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QUANTITATIVE ANALYSIS OF GLYCATION SITES ON HUMAN SERUM ALBUMIN USING 16O/18O-LABELING AND MATRIXASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

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QUANTITATIVE ANALYSIS OF GLYCATION SITES ON HUMAN SERUM ALBUMIN USING $^{16}O/^{18}O$-LABELING AND MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

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Abstract

Background—One of the long term complications of diabetes is the non-enzymatic addition of glucose to proteins in blood, such as human serum albumin (HSA), which leads to the formation of an Amadori product and advanced glycation end products (AGEs). This study uses $^{16}O/^{18}O$-labeling and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to provide quantitative data on the extent of modification that occurs in the presence of glucose at various regions in the structure of minimally glycated HSA.

Methods—Normal HSA, with no significant levels of glycation, was digested by various proteolytic enzymes in the presence of water, while a similar sample containing in vitro glycated HSA was digested in ^{18}O-enriched water. These samples were then mixed and the $^{16}O/^{18}O$ ratios were measured for peptides in each digest. The values obtained for the $^{16}O/^{18}O$ ratios of the detected peptides for the mixed sample were used to determine the degree of modification that occurred in various regions of glycated HSA.

Results—Peptides containing arginines 114, 81, or 218 and lysines 413, 432, 159, 212, or 323 were found to have $^{16}O/^{18}O$ ratios greater than a cut off value of 2.0 (i.e., a cut off value based on results noted when using only normal HSA as a reference). A qualitative comparison of the ^{16}O- and ^{18}O-labeled digests indicated that lysines 525 and 439 also had significant degrees of modification. The modifications that occurred at these sites were variations of fructosyl-lysine and AGEs which included 1-alkyl-2-formyl-3,4-glycoyl-pyrole, and pyrraline.

Conclusions—Peptides containing arginine 218 and lysines 212, 413, 432, and 439 contained high levels of modification and are also present near the major drug binding sites on HSA. This result is clinically relevant because it suggests the glycation of HSA may alter its ability to bind various drugs and small solutes in blood.

Keywords

Non-enzymatic glycation; human serum albumin; diabetes; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; $^{16}O/^{18}O$-labeling; quantitative proteomics

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Introduction

Glycation is a process that involves the non-enzymatic addition of sugar molecules to the amino groups of a protein, as occurs during diabetes [1]. The sites most susceptible to the initial stages of glycation are normally found on lysine residues, but this process can also occur at the *N*-terminus [1,2]. This initial phase involves the formation of a Schiff base, where a covalent bond forms between a carbonyl group on the sugar and an amine group on the protein. However, this Schiff base is an unstable intermediate that can go back to the initial reactants or rearrange to create a more stable Amadori product. The Amadori product may then degrade and rearrange through further reactions involving oxidation, dehydration, and cross-linking to form a mixture of amino acid-linked substances called “advanced glycation end products” (AGEs). AGEs are typically found on lysine and arginine residues, as well as the *N*-terminus [3,4]. AGEs that are formed during diabetes are believed to contribute to the chronic micro and macro vascular complications encountered in diabetes. Thus, there is a growing need for work that characterizes and quantifies modified residues on glycated proteins [5].

Human serum albumin (HSA) was examined in this current study because it is the most abundant protein in the serum and is known to undergo glycation [6–8]. This protein binds to many drugs and analytes in the body [7] and it has been suggested that glycation may lead to changes in the binding of some solutes with this protein [9]. There are a number of previous studies that have sought to characterize AGEs and to identify glycation sites on various proteins [10], including HSA [10–14]. Mass spectrometry is one tool that has been used to examine modifications that occur as a result of glycation on proteins [15,16] and peptides [16,17]. However, few quantitative studies have been reported using this approach for examining the modifications that occur in glycated HSA or in measuring early and late stage glycation products in this protein [18].

In this study, isotopic labeling and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) will be employed as tools for quantitative studies comparing HSA and minimally glycated HSA that have been digested by serine proteases in the presence of ^{16}O- or ^{18}O-enriched water (see general approach in Figure 1) [19–25]. The resulting digests will be mixed in a fixed ratio, and the $^{16}O/^{18}O$ ratios of peptides found in this mixed digest will be determined by using mass spectrometry, where all $^{16}O/^{18}O$ ratio calculations are based on the peak areas present within an isotopic cluster [21,26]. If a modification takes place in a given region of HSA due to glycation, an increase in the mass of peptides from this region should occur; the result is that less of the non-modified peptide would be seen in the glycated sample, causing the measured $^{16}O/^{18}O$ ratio to increase above levels expected when no modification is present. In addition, the size of the $^{16}O/^{18}O$ ratio should make it possible to compare the relative extent of glycation-related modifications that occur in different regions of HSA.

These experiments will be conducted by using these tools from quantitative proteomics to examine minimally glycated HSA that has been prepared *in vitro*. This previous research will be expanded upon in this current study by using $^{16}O/^{18}O$-labeling and MALDI-TOF MS to rank the degree of modification that is occurring at such sites in HSA. Minimally glycated HSA is of interest in this research because it is thought to mimic the extent of glycation seen in pre-diabetes or early stages of this disease [27,28]. The results should provide a better understanding of which regions on HSA are affected most by moderate levels of glycation and provide clues as to how these modifications alter some of the properties of HSA (e.g., the ability of this protein to bind drugs and small solutes).

2. Theory

The theory behind the determination of $^{16}O/^{18}O$ ratios when using ^{16}O- and ^{18}O-labeling has been described previously [19,29]. Two different methods for calculating $^{16}O/^{18}O$ ratios were used in this report. The first method employed mass spectra for a ^{18}O and ^{16}O mixed digest along with the expected relative intensities for unmodified peptides, as predicted by using MSIsotope [30]. The $^{16}O/^{18}O$ ratio for a peptide in this case was determined by using the following equation [29].

$$\frac{^{16}O}{^{18}O}=\frac{I_0}{\left(I_2-I_0\frac{M_2}{M_0}\right)+\left(I_4-I_0\frac{M_4}{M_0}-\left(I_2-I_0\frac{M_2}{M_0}\right)\frac{M_2}{M_0}\right)} \quad (1)$$

In this equation, the terms I_0 through I_5 represent the relative intensities for the M+0 to M+5 peaks in an isotope cluster, where M_0 through M_5 represent the expected relative intensities for a digest in which no isotopic label is present. The terms in the first parentheses on the left side of the denominator represent the ^{18}O contribution for a peptide with one ^{18}O label. The terms in the second parentheses represent the ^{18}O contribution for a peptide with two ^{18}O labels. This approach for determining $^{16}O/^{18}O$ ratios will be referred to as “Method 1” throughout this paper.

The second method employed an internal standard by using the mass spectra obtained by digesting HSA in ^{16}O-enriched water instead of using a theoretical digest. As described in the literature [19,23], a slightly modified form of this method can be used to simplify the determination of a $^{16}O/^{18}O$ ratio. When a ^{16}O digest and ^{18}O digest are mixed in a 1:1 ratio, an isotope peak in the mixed digest may be described as the sum of the contribution from the ^{16}O-labeled digest and the contribution from the ^{18}O-labeled digest, as summarized in the following equation.

$$I'_n+I''_n=I_n \quad (2)$$

In this equation, I'_n represents the contribution from the ^{16}O-labeled digest to the ^{16}O- and ^{18}O-labeled mixed digest (I_n). Similarly, I''_n represents the contribution from the ^{18}O-labeled digest to I_n.

When the relative intensities for the ^{16}O- and ^{18}O-labeled peptides are measured, these values can be used to estimate the contribution from both the respective digests to the intensity of any isotope peak in the mixed digest. The first step in this process is to rewrite eqn 2 in terms of the individual ^{16}O and ^{18}O contributions (see eqn 3) and then to rearrange this equation into the form given by eqn 4.

$$\frac{a_n}{a_0}I'_0+\frac{b_n}{b_4}I''_4=I_n \quad (3)$$

$$I'_0+\left(\frac{a_0b_n}{b_4a_n}\right)I''_4=\frac{a_0}{a_n}I_n \quad (4)$$

The terms a_0 through a_5 in these equations represent the relative intensities of the peaks in an isotope cluster for a given peptide in the ^{16}O-labeled digest, and the terms b_0 through b_5 represents the relative intensities for the same peaks in the ^{18}O-labeled digest. The intercept and slope of eqn 4 can be used to determine the ^{16}O-label contribution to the M+0 peak (i.e., I'_0) and the contribution of the ^{18}O-label to the M+4 peak (i,e., I''_4). Once I'_0 and I''_4 have been obtained, these values can be used to estimate the ^{16}O or ^{18}O contribution to any peak in the mixed spectra. An example is shown below, where the ^{18}O contribution to the M+0 peak is being determined.

$$I''_0 = I''_4 \left(\frac{b_0}{b_4}\right) \tag{5}$$

A correction must be made for incomplete ^{18}O incorporation during digestion because a mixture of peptides with different degrees of ^{18}O substitution will lead to overlapping isotope peaks. The contribution of these overlapping peptides can be corrected by factoring in the relative abundances from the ^{16}O digest for peptides that contain one ^{18}O atom (eqn 6) or two ^{18}O atoms (eqn 7).

$$I''_{2(c)} = I''_2 - I''_0 \left(\frac{a_2}{a_0}\right) \tag{6}$$

$$I''_{4(c)} = I''_4 - I''_{2(c)} \left(\frac{a_2}{a_0}\right) - I''_0 \left(\frac{a_4}{a_0}\right) \tag{7}$$

In eqn 6, a correction is made for the M+2 isotope in the unlabeled peptide. Eqn 7 includes two corrections, where the first correction is for the M+2 isotope in a peptide with one ^{18}O label and the second correction is for the M+4 isotope in a peptide with no ^{18}O label. The $^{16}O/^{18}O$ ratio can then be calculated by using these corrected variables as shown in the following expression.

$$\frac{^{16}O}{^{18}O} = \frac{I'_0}{\left(I''_0 + I''_{2(c)} + I''_{4(c)}\right)} \tag{8}$$

The concept behind eqn 8 is similar to eqn 1 but now uses the ^{16}O digest as an internal standard [23], with the relative abundances of these peaks (as well as those obtained from the ^{18}O digest) being used to estimate the ^{16}O or ^{18}O contribution to any given peak in the mixed spectra. The advantage of using this method is that it allows corrections to be made for variations in the actual extent of ^{18}O incorporation and the type of instrumentation being employed. The disadvantage of this method is that peptides have to be detected in all three digests for the $^{18}O/^{16}O$ ratio to be determined. The use of this approach to determine $^{16}O/^{18}O$ ratios will be referred to as “Method 2” in the remainder of this paper.

3. Materials and Methods

3.1. Materials

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): Des-Arg-bradykinin (97% pure), glu-fibrinopeptide (97%), angiotensin I (97%; acetate salt), HSA (99%, essentially fatty acid and globulin free), glycated HSA (95%; lot number 115K6108, containing 1.8 mol hexose/mol HSA), sequencing grade trypsin, sequencing grade Glu-C, sequencing grade Lys-C, guanidine HCl (99%), D/l dithiothreitol (99%), iodoacetamide (99%), formic acid (96%), 2,5-dihydroxybenzoic acid (98%), α-cyano-4-hydroxycinnamic acid (99%), ^{18}O-enriched water (97%), ^{16}O-enriched water (99.99%), tris-HCl (99%) and ammonium bicarbonate (99%). All of the chemicals that were used were reagent grade or better. The water used for these experiments (other than that utilized for ^{18}O-labeling) was obtained from a Nanopure water system (Barnstead, Dubuque, IA)

3.2. Apparatus

The following items were purchased from Thermo Fisher Scientific (Rockford, IL): Slide-A-Lyzer dialysis cassettes (7000 Da MW cutoff, 0.1 – 0.5 ml capacity) and a 0.5 – 10 μL digital pipette. Micro-C18 ZipTip pipette tips with 5.0 μg of bed material were obtained from Millipore (Billerica, MA). The overhead transparencies used for sample/matrix mixing prior to MALDI-TOF MS were purchased from C-Line Products (Des Plaines, IL). Mass spectra were acquired on a Voyager 6148 MALDI-TOF-MS system (Applied Biosystems, CA). The instrument settings were as follows: positive-ion delayed extraction reflection mode; delay time, 100 ns; accelerating voltage, 20 kV; guide wire voltage, 0.008% of accelerating voltage; grid voltage, 76% of accelerating voltage. The MSIsotope software was obtained from the UCSF Protein Prospector webpage [30].

3.3. Sample Pretreatment, Digestions & Peptide Fractionation

The minimally glycated HSA obtained from Sigma-Aldrich was prepared under proprietary conditions by incubating a fixed concentration of D-glucose with HSA at 37 °C for periods of time that were no longer than a week. The HSA samples were pretreated as described previously in work with immobilized HSA supports [19], but with the following modifications being made for this study. A 5 mg/ml HSA solution prepared in denaturing buffer was used as described in the previous study, however, the solution volume was now reduced from 1 ml to 300 μl to reduce the amount of HSA that was used. The ratio of HSA to dithiothreitol and iodoacetamide was kept the same as in Ref. [19] while the solution volumes were adjusted accordingly to compensate for the change in the volume of the HSA solution. Digestion was carried out with normal HSA being placed into ^{16}O-labeled water and glycated HSA being digested in ^{18}O-labeled water. Zip-tip fractionation was performed as described previously by using aqueous mixtures of 5, 10, 20, 30 and 50% acetonitrile for elution in a series of step gradients [19].

3.4. Mass Spectrometry & Data Acquisition

The details regarding the preparation of mass calibrants and matrix solutions are described elsewhere [19]. The mass spectrometer was externally calibrated using a mixture containing des-Arg-bradykinin (25 pmol/μl), glu-fibrinopeptide (32.5 pmol/μl), and angiotensin I (32.5 pmol/μl). A 4 μlL portion of this solution was mixed with 96 μl of α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid mixed matrix solution (i.e., giving a final concentration of 0.1–5 pmol/μl for the final calibration mixture), and the resulting mixture was spotted adjacent to each sample well on a MALDI plate. A mass spectrum was obtained for the calibration mixture using Voyager Control Panel software (Applied Biosystems, Foster City, CA) and the file was exported to the Data Explorer software (Applied Biosystems). Using Data Explorer, the monoisotopic M+0 peaks for des-Arg-bradykinin, glu-fibrinopeptide, and

angiotensin I were calibrated to masses of 904.4681 Da, 1570.6774 Da, and 1296.6853 Da, respectively. The calibration constants obtained were then saved and imported to the Voyager software. The mass spectrum for the sample was then obtained. The calibration process was repeated for every sample spot on the MALDI plate. The mass accuracy obtained when this method was used was typically <50 ppm.

3.5. Peak Selection Criteria and Data Sorting

The masses of the peptides detected in the HSA or glycated HSA digests were compared to the peaks predicted from a theoretical digest by using PEPTIDEMASS software [31,32]. When generating the theoretical digest, the following considerations were made: the maximum amount of missed cleavages was 2; all cysteine residues were assumed to be treated with iodoacetamide; variable oxidation of methionine residues was allowed; and only monoisotopic masses were selected. All peptides that could be matched within 50 ppm of the theoretical digest were selected for further analysis [26]. For all digests, only peptides having a signal-to-noise ratio greater than five were used for further quantitative studies. In the ^{18}O digests, the M+4 peak was used to determine the signal-to-noise ratio for the resultant peptides, while the M+0 peak was used to determine the signal-to-noise ratio for the ^{16}O digests. Information about the pK_a and the fractional accessible surface area (FAS) was used to predict the reactivity of a given amino acid residue in forming glycation products; the methods used for calculating these pK_a and FAS values have been described previously in the literature [11,33,34].

4. Results and Discussion

4.1. Sequence Coverage

Prior to looking at the relative extent of glycation at various regions on HSA, the sequence coverage for this protein was examined under the analysis conditions used in this study. When using only qualitative data and looking for *m/z* values that could be linked to specific peptides, the coverage obtained for the trypsin, Lys-C and Glu-C digests of HSA were found to be 83.6%, 61.0% and 54.0% respectively. The total coverage obtained when all three digests were considered was 92.3%, which is close to a previously reported value of 97.4% under similar pretreatment and analysis conditions for samples of soluble or immobilized HSA that were not glycated [19].

When Method 1 was used to obtain $^{16}O/^{18}O$ ratios, the sequence coverage in these quantitative studies was lowered to 51.5%, 53.9%, and 51.5% for the trypsin, Lys-C, and Glu-C digests respectively, which gave a total sequence coverage for HSA of 83.6% (see Supplemental Information). Similarly, when Method 2 was employed, the usable sequence coverage was 33.5%, 21.9%, and 42.1% for the trypsin, Lys-C, and Glu-C digests, respectively, which gave an overall sequence coverage of 66.3% for HSA. This reduction in usable sequence coverage in going from the qualitative to quantitative experiments was expected because several of the peptides that were detected in the qualitative studies did not have a high enough intensity to be used in the quantitative studies. This same type of reduction in sequence coverage has been observed in previous studies using quantitative proteomics for immobilized versus soluble HSA, where a total usable sequence coverage of 76.9% was obtained when quantitative data was considered [19].

When using both Methods 1 and 2 for the quantitative studies, $^{16}O/^{18}O$ ratios could be determined for peptides that encompassed 42 lysine and 21 arginine residues in HSA. By using a qualitative comparison between the ^{16}O- and ^{18}O-labeled samples, K525 (found in residues 525–534 in the tryptic digest obtained in this study), was also found to have significant levels of modification. The remaining 16 lysine and 3 arginine residues that were not included in these peptides are located within residues 58–61, 160–163, 191–206, 295–317, 355–359, 520–

524, 535–543 and 558–585. None of these residues, with the exception of K199 (a known site of major glycation), is thought to undergo any significant amounts of modification during the glycation of HSA [11,18,19,35]. Ongoing studies are currently being conducted to modify the approach used in this work to include K199; however, it is also already known that this residue is a prominent glycation site on HSA and is located at or near Sudlow site 1 [36].

4.2. Measurements of $^{16}O/^{18}O$ Ratios for Peptide Digests from Glycated HSA

Peptides containing a $^{16}O/^{18}O$ ratio higher than a cutoff value of 2.00 were selected and treated as potential modification sites in the digests of glycated HSA. This cutoff value was determined using a cumulative sums plot (Figure 2), where it was determined that 6% of the detected peptide peaks had a $^{16}O/^{18}O$ ratio greater 2.00. This cutoff value is similar to an upper reference value for $^{16}O/^{18}O$ ratios of 2.0–3.0 (depending on the type of digest being used) that has been previously reported for this digestion and labeling approach with normal HSA [19]. The $^{16}O/^{18}O$ ratio range of 2.0–3.0 was previously obtained by comparing two control samples of the same preparation of normal HSA [19]. When both Methods 1 and 2 were used for quantitative analysis, the $^{16}O/^{18}O$ ratios for 83 peptides were determined. Of these peptides, eight peptides containing four lysine residues and five arginine residues were identified as major sites of modification because their $^{16}O/^{18}O$ ratios were above the selected cutoff ratio of 2.0. Two peptides that encompass residues 525–534 and 415–439 on HSA were found to have significant levels of modification; however, the extent of modification was too large to be quantified by this current approach.

There were 2 cases where the $^{16}O/^{18}O$ ratio was calculated for peptides with identical residues where one peptide had a cysteine that had been modified by iodoacetamide to form carbamidomethyl-cysteine (CAM-cysteine) and the other peptide remained unmodified. These two peptides were detected in the Glu-C digest and corresponded to residues 466–479 and residues 101–119. In this situation, a pair of $^{16}O/^{18}O$ ratios was calculated for each residue, where one ratio corresponds to the CAM-modified peptide and the other to the unmodified peptide. In both cases, the unmodified peptides had a much higher $^{16}O/^{18}O$ ratio (1.27–1.86) than their CAM-modified counterparts (0.31–0.37). This anomaly may indicate that CAM-incorporation was not consistent between the glycated HSA and the reference HSA samples. Because of this, the $^{16}O/^{18}O$ ratio for the CAM-modified peptide in this case was assumed to be the $^{16}O/^{18}O$ ratio that most closely represented glycation.

The $^{16}O/^{18}O$ ratios obtained for the tryptic digest using Method 1 and 2 are given in Tables 1 and 2, respectively. In the tryptic digest, five peptides were found to have $^{16}O/^{18}O$ ratios >2.00. A total of 3 peptides were also noted in the Lys-C digest that had $^{16}O/^{18}O$ ratios greater than 2.00, but the Glu-C digest gave no peptides with $^{16}O/^{18}O$ ratio above this cutoff value (see Supplemental Information).

Table 3 shows the ranking of the peptides that were found in the quantitative studies to have major degrees of modification, as listed according to the order of their $^{16}O/^{18}O$ ratios. These peptides contained amino acids R114, R81, R218, K413, K432, K159, K212 and/or R209, and K323. Of these residues R218, K159 and R209 were found to be modified to produce the AGEs 1-alkyl-2-formyl-3,4-glycoyl-pyrole (AFGP), pyrraline (Pyr) and AFGP, respectively. Similarly, residues 212, 413 and 432 were modified to form fructosyl-lysine (FL), FL-$2H_2O$ and FL-$2H_2O$, respectively, all of which are early stage glycation products. The procedure used for identifying these modifications was based on a Perl-script algorithm, as described in Ref. [11]. In some cases, a manual comparison was also performed using a simplified search list (i.e., only one modification allowed per peptide) as based on previously-identified modifications [11].

There were 2 instances where the *m/z* values for a given peptide were clearly detected in the ^{16}O digest, but the same *m/z* values were not found in the ^{18}O digests or mixed labeled digest (see Table 3). The absence of these characteristic peptides in the ^{18}O digests is indicative of significant glycation, because the concentration of the unmodified forms of these peptides were too low to be detected at the selected signal-to-noise threshold of 5 (i.e., only peaks with intensities below this threshold were noted in the ^{18}O and mixed oxygen labeled digests). Low intensity *m/z* values corresponding to residues 525–534 (1128.69 Da) in the tryptic digest and residues 415–439 (2629.42 Da) in the Lys-C digest were found only in the ^{16}O-labeled digest. Interestingly, these residues also correspond to lysines 439 and 525, which are amongst the most commonly cited glycated lysine residues in HSA [18]. Supporting evidence of this was found previously in that K525 was shown to be modified to form N_{ε}–carboxyethyl-lysine (CEL) and K439 was shown to be modified to form FL [11]. In addition to these peptides, a section from residues 226–233 is also believed to have significant levels of modification. A set of *m/z* values corresponding to this peptide (880.44 Da), were clearly identified in the ^{16}O- and ^{18}O-labeled digests; however, the corresponding *m/z* value in the mixed digest seemed to shift to 881.29 Da. This shift would result in a value that falls outside the assigned mass accuracy. Nevertheless, a $^{16}O/^{18}O$ ratio was calculated for this peptide, giving an $^{16}O/^{18}O$ ratio of 4.32, indicating that this peptide could potentially contain high levels of glycation. Previous studies have indicated that K233, which is found within residues 226–233 of HSA, may be glycated [18].

The $^{16}O/^{18}O$ ratios for other modifications that occur on glycated HSA which did not exceed the 2.00 cutoff value were also considered, based on possible modifications that have been noted in the literature in qualitative studies with HSA [11]. If multiple $^{16}O/^{18}O$ ratios could be linked to a given lysine or arginine residue, the resulting $^{16}O/^{18}O$ ratios that are modified to form CAM-cysteine and peptides that have a minimum combination of lysine and/or arginine residues were used to assign the most likely glycation sites. This was done to provide the most realistic representation of the $^{16}O/^{18}O$ ratio for a given amino acid, without the influence of CAM modified cysteine or assigning $^{16}O/^{18}O$ ratios that result from multiple minor modifications to a single amino acid. When CAM modification and lysine/arginine heterogeneity are factored in, the range of $^{16}O/^{18}O$ ratios for lysines 12 and 51 (or the *N*-terminus) is 1.18–1.32 and 1.28–1.78 respectively. These results indicate that K51 (or the *N*-terminus) contains the most early glycation products, followed by K12. These amino acid residues have previously been linked to the formation of early stage glycation products (i.e., FL-related modifications). Similarly, the range of $^{16}O/^{18}O$ ratios for AGE-linked peptides [11] containing K159, K286, K378, R472 and R222 is 1.80–2.40, 0.26–0.28, 0.45–1.29, 0.31–0.50 and 1.62–1.86, respectively. When these residues are ranked in order of decreasing $^{16}O/^{18}O$ ratios, K159 appears to have the largest extent of AGE-modification followed by R222, K378, R472 and K286. Lysines 159, 378, and 286 were linked to Pyr, CEL, and CML respectively. Similarly, arginines 222 and 472 were linked to G-H1 and ArgP respectively.

The peptide with the highest detected $^{16}O/^{18}O$ ratio of 3.77 was detected in the trypsin digest and had a mass of 940.45 Da. This corresponds to residues 107–114 on HSA, which indicates that R114 is modified to form AGEs. The particular type of modification could not be identified by *m/z* shifts in this study or in our previous study [11], however, early glycation does not occur on arginine residues so it likely being modified to form AGEs. Similarly, a peptide (933.52 Da) with a high $^{16}O/^{18}O$ ratio of 3.68 was detected in the trypsin digest. This corresponds to residues 74–81 on HSA, indicating that R81 may be involved in AGE formation. Several peptides were identified in this analysis that had high $^{16}O/^{18}O$ ratios with corresponding glycation related *m/z* shifts as identified previously [11]. For example, a high $^{16}O/^{18}O$ ratio peptide corresponding to residues 213–218 (673.38 Da) on HSA has previously been shown

to be modified by AFGP. Similar cases where high $^{16}O/^{18}O$ ratios and glycation related modifications can be linked are shown in Table 3.

4.3. Peptides with High $^{16}O/^{18}O$ Ratios Representing Early or Advanced Glycation Products

Some of the peptides containing $^{16}O/^{18}O$ ratios above the cutoff ratio had multiple sites for possible modification. As a result it was difficult to link an increased $^{16}O/^{18}O$ ratio to a particular amino acid in the respective peptide. For peptides with several possible modification sites, the detected mass shifts were used to help assign the sites that were being modified. For instance, peptide 403–413 (1352.77 Da) detected in the Lys-C digest had a $^{16}O/^{18}O$ ratio of 3.29 and contained both K413 and R410. A separate peptide was found from the same region (residues 403–413) that had a mass shift of 126 Da. This mass shift corresponds to modification by FL-$2H_2O$, suggesting that K413 is being modified during glycation to form this type of adduct. Similarly, lysines 432 and 212 were clearly identified as likely modification sites because the *m/z* shift data that was obtained previously [11] show that these amino acids were being modified by early glycation products.

There were some glycation-related mass shifts that were identified in this study (Table 4) and linked to peptides with intermediate $^{16}O/^{18}O$ ratios. The resulting mass shifts were similar to the mass shifts that were obtained previously [11]. For instance, amino acids K106, K136, K240, K174, K466,, and R336 are suspected to be modified to produce CML, FL-$2H_2O$, FL-$1H_2O$, FL and CEL, CEL, and AFGP respectively. Of these residues, lysines 136, 174, and 240 were previously shown to be modified by FL-$1H_2O$, CEL, and FL-$1H_2O$, respectively [11]. All of the other modifications that were linked to intermediate $^{16}O/^{18}O$ ratios are new to this current study. Of these residues, K432 and K413 were linked to $^{16}O/^{18}O$ ratios that were above the cutoff ratio of 2.00. The number of *m/z* shifts that were linked to intermediate $^{16}O/^{18}O$ ratios in this study and in Ref. [11] illustrates the heterogeneous nature of glycation-related modifications and the need for improved characterization techniques.

4.4. Correlation of Modifications with Local pK_a or FAS Values

Additional sources of information can be used to determine which lysine or arginine residues on HSA might be prone to modifications by glycation. This information includes the estimated pK_a values for the individual amino acid side chains, the FAS (i.e., fractional accessible surface area) for each of these residues, and the location (surface or buried) of the amino acid residue. Lysine or arginine residues on HSA having low pK_a values would be expected to be more susceptible to glycation reactions because a larger portion of these residues would exist in a non-protonated state, favoring nucleophilic addition/substitution [11]. Amino acid side chains with high FAS values and residues that occur on the surface of a protein such as HSA could be more reactive because they have greater access to reactants compared to sites buried within the protein's structure. Another factor to consider is that HSA is a flexible protein [7] in which changes in confirmation may facilitate or hinder glycation reactions. As a result, the aforementioned variables are not static, and residues with low FAS values, high pK_a values or that are normally buried within the protein may become more reactive as glycation-induced conformational changes occur.

Of the various residues with high $^{16}O/^{18}O$ ratios that were noted in this study (Table 3), residues R218, R410, and R428 are all buried within the structure of HSA, while the majority of the remaining residues are located on the surface of HSA. In addition, lysines 233, 439, and 525, which are believed to have undergone significant glycation based on a qualitative comparison of the digest, are all found on the surface of HSA. This result makes sense because the majority of modification that occurs on HSA is expected to occur with residues that are easily accessible to sugars in the surrounding medium. When looking at the modifications which were identified by mass shifts (Table 4), six of these modifications occur on residues that are found on the

surface of the protein and five occur on residues that are more buried in the structure of HSA. The location of AGEs or glycation products in the buried locations could be explained by changes in the structure of HSA when glycation occurs. Changes in the confirmation of HSA that was glycated both *in vitro* and *in vivo* were observed in a prior study [35]. Further evidence of this was indicated previously, when K199 was identified as a prominent glycation site. Even though the location of this residue is buried within the structure of HSA, it has consistently been identified as a glycation site that occurs on HSA [11,37]. Additional information, such as the calculated pK_a and FAS values for these residues, were next used to provide further insight into these modification processes.

The range of calculated pK_a values for the lysine and arginine residues on HSA are 6.23–11.11 and 7.56–16.02, respectively, where the lysine residues have an average pK_a value of 10.08 and the arginine residues have an average pK_a of 12.34 (Note: details on how these calculated results were obtained are provided in the literature [19]). The peptides listed in Table 3 appear to follow a trend in which the most frequently occurring modifications took place on residues that either have pK_a values below the average or have high FAS values. For instance, R114, R81, and R209 have FAS values of 0.80, 0.76, and 0.61, respectively, which are on the high end of the range of FAS values for arginine. These residues are also suspected modification sites based on their $^{16}O/^{18}O$ ratio values. R218 has the highest FAS value of all arginines on HSA, a pK_a value which is lower than average, and the highest calculated $^{16}O/^{18}O$ ratio in this study. All of these features provide strong evidence that this residue is a likely site for modification due to glycation-related reactions. Similarly, lysines 413, 432, and 212 were assigned as glycation sites because of the corresponding low pK_a values, lysine specific modifications were identified on these peptides, and peptides corresponding to these residues all had high $^{16}O/^{18}O$ ratios. Similar assignments were made for *m/z* shifts that were identified in this study that corresponded to early or late stage glycation adduct formation (Table 4). Of the glycation sites that were identified through a qualitative comparison of the digests, lysine 439 had a high FAS value of 0.94, which partially explains the reactivity of this residue. The reactivity of K233 and K525, however, cannot be explained based on the pK_a or FAS values alone because these values are more typical of those seen for other lysines on HSA. It is therefore likely that some other process such as a conformational change upon glycation [35] or localized acid-base catalysis [18] may increase the amount of modification that occurs on these residues.

4.5. Location of Modified Sites versus Major Drug Binding Sites on HSA

It has been suggested that the extent of glycation of HSA may affect how tightly HSA binds drugs and small solutes in the body [7,38–40]. The two major drug binding sites on HSA, commonly referred to as the Sudlow sites 1 and 2, contain several amino acids which facilitate the binding of these compounds to HSA [7,38–40]. When the results from Tables 1 and 2 are compared to the location of these binding sites [7], many of the potential glycation sites are at or near the same regions on HSA that facilitate drug and solute binding. For instance, K212, K233, R209, and R218 (noted in Table 3 to be potentially important sites for modifications due to glycation) occur in the same vicinity as the key residues W214 and F211 that are found in Sudlow site 1. Similarly, K413, K432, K439, are located in the same region of HSA as R410 and Y411, which are important residues taking part in drug interactions at Sudlow site 2. Of the major glycation adducts that were noted in this study (Table 4), K432 and K240 occur in the same region of HSA as L430 (Sudlow site 2) and L234/l238 (Sudlow site 1), respectively. These results suggest that the glycation of HSA, even at the minimal levels examined in this report, may have a potential effect on the binding of drugs and other small solutes at these sites on HSA.

5. Conclusions

Figure 3 shows an image of HSA (as created using the Visual Molecular Dynamics software [41]) that summarizes the results found in this study. This figure includes the major glycation/modification sites identified in this study and lists their $^{16}O/^{18}O$ ratios. When ranked in order of decreasing $^{16}O/^{18}O$ ratios, R114, R81, R218, K413, K432, K159, K212, and K323 were found to give significant changes in their $^{16}O/^{18}O$ ratios due to glycation or a reaction that produced AGEs. In addition to these peptides, lysines 439, 525, and 233 are also believed to contain significant amounts of modification based on this work. These possible modification sites were obtained by combining information on the observed mass shifts for the affected peptides in the digests, the $^{16}O/^{18}O$ ratios for these peptides, the location of the peptides in HSA, and the corresponding pK_a and FAS values that were calculated for the given residues.

The approach discussed in this report is novel because it allows for the simultaneous quantification of glycation-related products that occur on lysine and arginine residues in HSA. It was clearly established in this work that significant glycation and AGE-modifications occur at or near the two major drug binding sites of HSA, which explains the changes in binding affinity that have been noted for some endogenous solutes with glycated HSA (e.g., bilirubin and fatty acids) [35,40,42]. These modifications also explain, in part, changes in the binding of various drugs with HSA or in serum during diabetes [43–45]. The clinical implication of these findings is that protein glycation and related modifications might affect the protein binding and, thus, the drug activity that occurs in persons with diabetes compared to non-diabetic patients. The results of this work also complement and build on previous work in the analysis of glycation-related modifications [5,12,13] by providing a relative ranking of the amount of glycation that is found in various portions of minimally glycated HSA.

Future work will explore the extension of this approach to HSA containing various levels of glycation and to glycated HSA that is found *in vivo*. A comparison of the results of such studies with this current report and past studies should make it possible to get a more complete picture of how glycation patterns may vary for HSA in clinical samples or under various reaction conditions. Modification of the techniques used in this study to allow a higher sequence coverage of HSA (e.g., including lysine 199) [46] are also being considered and experiments are planned that will examine the rates at which various portions of HSA are modified as a result of glycation. Studies with various pharmaceutical agents are also in progress to see how drug-protein interactions change for various preparations of glycated HSA. The expected result is information that can be used by physicians in personalized medicine to help design and modify treatment drug treatments for patients with diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

AFGP	1-alkyl-2-formyl-3,4-glycoyl-pyrole
AGE	advanced glycation end product

CAM	carbamidomethyl
CEL	N_{ε}–carboxyethyl-lysine
CHCA	α-cyano-4-hydroxycinnamic acid
DHB	2,5-dihydroxybenzoic acid
FAS	fractional accessible surface area
FL	fructosyl-lysine
HSA	human serum albumin
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
Pyr	pyrraline
TFA	trifluoroacetic acid

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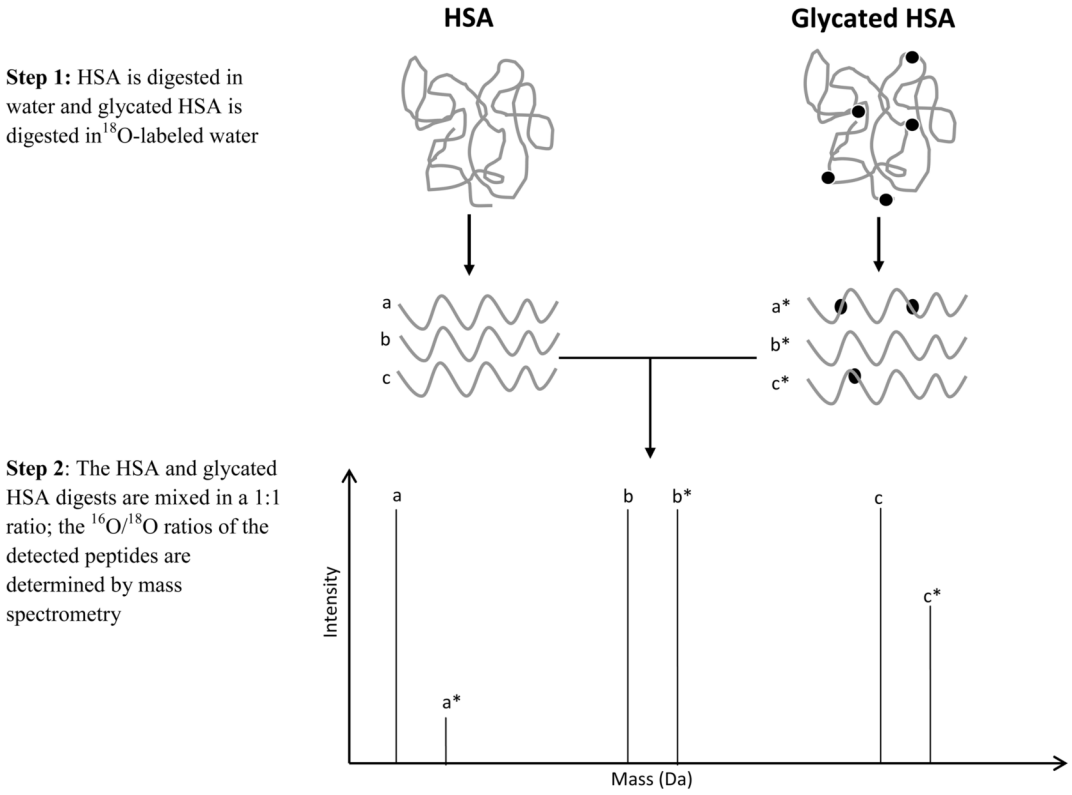

Figure 1.

General procedure for obtaining quantitative estimates of glycation based on ^{18}O-labeling MALDI-TOF MS. The non-modified peptides from HSA are represented by a–c, while a*–c* represent modified peptides from the same regions of glycated HSA.

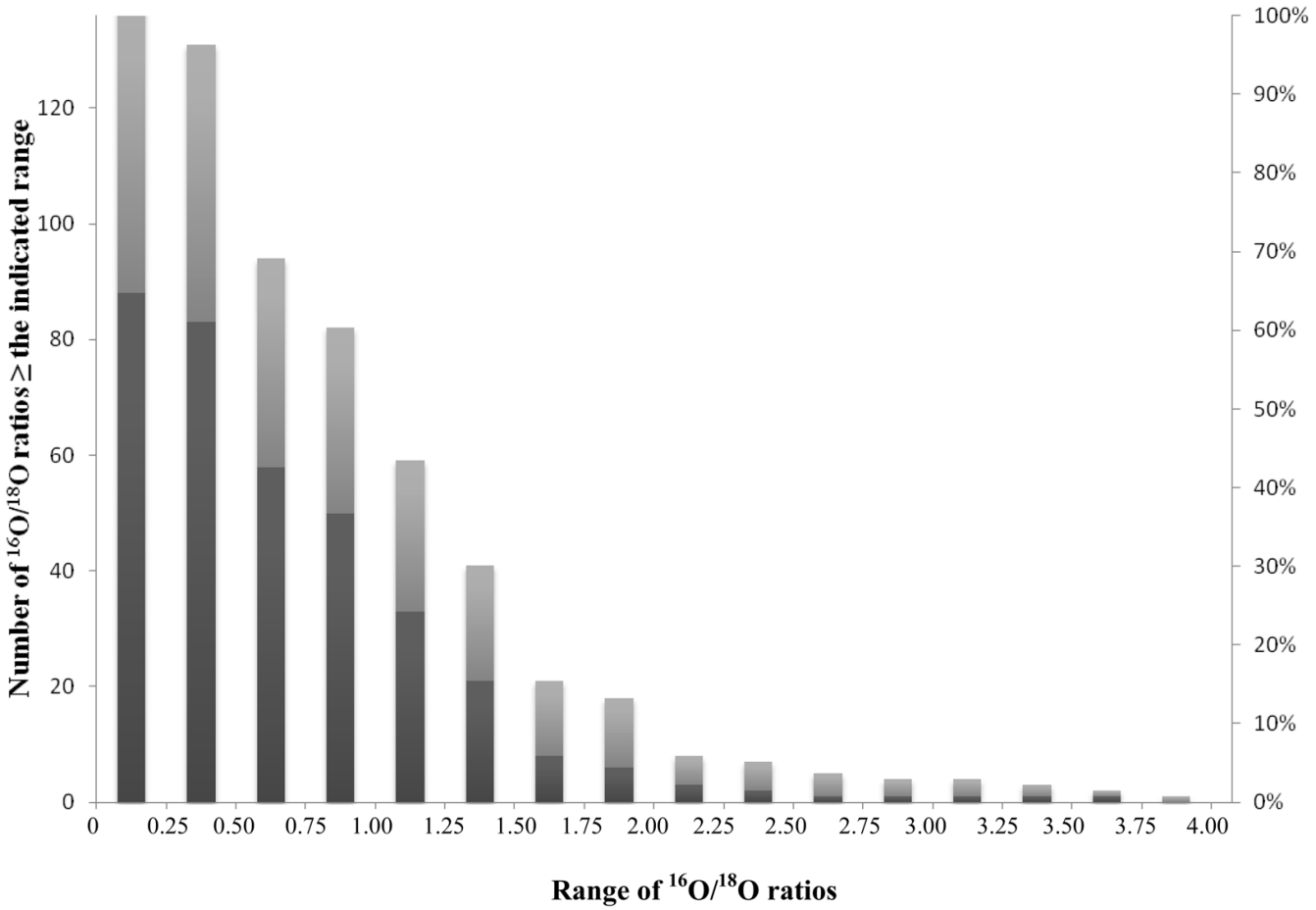

Figure 2.

Bar chart showing the cumulative sum of all the detected peaks as a function of $^{16}O/^{18}O$ ratio range for Methods 1 (■) and 2 (■). The number of $^{16}O/^{18}O$ ratios that were greater than or equal to the indicated $^{16}O/^{18}O$ ratio range were used for this plot. The relative amount of the number of $^{16}O/^{18}O$ ratios is also given as a percentage.

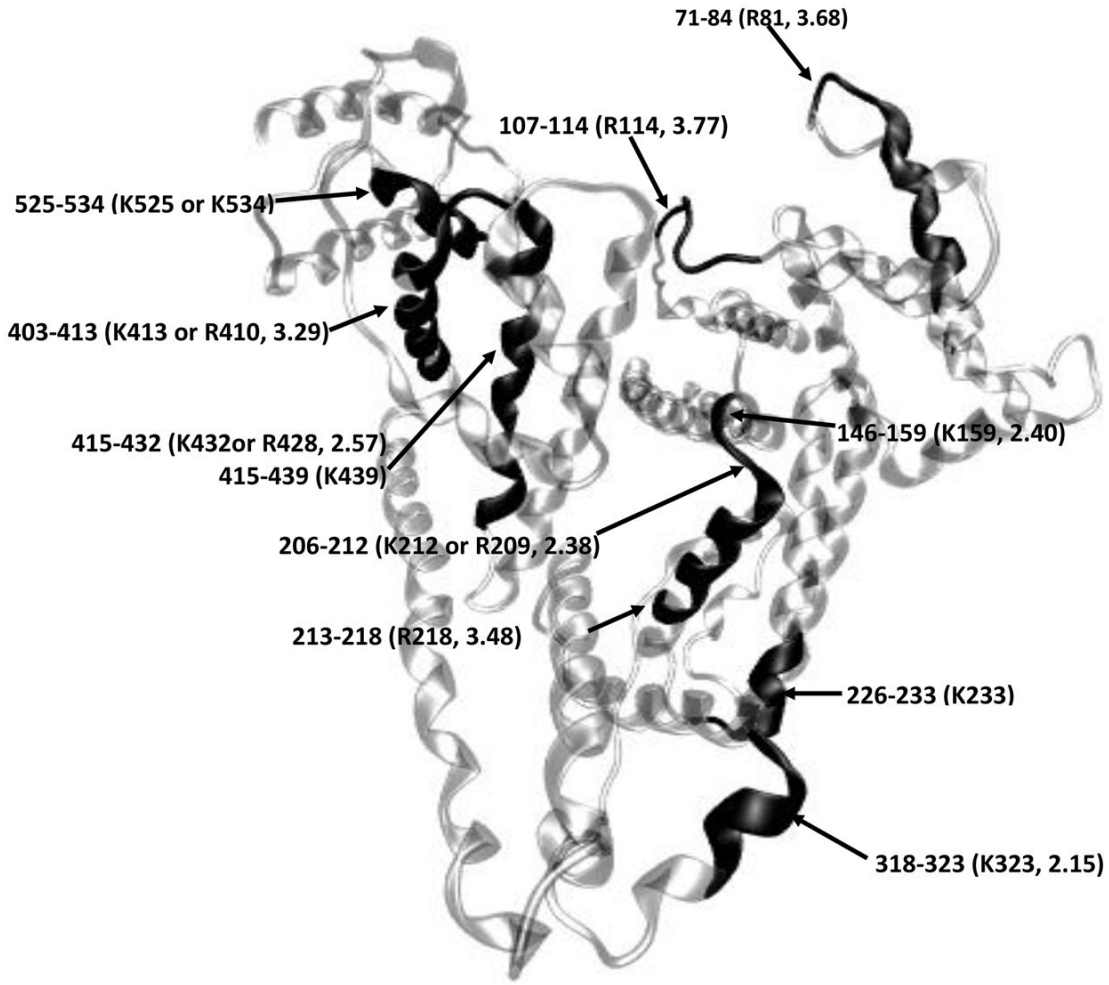

Figure 3.

Structure of HSA, showing the peptides with significant glycation-related modifications that were found in minimally glycated HSA and the corresponding arginine or lysine that was modified in this region. The values of the measured indicated $^{16}O/^{18}O$ ratios are also provided, where applicable. This image was generated from a PDB file with accession number 1AO6 [46].

Table 1

Calculated $^{16}O/^{18}O$ ratios for the tryptic digest using Method 1[a]

Residue	Predicted mass (Da)	Observed peak ratios for each peptide at given % ACN in water					Average $^{16}O/^{18}O$ ratio
		5% ACN	10% ACN	20% ACN	30% ACN	50% ACN	
74–81	933.52	3.68					3.68
146–159	1742.89				2.40		2.40
318–323	695.34	2.15					2.15
324–336	1623.79					1.94	1.94
1–10/42–51	1149.62	1.43					1.43
145–159	1899.00				1.30	1.17	1.23 (± 0.09)
337–348	1467.84	1.08	1.39	1.11	1.18	1.12	1.17 (± 0.13)
65–81	1932.04				1.07	1.10	1.09 (± 0.02)
11–20	1226.61	1.07					1.07
213–218	673.38	1.02					1.02
65–73	1017.54	0.81	1.15				0.98 (± 0.24)
138–144	927.49		0.97	0.94			0.95 (± 0.02)
403–410	960.56	0.93	0.93	0.96			0.94 (± 0.02)
414–428	1639.94		1.12	0.75			0.94 (± 0.26)
546–557	1342.63			0.93			0.93
373–389	2045.10			0.85	0.95		0.90 (± 0.07)
107–114	940.45	0.89					0.89
338–348	1311.74			0.87	0.80		0.84 (± 0.05)
13–20	951.44	0.83					0.83
137–144	1055.59	0.68	0.78				0.73 (± 0.07)
263–274	1443.64	0.70					0.70
234–240	789.47	0.51					0.51
82–93	1434.53	0.36					0.36
182–186	645.36	0.34					0.34
433–444	1400.68	0.34					0.34
390–402	1657.75			0.32			0.32
275–286	1546.80	0.26					0.26
115–136	2778.36			0.20	0.12	0.39	0.24 (± 0.13)

Residue	Predicted mass (Da)	Observed peak ratios for each peptide at given % ACN in water					Average $^{16}O/^{18}O$ ratio
		5% ACN	10% ACN	20% ACN	30% ACN	50% ACN	
94–106	1714.80	0.21	0.12				0.16 (± 0.06)
485–500	1910.93			0.13	0.17		0.15 (± 0.03)
163–174	1371.57	0.10					0.10
115–136	2650.26					0.10	0.10

[a]The standard deviations shown were calculated using the $^{16}O/^{18}O$ ratios obtained in the different ACN fractions.

Table 2

Calculated $^{16}O/^{18}O$ ratios for the tryptic digest using Method 2[a]

Residue	Predicted mass (Da)	Observed peak ratios for each peptide at given % ACN in water					Average $^{16}O/^{18}O$ ratio
		5% ACN	10% ACN	20% ACN	30% ACN	50% ACN	
107–114	940.45	3.77					3.77
213–218	673.38	3.48					3.48
145–159	1899.00				1.80		1.80
414–428	1639.94		1.41	1.80			1.60 (± 0.28)
137–144	1055.59	1.35	1.28				1.32 (± 0.05)
138–144	927.49	1.25	1.27				1.26 (± 0.14)
403–410	960.56	1.25	1.27				1.26 (± 0.14)
21–41	2490.29					0.94	0.94
373–389	2045.10			0.90	0.87		0.88 (± 0.02)
337–348	1467.84			0.81			0.81
94–106	1714.80	0.43	0.43				0.43 (± 0.00)
338–348	1311.74			0.41			0.41
11–20	1226.61	0.39					0.39
390–402	1657.75			0.36			0.36
433–444	1400.68	0.35					0.35
485–500	1910.93			0.23	0.19		0.21 (± 0.03)
115–136	2778.36			0.14	0.12		0.13 (± 0.01)

[a]The standard deviations shown were calculated using the $^{16}O/^{18}O$ ratios obtained in the different ACN fractions.

Table 3

Peptides corresponding to the top eight peak ratios (using a cutoff ratio of 2.0) and their potential modifications[a]

Amino Acids	pK_a/FAS[b]	Residue (digest, method)	Peak ratio	Residue [11][c]	Modification
R114	12.29/0.80	107–114 (Trypsin, 2)	3.77	n/a	none detected
R81	12.50/0.76	71–84 (Trypsin, 1)	3.68	n/a	none detected
R218	9.64/0.20	213–218 (Trypsin, 2)	3.48	200–218 (Try.)	AFGP [11]
K413, R410	10.01/0.23, 12.46/0.33	403–413 (Lys-C, 2)	3.29 (± 0.89)	403–413	FL-2H_2O
K432, R428	9.92/0.36, 11.29/0.18	415–432 (Lys-C, 2)	2.57 (± 1.48)	426–442 (Glu-C)	FL [11]
				415–432	FL-2H_2O
K159	9.94/0.44	146–159 (Trypsin, 1)	2.4	145–160 (Try.)	PYR [11]
K212, R209	10.43/0.40, 12.08/0.61	206–212 (Lys-C, 2)	2.32 (± 0.76)	200–218 (Try.)	AFGP [11]
				206–225 (Lys-C)	FL
K323	10.50/0.54	318–323 (Trypsin, 1)	2.15	n/a	none detected
Significantly modified peptides as indicated by a qualitative comparison					
K233	10.29/0.43	226–233 (Lys-C, 1)	4.32[d]	n/a	none detected
K436, **K439**	9.67/0.51, 10.50/0.94	415–439 (Lys-C)	n/a	426–442 (Glu-C)	FL [11]
K525, K534	10.06/0.07, 11.11/0.12	525–534 (Trypsin)	n/a	520–525 (Lys-C)	CEL [11]

[*] The values in bold represent the most likely modification sites. Modifications that correspond to peptides with high $^{16}O/^{18}O$ ratios are reported here. These modifications were identified by looking for m/z values that corresponded to glycated peptides, as previously described in Ref. [11] using a Perl-script algorithm, and m/z values that didn't correspond to those predicted for a theoretical digest of non-glycated HSA.

[b] This column shows the pK_a and fractional accessible surface area for the protein calculated using the software programs PROPKA [33] and VADAR [34] respectively.

[c] This column indicates whether a similar amino acid or peptide was detected previously [11] or in this current study. The relevant residue, mass shift, and potential modification are also shown. A manual comparison was carried out using a simplified search list based on modifications identified in a previous report [11]. This manual comparison was used to identify modifications that occurred on lysines 413, 432, and 212.

[d] The peptide in the mixed digest that was used to calculate this $^{16}O/^{18}O$ ratio had a mass accuracy that was outside the 50 ppm threshold.

Table 4

Glycation adducts identified and linked to peptides with measured $^{16}O/^{18}O$ ratios[a]

Amino Acids	Residue	Detected Digest	Mass Shift	Modification	$^{16}O/^{18}O$ (Method 1)	$^{16}O/^{18}O$ (Method 2)
K413, R410	403–413	Lys-C	126.03	FL-2H_2O	1.29 (± 0.19)	3.29 (± 0.89)
K106, R98	94–106	Lys-C	58.01	CML	0.77 (± 0.08)	1.42 (± 0.13)
K174	163–174	Lys-C	162.05	FL	0.72 (± 0.06)	na
K174	163–174	Lys-C	72.02	CEL	0.72 (± 0.06)	na
K432, R428	415–432	Lys-C	126.03	FL-2H_2O	1.37 (± 0.35)	2.57 (± 1.48)
K106, R114, R117	101–119	Glu-C	58.01	CML	1.27 (± 0.24), 0.37 (± 0.29)	0.47 (± 0.20)
K466, R472, K475	466–479	Glu-C	72.02	CEL	1.86 (± 1.39), 0.31 (± 0.14)	1.19 (± 0.14)
K240	234–240	Trypsin	144.04	FL-1H_2O	0.51	na
R336	324–336	Trypsin	270.07	AFGP	1.94	na
K136, R117	115–136	Trypsin	126.03	FL-2H_2O	0.39, 0.10	0.13

[a] A manual comparison was also performed using a simplified search list (i.e., with only one modification allowed per peptide), as based on modifications that have been identified previously [11]. This manual comparison was carried out because the ^{18}O-labeling of peptides complicated the determination of modifications via the Perl-script approach.