSA have not been reported in previous work. These newly discovered modification sites included lysines K51, K159, K205, K286, and K538, as well as arginines R160, R222 and R472, among others.

Figure 6 shows the locations of these various modification sites on the three dimensional structure of HSA. This figure also includes the known locations of the 2 major drug binding sites on HSA (i.e., Sudlow sites I and II). As is shown in this figure, modifications that occur at K199, R222 and K286 would be located at or near Sudlow site I, while a modification at K439 would be close to Sudlow site II. In addition, a modification at K378 would be located on the same helix (h1 in Subdomain III of HSA) as residues P384, L387, I388 and N391, which are all involved in Sudlow site II.

The results in Figure 6 support the belief that glycation or AGE formation at some of these residues could lead to altered binding of drugs and small molecules at Sudlow sites I and II. These changes in binding might occur due to a change in local charge or secondary structure as a result of glycation [39,40]. This may explain why several drugs used to probe binding at Sudlow site II (e.g., dansylproline, ibuprofen and flufenamic acid) and Sudlow site I (e.g., warfarin and phenylbutazone) have been observed to give different binding behavior for glycated HSA vs normal HSA [23,24]. These results suggest that further research is warranted in examining the effects of glycation on the binding of drugs to HSA and on the changes that may occur in drug behavior as a result of such modifications.

4. Conclusions

In this work, MALDI-TOF MS was used to characterize the modification sites and structures of glycation and AGE products on minimally glycated HSA that was prepared *in vitro*. The use of a multiple enzyme digestion and peptide fractionation before MALDI-TOF MS analysis allowed 98.8% of the primary sequence of HSA to be examined in this study. A comparison of the results for glycated and non-glycated HSA lead to the identification of several specific lysine and arginine residues as likely modification sites in minimally glycated HSA. These residues included K12, K51, K159, K199, K205, K286, K378, K439, K538, R160, R222 and R472.

The types of modifications that were occurring at each of these residues were also determined. For instance, it was found that K12, K51, K199, K205, K439 and K538 were modified through the formation of fructosyl-lysine (with or without the loss of one or two molecules of water).

Modifications at residues K159 and K286 involved the formation of pyrraline and N_ε-carboxymethyl-lysine, respectively. Lysine K378 was modified by N_ε-carboxyethyl-lysine in some forms of glycated HSA but underwent a fructosyl-lysine modification in other forms of this same protein. Residues R160 and R472 were modified by reaction with G-H1. Lysine R222 was modified to give argpyrimidine in some forms of glycated HSA but was modified to give 3-DG-H1 or THP in other forms. Further studies based on quantitative proteomics are currently in progress to determine the relative extent to which each of these sites is modified in glycated HSA. The extension of these studies to *in vivo* glycated HSA is also being considered.

With the exception of K12, K199, K378, K439 and K525, all of the observed modification sites for minimally glycated HSA are new to this current study. The locations of these modification sites were also noted on the three dimensional structure of HSA and compared to the known locations of the two major drug binding sites on HSA. It was found from this comparison that many of these modifications were near these drug binding sites. This could explain why some differences have been previously noted in the binding of certain drugs to glycated vs non-glycated HSA. The results in this paper will also provide important structure information in examining the effects of glycation on the binding of drugs to HSA and on the changes that may occur in drug behavior as a result of such modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

AGE
advanced glycation endproduct

AFGP
1-alkyl-2-formyl-3,4-glycosyl-pyrrole

CEL
N_ε-carboxyethyl-lysine

CML
N_ε-carboxymethyl-lysine

3-DG-H1
N_ε-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine

FL
fructosyl-lysine

G-H1
N_ε-(5-hydro-4-imidazolon-2-yl)ornithine

HSA

human serum albumin

MG-H1

N_{ε}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine

THP

tetrahydropyrimidine

TFA

trifluoroacetic acid

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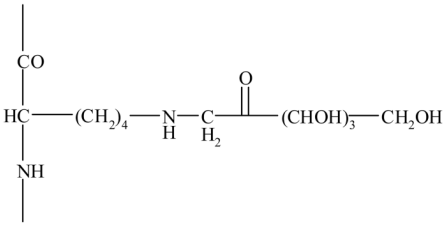
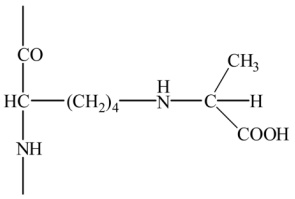
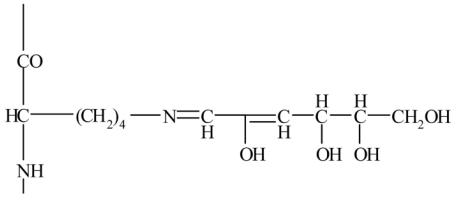
(a) Early and advanced glycation adducts involving lysine residues



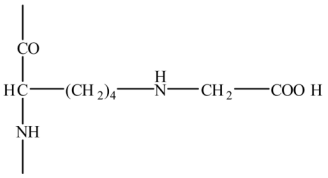
Fructosyl-lysine
(FL; ΔM = 162.0528 Da)



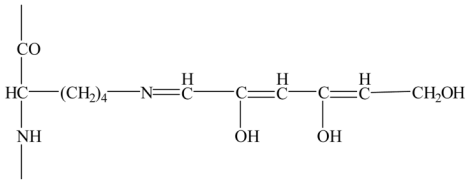
N_{ε}-Carboxyethyl-lysine
(CEL; ΔM = 72.0211 Da)



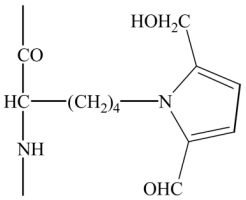
Fructosyl-lysine-1 H_2O
(FL-1 H_2O; ΔM = 144.0423 Da)



N_{ε}-Carboxymethyl-lysine
(CML; ΔM = 58.0055 Da)

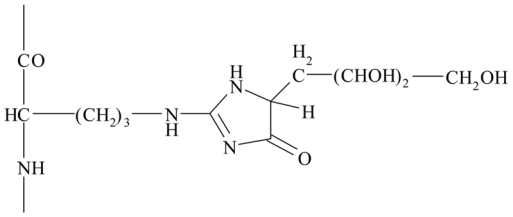


Fructosyl-lysine-2 H_2O
(FL-2 H_2O; ΔM = 126.0317 Da)

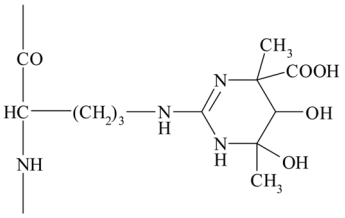


Pyrraline
(ΔM = 108.0211 Da)

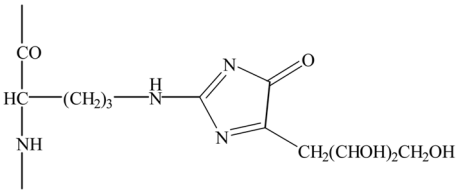
(b) Advanced glycation adducts involving arginine residues



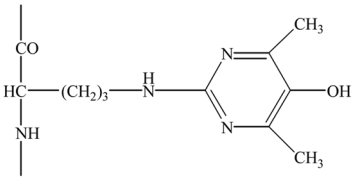
N_{ε}-[5-(2,3,4-Trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine
(3-DG-H1; ΔM = 144.0423 Da)



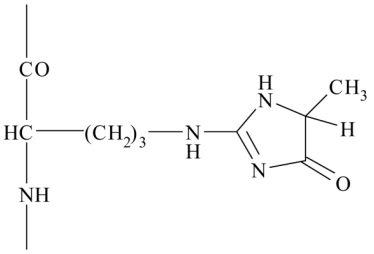
Tetrahydropyrimidine
(THP; ΔM = 144.0423 Da)



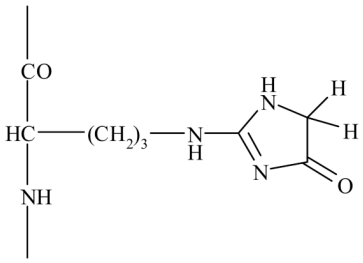
Imidazolone B
(ΔM = 142.0266 Da)



Argpyrimidine
(ΔM = 80.0262 Da)

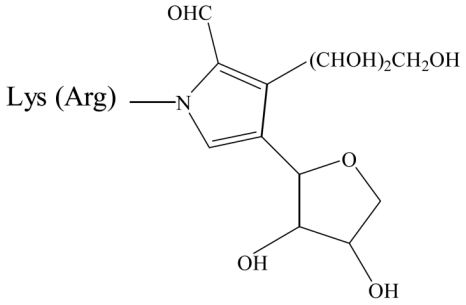


N_ε-(5-Hydro-5-methyl-4-imidazolon-2-yl)ornithine
(MG-H1, ΔM = 54.0106 Da)



N_ε-(5-Hydro-4-imidazolon-2-yl)ornithine
(G-H1, ΔM = 39.9949 Da)

(c) Advanced glycation adducts involving lysine or arginine residues



1-Alkyl-2-formyl-3,4-glycosyl-pyrrole
(AFGP; ΔM = 270.0740 Da)

Figure 1.
Possible early and advanced glycation adducts that involve lysine residues (a), arginine residues (b) or either lysine or arginine residues (c) on a protein.

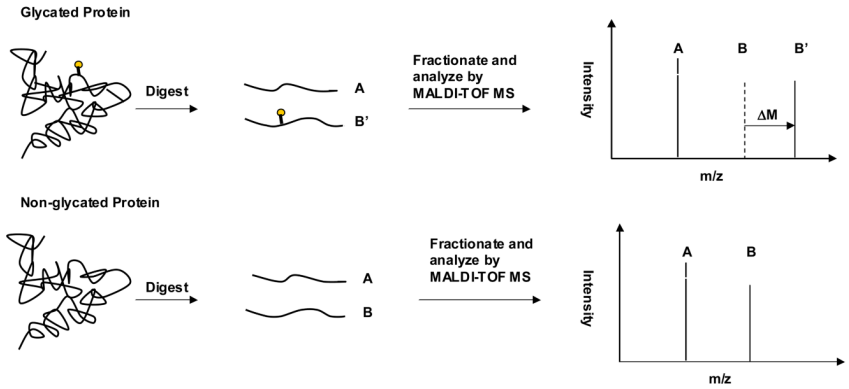


Figure 2.
Use of MALDI-TOF MS and peptide mapping to study modification sites on a glycated protein.

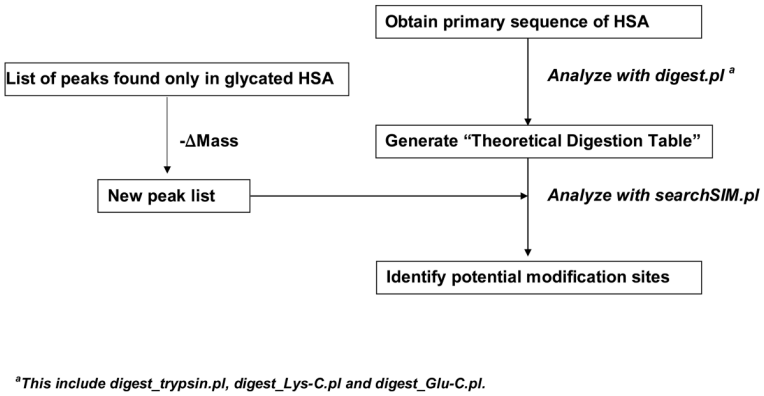


Figure 3.
Strategy used for identifying potential modification sites on glycated HSA

(a) Residue No. Amino Acid Sequence

```
  1-50   DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV KLVNEVTEFA
         ****** ********** ********** ********** **********
 51-100  KTCVADESAE NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE
         *******  ********** ********** ********** **********
101-150  CFLQHKDDNP NLPRLVRPEV DVMCTAFHDN EETFLKKYLY EIARRHPYFY
         ********** ********** ********** ********** **********
151-200  APELLFFAKR YKAAFTECCQ AADKAACLLP KLDELRDEGK ASSAKQRLKC
         ********** ********** ********** ********** **********
201-250  ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK VHTECCHGDL
         ********** ********** ********** ********** **********
251-300  LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA
         ********** ********** ********** ********** **********
301-350  DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLLRLA
         ********** ********** ********** ********** **********
351-400  KTYETTLEKC CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE
         ********** ********** ********** ********** **********
401-450  YKFQNALLVR YTKKVPQVST PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE
         ********** ********** ********** ********** **********
451-500  DYLSVVLNQL CVLHEKTPVS DRVTKCCTES LVNRRPCFSA LEVDETYVPK
         ********** ********** ********** ********** **********
501-550  EFNAETFTFH ADICTLSEKE RQIKKQTALV ELVKHKPKAT KEQLKAVMDD
         ********** ********** ********** ********** **********
551-585  FAAFVEKCCK ADDKETCFAE EGKKLVAASQ AALGL
         ********** ********** ********** ******
```

(b) Lysines in HSA (c) Arginines in HSA

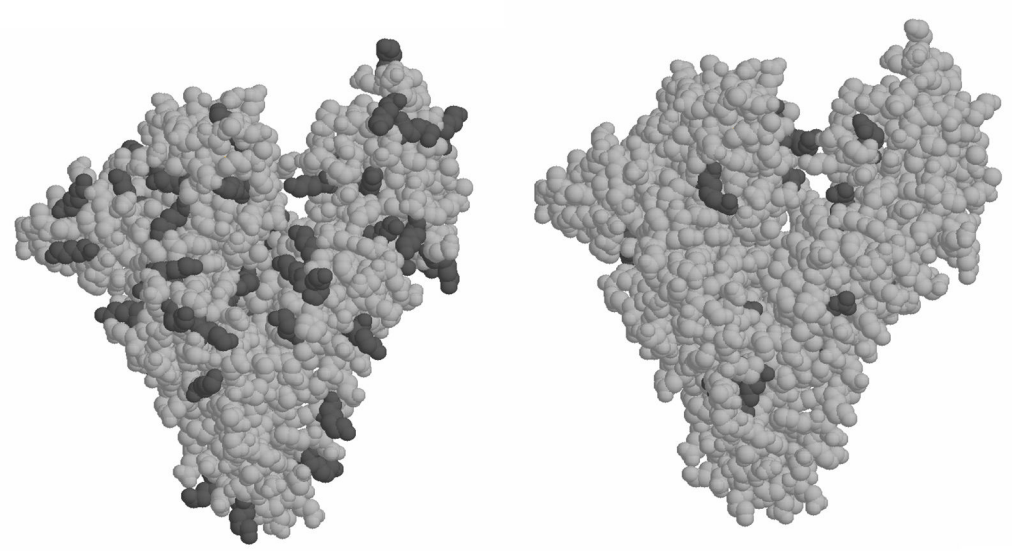
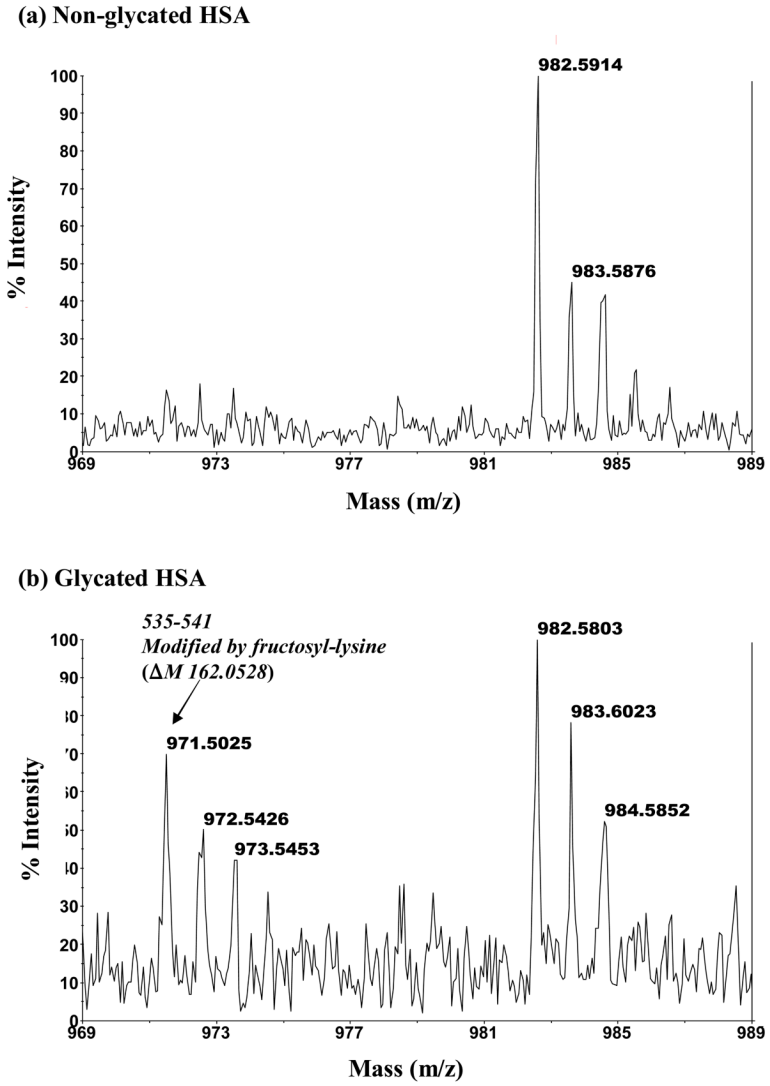


Figure 4.

(a) Primary sequence and (b–c) crystal structure of HSA. The lysine residues (K) are given in italics in (a) and as darkened regions in (b). The arginine residues (R) are given in bold in (a) and as darkened regions in (c). The sequence in (a) shown the regions of the primary sequence that could be identified by MALDI-TOF MS for normal HSA (___) and minimally glycated HSA (***). The plots (b) and (c) are based on PDB file 1AO6.



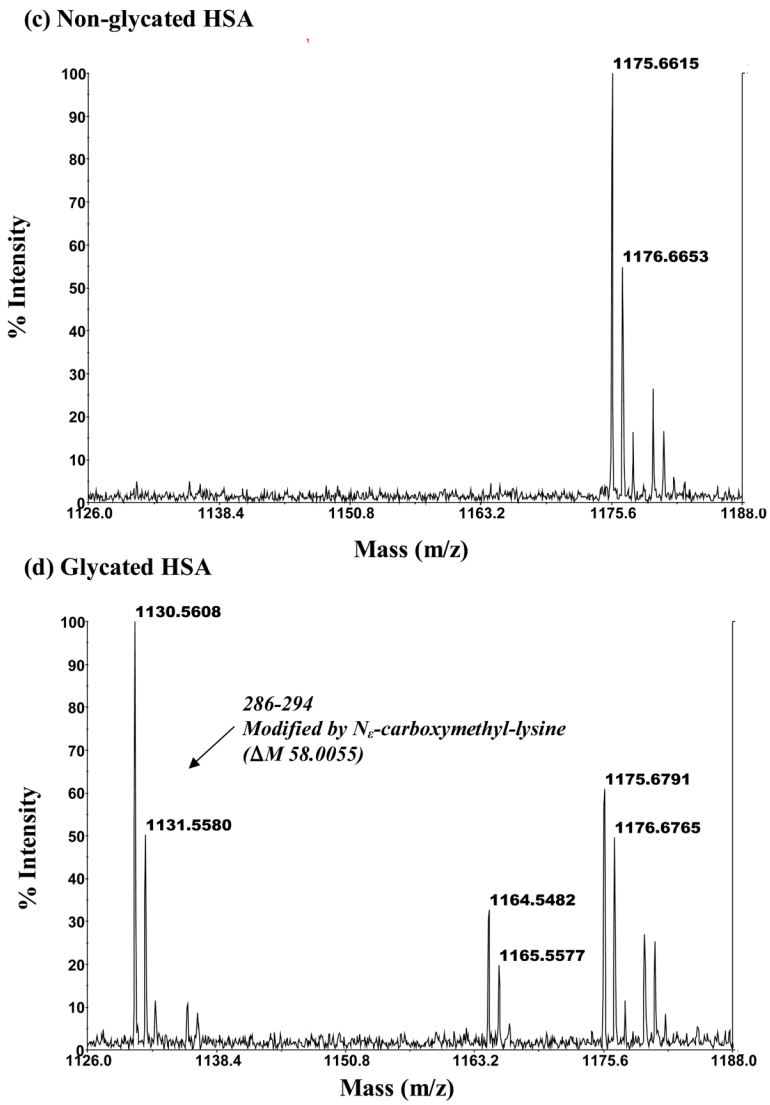


Figure 5.

MALDI-TOF MS mass spectra for modified peptides found in digests of glycated HSA but absent in digests of normal HSA. The mass spectrum in (b) is for a modified peptide with a mass of 971.50 Da that originates from the 535–541 region of HSA and represents a fructosyl-lysine modification (ΔM, 162.05 Da). The mass spectrum in (d) is for a modified peptide with a mass of 1130.56 Da that corresponds to the 286–294 region of HSA and represents a N_{ε}-carboxymethyl-lysine modification (ΔM, 58.01 Da). The mass spectra in (a) and (c) show the absence of these modified peptides in digests of normal HSA.

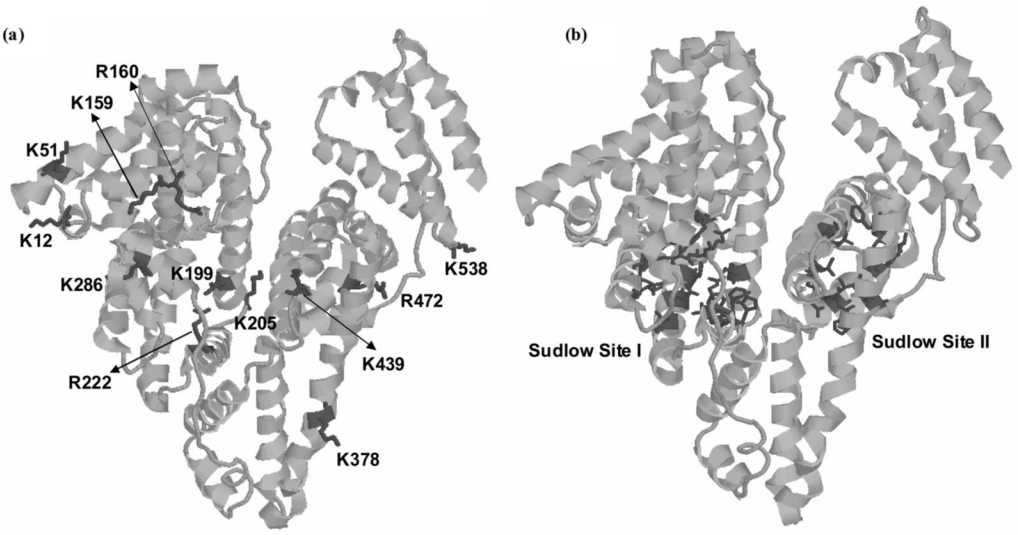


Figure 6.

The 3-dimensional structure of HSA, showing (a) the sites of both early and advanced glycation adducts on HSA and (b) the locations of Sudlow sites I and II. These structures are based on PDB file 1AO6.

Table 1

Peptides only containing one type of modification in minimally glycated HSA

Location in HAS	Digests in which identified	Type of modification[a]	Possible modification sites in peptide
7–17	Glu-C	FL	K12
49–57	Glu-C	FL-2 H_2O	K51
145–160	Trypsin	Pyrraline	K159
160–181	Trypsin	G-H1	R160
196–209	Trypsin	2 FL	K199 & K205
219–225	Trypsin	Argpyrimidine	R222
286–294	Glu-C	CML	K286
377–382	Glu-C	FL-2 H_2O	K378
377–383	Glu-C	CEL	K378
467–484	Trypsin	G-H1	R472

[a] See Figure 1 for the full names and structures of these various modification classes.

Table 2

Peptides in minimally glycated HSA containing several potential modification sites

Location in HSA	Digests in which identified	Type of modification[a]	Possible modification sites[b]	Estimated pK_a	Estimated fractional solvent accessible surface areas
133–141	Glu-C	FL-1 H_2O	K136 or K137	10.27/10.43	0.27/0.52
154–167	Glu-C	FL	K159 or K162	9.94/10.43	0.44/0.43
168–188	Glu-C	CEL	K174 or K181	10.22/10.01	0.34/0.37
191–205	Trypsin	FL-1 H_2O or 3-DG-H1/THP	K195, **K199** or R197	10.76/**7.47** 14.37	0.50/**0.16** 0.13
200–218	Trypsin	AFGP	K205, R209 or K212	10.43/12.08/10.43	0.58/0.61/0.40
206–225	Lys-C	FL	K212 or K225	10.43/9.80	0.40/0.44
219–240	Trypsin	FL-1 H_2O or 3-DG-H1/THP	K225, K233 or **R222**	9.80/10.29 **7.56**	0.44/0.43 **0.16**
349–372	Trypsin	FL-1 H_2O	K351 or K359	10.15/10.50	0.61/0.81
377–393	Glu-C	Pyrraline	K378 or K389	10.50/10.50	0.71/0.68
426–442	Glu-C	FL	K432, K436 or **K439**	9.92/9.67/**10.50**	0.36/0.51/**0.94**
520–525	Lys-C	CEL	K524 or K525[c]	10.43/10.06	0.54/0.07
535–541	Trypsin	FL	K536 or **K538**	10.08/**10.50**	0.24/**0.96**
572–585	Glu-C	Pyrraline	K573 or K574	10.50/10.50	0.72/0.82

[a] See Figure 1 for the full names and structures of these various modification classes.

[b] The residues in bold are the suspected modification sites in minimally glycated HSA. The pK_a and accessible surface areas for these sites are also given in bold.

[c] K525 has been reported in previous *in vivo* work to be a major site of modification for HSA with low levels of glycation [36].