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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.

1. 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

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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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 Fig. 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*. 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 various 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 an ^{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 Eq. (2) in terms of the individual ^{16}O and ^{18}O contributions

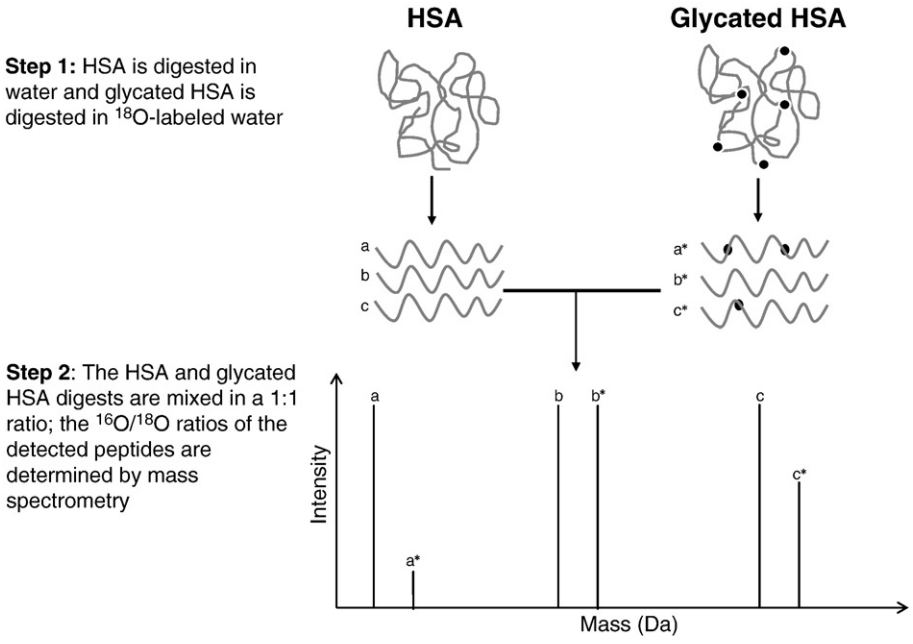

Fig. 1. General procedure for obtaining quantitative estimates of glycation based on $^{16}O/^{18}O$-labeling MALDI-TOF MS. The non-modified peptides from HSA are represented by *a*–*c*, while *a**–*c** represent modified peptides from the same regions of glycated HSA.