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Determining the glycation site specificity of human holo-transferrin

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ABSTRACT

Understanding the effect of glycation on the function of transferrin, the systemic iron transporter, is fundamental to fully grasp the mechanisms leading to the loss of iron homeostasis observed in *diabetes mellitus* (DM). The spontaneous reaction with protein amino groups is one of the main causes of glucose toxicity, but the site specificity of this reaction is still poorly understood. Here in, an *in vitro* approach was used to study human holo-transferrin glycation in detail. Lysine residues 103, 312 and 380 proved to be the most reactive sites, and overall glycation specificity was found to be remarkably different from that described for apo-transferrin. A computational biochemistry approach was subsequently applied to rationalize lysine reactivity. Even though pK_a values, solvent accessible surface area, hydrogen bonds or the presence of nearby charged/polar residues could be related to lysine reactivity, these parameters do not suffice to describe glycation site specificity in holo-transferrin. Furthermore, analysis of the most reactive residues suggests that the correct lysine side chain orientation may play a fundamental role in reactivity. Nevertheless, in holo-transferrin, glycation occurs away from the iron-binding sites and, despite the observed iron release, the modification of apo-transferrin should play a more relevant role for the loss of iron-binding capacity observed in the blood serum of DM patients.

1. Introduction

Protein glycation, the non-enzymatic modification of proteins by reducing sugars, is one of the main causes of hyperglycemia related-toxicity in *diabetes mellitus* (DM) [1]. In fact, levels of protein glycation have been shown to be directly related to glucose blood plasma concentration [2] and modification by glucose has been demonstrated to compromise the functioning of a series of enzymes, eventually leading to a progressive decline in tissue and organ function [3,4]. Glycation is a site specific reaction, initiated by the reversible nucleophilic attack of a free amino group (mainly in lysine side chains) on a carbonyl moiety to yield a Schiff base, which may rearrange to form the more stable Amadori product (AP). Further oxidation and dehydration reactions render irreversible modifications collectively named as advanced glycation end-products [3,5]. The diverse susceptibility to modification by electrophilic carbonyls of the various amino groups within a protein may be essential to determine its loss of function [6,7], but the structural determinants favoring glycation of particular amino acid residues are still poorly understood. Lysine side chains presenting lower pK_a values have been shown to be more reactive [7,8], but surface exposure [9] and the catalysis of Amadori rearrangement by neighboring amino acid residues have also been proposed to play a major role in determining glycation site specificity [6,10,11]. *In vitro* model studies indicated that histidine, threonine, lysine and aspartate residues favor AP formation in proximal lysine residues [6,12,13], while hydrogen bonds and strong electrostatic interactions seem to protect lysine residues from being modified [10,12].

Transferrin (Tf), the systemic iron transporter, is among the proteins whose function is affected by glycation, and its modification has been described to impair its iron-binding capacity [7,14], contributing to increased oxidative stress [15] and the occurrence of non-transferrin-bound iron species (NTBI), which has been described in diabetes [16]. Due to the biological relevance of Tf and its implication in the pathology of DM, we have decided to thoroughly study the structural alterations induced by glycation. Recently, we have produced a detailed description of apo-Tf glycation, revealing that the presence of a sugar residue (or AP) at the two most favorable glycation sites (K534 and K206) blocks access to the two iron-binding sites [7]. In addition, glycation site reactivity was shown to be favored by low lysine pK_a values and low lysine side-chain solvent accessibility (SASA – solvent accessible surface area).

Herein, we try to increase knowledge about glycation site specificity

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by studying iron saturated Tf (holo-Tf). Tf has a bilobal structure which undergoes significant changes upon iron-binding, despite the extensive conservation of secondary structure motifs between iron isoforms [17–19]. We have used mass spectrometry (MS) to identify glycation sites in holo-Tf and determine their different susceptibility to this reaction. Further, a computational biochemistry approach has been used to compare the two structures and rationalize the significant differences encountered in glycation specificity for the two Tf isoforms.

2. Materials and methods

2.1. Reagents

Most reagents were acquired from Sigma-Aldrich and were used without further purification. Human holo-transferrin was purchased from Sigma (T4132), while MS-grade TPCK-treated porcine trypsin was obtained from Thermo Scientific Pierce (90057). Iron standard for determination iron concentration was from Fluka Analytical (43149). Chromasolv grade acetonitrile (Sigma-Aldrich) was used in LC-MS studies and MilliQ water was used throughout the study.

2.2. Holo-transferrin glycation

Human holo-serotransferrin at a concentration of 2.5 g·L^{-1} (32 μM) was incubated at 37 °C in pH 7.4 buffer containing 20 mM disodium hydrogen phosphate, 25 mM sodium hydrogencarbonate, 0.02% sodium azide and variable concentration of D-glucose (0, 10, 20, 100 and 500 mM). Glucose concentrations were chosen in order to represent a pre-diabetic condition (10 mM) and hyperglycemia (20 mM and 100 mM) [20]. 500 mM glucose aimed at showing an extreme condition. 200 μL aliquots were collected after 7 or 14 days incubation, a period comprising the half-life of serum transferrin (9 days). Excess glucose was removed using cycles of concentration/dilution in glucose less buffer, utilizing 10 kDa molecular weight cut-off (MWCO) ultrafiltration units (Amicon). After the first centrifugation, the ultra-filtrate was stored, in order to allow quantification of iron released by transferrin during incubation. Tf samples were conserved at −80 °C until proteomic analysis. Triplicate reactions were carried out.

2.3. Ferrozine assay for iron quantification

Iron released from holo-Tf during incubation with glucose was quantified after ultra-filtration in 10 kDa MWCO ultra-filtration units, using the colorimetric ferrozine assay [21] adapted to a 96 microwell plate reader. Briefly, 200 μL samples were ultracentrifuged at 10000 *g* for 30 min, to remove transferrin. Subsequently, 100 μL of the ultra-filtrate was mixed with 50 μL of a 4 mM ascorbic acid solution in 0.2 mM formic acid at pH = 3, and incubated at room temperature for 5 min. Subsequently, 50 μL of a 1 mM ferrozine solution in formic acid were added and the mixture was incubated for 30 min at room temperature. Absorbance was read at 562 nm in a microwell plate reader (Bio-Tek). Iron concentration was calculated from a standard curve prepared by serial dilutions of an analytical Fe standard in Tf incubation buffer. Statistical analysis of variance was performed in the GraphPad Prism 5.00 software package. Two-away ANOVA followed by the Bonferroni's post-test was employed to evaluate significance of differences between linear correlations along time or glucose concentration. One-way ANOVA followed by the Tukey's post-test was used to evaluate differences between all pairs of samples, when these were considered independently.

2.4. Triptic digestion

Glycated Tf samples were diluted to a final concentration of 1 g·L^{-1} in 50 mM ammonium bicarbonate buffer (pH = 8.0) containing 8 M urea and 0.1% sodium dodecylsulphate (SDS). Dithiotreitol (DTT) was added to a final concentration of 20 mM and samples were incubated at 60 °C for 1 h. After, samples were incubated with 40 mM iodoacetamine (IAA) and kept in the dark for 30 min. Excess IAA was quenched with a further addition of DTT (to a final concentration of 10 mM). Samples were diluted to a Tf concentration of 0.1 g·L^{-1} and trypsin was added in a 1:20 protein mass ratio. Digestion was allowed to proceed overnight at 37 °C.

2.5. Glycation site identification by mass spectrometry

Tryptic digests were analyzed with a nanoHPLC system (Dionex, 3000 Ultimate RSLCnano) coupled on-line to a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) equipped with a nano-electrospray ion source (Thermo Scientific, EASY-Spray source). Eluent A was aqueous formic acid (0.1%) and eluent B was formic acid (0.1%) in acetonitrile. Samples (10 μL) were injected directly into a trapping column (C18 PepMap 100, 5 μm particle size) and washed over with an isocratic flux of 98% eluent A and 2% eluent B at a flow rate of 30 μL·min^{-1}. After 3 min, the flux was redirected to the analytical column (EASY-Spray C18 PepMap, 100 Å, 150 mm × 75 μm ID and 3 μm particle size) at a flow rate of 0.3 μL·min^{-1}. Column temperature was set at 35 °C. Peptide separation occurred using a two-step gradient: linear gradient of 2–35% eluent B over 12 min followed by 35–60% eluent B over 45 min. Eluent B was then raised to 90% and the column was washed for 10 min.

The mass spectrometer was operated in the positive ion mode, with a spray voltage of 1.9 kV and a transfer capillary temperature of 250 °C. Tube lens voltage was set to 120 V. MS survey scans were acquired at an Orbitrap resolution of 60,000 for an *m/z* range from 300 to 2000. Tandem MS (MS/MS) data were acquired in the linear ion trap using a data dependent method with dynamic exclusion: The top 3 most intense ions were selected for collision induced dissociation (CID). CID settings were 35% normalized collision energy, 2 Da isolation window, 30 ms activation time and an activation Q of 0.250. A window of 45 s was used for dynamic exclusion. Automatic Gain Control (AGC) was enabled and target values were 1.00e + 6 for the Orbitrap and 1.00e + 4 for LTQ MSn analysis. Data were recorded with Xcalibur software version 2.1.

MS data were analyzed using the software Proteome Discoverer 1.4 from Thermo Scientific. Searches were performed against the Human Proteome in the SwissProt database (22/11/2015). Sequest HT algorithm was used, allowing for 2 missed cleavages and mass tolerances 10 ppm for precursor ion and 0.8 Da for fragment ions. Carbamidomethylation of cysteine (+57.02146) was considered as a fixed modification and oxidation of methionine (+15.99491) and Amadori Product formation (+162.05282) were considered as a variable modification in all database searches. Formation of advanced glycation end-products (AGEs) was also considered (Supplementary Table S1). Identified glycated peptides were manually validated. Carboxymethylation of peptide CLVEKGDVAFVK was ignored, because it was found to correspond to a mis-assignment of the homologous peptide CLKDGAGDVAFVK. All results were curated manually and Sequest annotated modifications for which the best PSM was considered not to contain sufficient information to validate the identification were disregarded as false positives. A modification was considered whenever it was identified by the software in any given sample and its presence could be ascertained in its two additional experimental replicates based on accurate mass and chromatographic retention time. Supplementary Table S2 compiles Sequest HT results for the best PSMs of modified peptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE [22] partner repository with the dataset identifier PXD009345. Throughout this manuscript, notation of modified amino acid residues refers to their location within the processed protein (without signal peptide), thus presenting a 19 residue deviation to their position in the Uniprot curated sequence (**P02787**).

Results were compared with glycation site prediction by the

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