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Massive glycation of protein HC, a low molecular weight lipocalin, in non-diabetic individuals

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Abstract Human protein HC is a member of the lipocalin superfamily with unique properties since it carries a covalently bound fluorescent chromophore mediating the linkage of the major part of protein HC to several plasma proteins, with IgA as the dominating complex partner. Native protein HC displays characteristic absorption and fluorescence spectra similar to those of glycated proteins with advanced glycosylation end products (AGEs). In vitro glycation of protein HC induces the formation of fibril aggregates with a corresponding increase of absorption in the visible region of the spectrum. Boronate-affinity chromatography and a novel galactosyltransferase assay indicate that protein HC is modified with residues of glucose exposed in a terminal non-reducing position which is typical of glycated proteins. The glycation level of several isolated batches of protein HC as measured by both assays was around 35%, which represents the highest level described for human plasma-derived proteins from healthy individuals.

Key words: Protein HC; Glycation; Lipocalin

1. Introduction

Protein HC (human complex-forming glycoprotein heterogeneous in charge) is a low molecular weight plasma protein that belongs to the lipocalin superfamily of hydrophobic ligand-binding proteins. It is unique among the large lipocalin family since its major brown fluorescence ligand displays extensive charge and size heterogeneity [1–6]. Its chromophore is covalently linked to the polypeptide chain by a novel non-thioester reduction-resistant bond involving the cysteine residue 34 [5] giving the protein its spectroscopic properties and its heterogeneity. A related reduction-resistant linkage has been reported in the HC-IgA complex with the chromophore acting as an intermolecular bridge linking cysteine 34 on protein HC to the penultimate cysteine residue in the carboxy-terminal end of one of the IgA light chains [6]. Although the structure of the protein HC chromophore has not yet been elucidated, the chromophore displays characteristic absorption and fluorescence spectra [1–6] which are closely related to those of glycated proteins carrying advanced glycosylation end products (AGE) [7].

Protein glycation involves the non-enzymatic covalent binding of glucose and other reducing monosaccharides to α- and ε-amino groups of polypeptide chains to yield Schiff-base intermediates which via Amadori rearrangement form more stable ketoamine derivatives. These derivatives may subsequently undergo slow and only partially characterized reactions to form heterogeneous fluorescent and non-fluorescent AGE. The formation of AGE affects mainly longlived proteins, e.g. collagen, laminin and fibronectin of the extracellular matrix [8,9], although recently there have been an increasing number of reports concerning the presence of glycation in several non-longlived plasma proteins and tissue proteins as well [10–15]. The modified proteins usually develop inter- and intra-polypeptide chain linkages, become protease-resistant and detergent-insoluble and display characteristic fluorescence spectra [16]. The glycation and subsequent generation of AGE is believed to be pivotal in the normal human aging process and in the production of the abnormal protein deposits occurring in the brains of Alzheimer patients [17,18]. The raised blood glucose level in diabetes causes an increased rate of glycation of proteins and is thus assumed to be involved in the pathogenesis of late diabetic complications. In healthy persons, the serum albumin and hemoglobin glycation levels are, expressed as percentage of glycated protein, below 1–6% [19] and 4–5% [20], respectively. In the present work we demonstrate by boronate-affinity chromatography, and by a colorimetric assay and a novel galactosyltransferase assay, that protein HC of non-diabetic individuals is glycated to more than 30%.

2. Materials and methods

2.1. Isolation of protein HC

Protein HC was purified from the urine of non-diabetic patients as described earlier [1,2].

2.2. Boronate-affinity chromatography

Glyco-Gel II from Pierce was used in affinity chromatography to separate and quantify glycated forms of native and deglycosylated protein HC (100 μg). Bound proteins were eluted with a buffer containing sorbitol following the manufacturer's instructions. The relative ratios of bound and non-bound fractions (500 μl) were quantified by amino acid analysis of the pooled fractions.

2.3. Galactosyltransferase assay

The UDP-galactose:glycoprotein galactosyltransferase (EC 2.4.1.38) was assayed essentially as described [18] using native and deglycosylated HC as an exogenous acceptor. The samples assayed were the pooled fractions (retained and non-retained) obtained from the Glyco-Gel II chromatography.

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2.4. Colorimetric glycation assay

For the colorimetric determination of protein HC glycation, a method recently developed by Kennedy and coworkers [21] was carried out with and without modifications (under borohydride reduction) using in vitro glycated fetuin as a standard in order to avoid interference due to the protein HC glycosylation. For the calibration curve, mixtures of in vitro glycated and non-glycated fetuin in varying proportions were prepared so that the total concentration of protein, and hence glycosylation interference, remained constant. The in vitro modified fetuin bound to the boronic acid column was considered to represent 100% glycated fetuin.

2.5. Glycation of proteins in vitro

Samples of fetal calf fetuin (5 mg/ml) and protein HC (1.0 mg/ml) were incubated with 500 mM glucose in 50 mM phosphate buffer, pH 7.0, for 20 and 110 days, respectively, at 37°C. Alternatively, 50 μg of protein HC was incubated with 1 μCi of D-[^{14}C]glucose (56.4 mCi/mmol) in 50 mM phosphate buffer for up to 16 days.

2.6. High performance liquid chromatography (HPLC)

Size-exclusion HPLC was performed in a Pharmacia Smart system using a Superdex 75 PC 3.2/3 micro-column (3.2×300 mm) connected on line with a Waters 996 Photodiode Array Detector. The column was equilibrated and eluted with 50 mM phosphate buffer, pH 7.0, at room temperature and at a flow rate of 0.04 ml/min.

2.7. Enzymatic removal of glycans

Treatment of protein HC with β-*N*-acetylhexosaminidase (Jack Bean, EC 3.2.1.30, Oxford Glycosystems, Oxford, UK), PNGase F (*Flavobacterium meningosepticum*, Oxford Glycosystems) and endo-α-*N*-acetylgalactosaminidase (*O*-glycanase, *Streptococcus pneumoniae*, EC 3.2.1.97, Oxford Glycosystems) was carried out according to the manufacturer's instructions.

2.8. Electron microscopy

For electron microscopy, 5 μl of in vitro glycated and native HC (1 μg/μl) was applied to Formvar-coated nickel grids, negatively stained with 2% (w/v) uranyl acetate, and observed in an electron microscope model 410 (Philips Electronic Instruments, Mahwah, NJ) at 80 kV.

3. Results

3.1. Boronate-affinity chromatography of protein HC

Boronate-affinity chromatography is routinely used to assay glycated proteins since only those glycated proteins bind to and are retained by the matrix. Thus, five preparations of protein HC isolated from the urine of non-diabetic patients were tested for glycation by binding to a boronate-affinity chromatography column. Each preparation was applied to a 2-ml Glyco-Gel column and the retained fractions of protein HC were eluted with sorbitol-containing buffer. The retained and non-retained fractions were quantified by amino acid analysis (using norleucine as internal standard) after hydrolysis. Table 1 shows that all protein HC preparations contain a high proportion (25–48%) of molecules containing *cis*-diol groups and hence are retained by the column and eluted by sorbitol as described to be characteristic for glycated proteins. Deglycosylated protein HC behaves on the phenylboronate column as native protein HC, suggesting that protein glycosylation does not determine the binding of protein HC to the affinity resin (results not shown).

Table 1
Glycation analysis of protein HC by GlycoGel system and colorimetric assay

Lot	% HC bound[a]	cpm [^{3}H]Gal linked to HC[b]	% of [^{3}H]Gal-HC bound[c]
87	32.4	7501	66.6
88	48.1	8412	72.3
90	25.3	12523	84.3
91	36.2	11231	76.2
92	34.3	10737	83.5

[a]% of protein HC bound to the GlycoGel column.
[b]Radioactivity associated with protein HC after labeling via the galactosyltransferase assay.
[c]% of protein HC bound to the GlycoGel after treatment with PNGase and β-hexosaminidase followed by labeling via the galactosyltransferase assay.

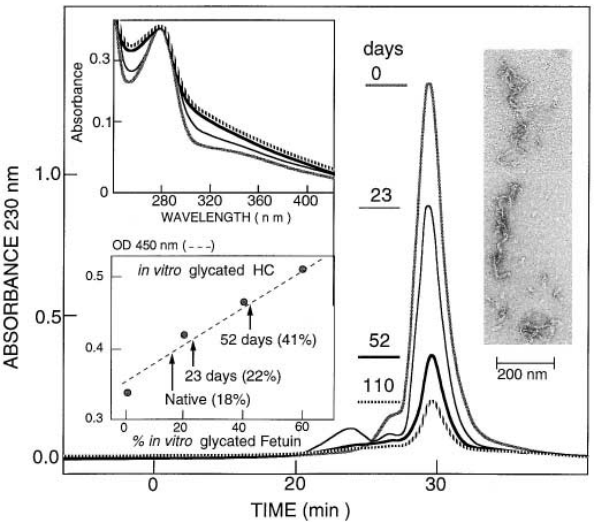

Fig. 1. In vitro glycation of protein HC as revealed by the formation of aggregates. Incubation in 500 mM D-glucose took place for 110 days. At the indicated times, the samples were centrifuged at 14000 rpm for 3 min and the supernatant was subjected to size-exclusion HPLC. The absorption spectra normalized to 280 nm, automatically determined at the peak maxima of the monomeric peak, are shown above. The inset shows the electron microscopy image showing fibril aggregates stained with 2% uranyl acetate. The colorimetric glycation assay of protein HC using fetuin as a standard curve is also shown in an inset. Arrows show the OD values obtained for the three samples of native and in vitro glycated protein HC at two different durations of glycation, 23 and 52 days. The percentage glycation is indicated in parentheses.

3.2. Galactosyltransferase assay of protein HC

A novel galactosyltransferase assay designed to demonstrate the presence of free non-glycosylation (*N*-, *O*-, or *O*-GlcNAc) terminal residues of glucose and/or NAcGlc in proteins by the transfer of [^{3}H]galactose to such groups was applied to protein HC preparations. Table 1 demonstrates that all preparations of protein HC incorporate relevant amounts of radiolabeled galactose. As protein HC contains two *N*-glycans per molecule, the protein HC preparations were treated with PNGase to completely remove the *N*-glycans with putative terminal GlcNAc residues which could act as acceptors for [^{3}H]galactose. This treatment reduced the protein HC-linked [^{3}H]galactose counts by less than 18% in all preparations, indicating that more than 80% of the incorporated [^{3}H]galactose is in a non-glycosylation-derived residue. Furthermore, pretreatment of the samples with β-hexosaminidase to specifically remove terminal GlcNAc residues present in any glycan diminished the transfer of [^{3}H]galactose to protein HC by the same magnitude as the PNGase pretreatment (less than 22%). This result points to a specific linkage of the [^{3}H]galactose to other residues than those from *N*-glycans. In addition, treatment of the radiolabeled samples with endo-α-*N*-acetylgalactosaminidase (*O*-glycanase) had no effect

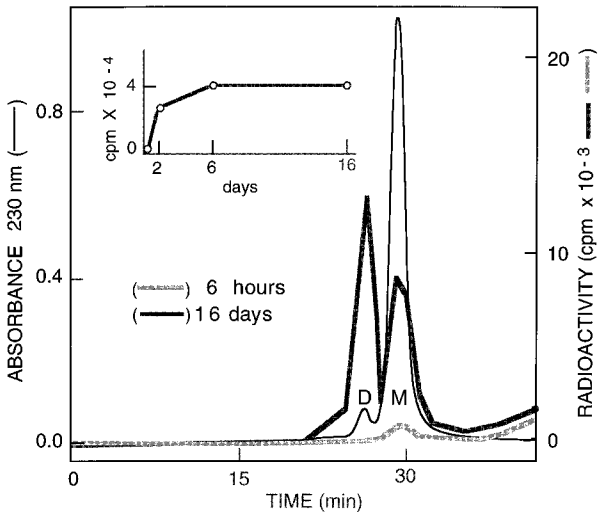

Fig. 2. Radioactivity profile of size-exclusion HPLC of protein HC glycosylation with D-[^{14}C]glucose after 16 days. Time courses of total radioactivity (monomer, M and dimer, D) are shown in the inset.

on the labeling efficiency, thus ruling out any possibility that the radioactive sugar is transferred to the *O*-glycans. It is important to mention that the galactosyltransferase assay was done in the presence of lactalbumin so that the transfer of radiolabeled galactose is towards glucose residues and not to GlcNAc residues.

The results of boronate-affinity chromatography and the galactosyltransferase assay clearly indicate that a large proportion of the investigated protein HC populations are modified by glucose residues attached to protein molecules and exposed in a terminal non-reducing position. As an additional control to check that the components mediating the binding of protein HC to the boronate column are the same as those acting as acceptors for [^{3}H]galactose residues, protein HC samples, previously deglycosylated and terminal GlcNAc removed by treatment with PNGase and β-hexosaminidase and then [^{3}H]galactose-labeled, were tested by boronate-affinity chromatography. The results presented in Table 1 show that a large part of the radioactivity was retained by the column, hence providing further evidence supporting the notion that the presence of terminal non-glycosylation-derived glucose residues on protein HC molecules may cause their binding to boronate columns. It should be emphasized that [^{3}H]galactose labeling of in vitro glycated native and deglycosylated bovine serum albumin, IgG, RNAse and ovalbumin does not significantly affect the binding of those proteins to the boronate-affinity chromatography (Bonay et al., manuscript in preparation).

3.3. Colorimetric assay of glycation

During the course of this investigation we decided to test another assay of glycation, free of interference from other post-translational modifications like glycosylation [21], in order to rule out the possibility that modifications other than glycation are responsible for the high binding values obtained in the boronic acid assay. This assay requires the reduction of the proteins with sodium borohydride prior to periodate oxidation, thus increasing the amount of formaldehyde released proportional to the glycation without interference from sugars in the glycosylated glycans. For the calibration curve we used in vitro glycated fetuin as a standard, since it is a moderately glycosylated protein, like protein HC. The results of applying this colorimetric assay to protein HC are shown in Fig. 1, which clearly demonstrates that native protein HC is glycated since it gives a readout equivalent to a fetuin glycation level of 18% (Table 1). Furthermore, as an internal validation of the method, in vitro glycated protein HC after two different incubation periods (23 and 52 days) was also tested in the colorimetric assay giving consistent and repeatable values of equivalent fetuin glycation of 22 and 41% respectively. Those values fit with the other parameters used to evaluate the in vitro glycation of protein HC and confirm the high basal glycation state of native protein HC. The same assay done with in vitro glycated bovine serum albumin as a standard protein provides similar values of glycation (results not shown).

3.4. Incorporation of [^{14}C]glucose into protein HC

Protein HC (0.34 mg/ml) was incubated with a low concentration of [^{14}C]glucose for various periods and then analyzed by size-exclusion HPLC. The virtually identical chromatograms obtained after incubation periods of 6 h and 16 days (Fig. 2) demonstrated that the two protein HC populations contained a predominant monomeric and a small dimeric fraction and displayed the same dimer/monomer ratio. However, while the short incubation period of 6 h led to incorporation of glucose only into the monomeric fraction, incubation periods of 6–16 days produced glucose incorporation also into the dimeric fraction, which contained about 60% of the glucose after the longer incubation periods (Fig. 2). The total amount of radiolabeled glucose incorporated increased with the incubation period and reached a plateau level after 6 days (Fig. 2, inset).

3.5. Glucose-induced oligomerization of protein HC

In a second incubation experiment, protein HC (1.0 mg/ml) was incubated with a high concentration of glucose (500 mM) and the mixture analyzed by gel chromatography after various periods of incubation up to 110 days. Fig. 1 shows a reduction in the light absorption of the protein HC solution as a consequence of the characteristic formation of fibrils and amorphous aggregates typical of in vitro glycated proteins. The absorption spectra of each of the soluble in vitro glycated protein HC fractions, recorded at the peak maxima, were found to be similar, displaying significant absorption in the visible region like native protein HC, but with increased 330/280 nm absorbance ratios. The absorbance ratio increased with the incubation period and reached a plateau level after approximately 50 days, much later than the equilibrium reached by the radiolabeled glucose incorporation, indicating the formation of post-Amadori products.

4. Discussion

The present work presents evidence indicating that protein HC is modified in such a way that glucose/glucosamine residues are exposed and accessible in a non-reducing terminal position characteristic non-exclusively of glycated proteins. The behavior of protein HC on boronic acid affinity chromatography makes that modification compatible with glycation. In addition, protein HC is a good acceptor as revealed by a

recently described radiometric assay developed to quantify glycation modified proteins [18]. To rule out any interference from other possible modifications, like *N*- or *O*-glycosylation, *O*-GlcNAc, which could artifactually affect the behavior of proteins on the affinity resin, we also tested the putative glycation of protein HC by a colorimetric assay claimed to be free of interference from glycosylation [21]. The results derived from the three assays support the same conclusion that protein HC contains covalently linked and terminally exposed glucose residues other than those present on the *O*- and *N*-glycosylation sites already described in protein HC [22]. The most appropriate conclusion that can be derived from the evidence presented here and the analysis of post-translational modifications of proteins found in the literature is the occurrence of glycation until further analysis is done. The apparent discrepancy in the levels of native protein HC glycation as determined by the affinity chromatography method (about 35%) and the colorimetric assay (about 18%) can be easily explained since the latter value is a relative one based upon the glycation of a standard glycosylated protein like fetuin. In contrast, the affinity chromatography assay gives an estimation of the actual proportion of glycated protein HC molecules in the entire molecular population of protein HC. As the density of glucose residues on the polypeptide chain is known to affect the binding to the boronic acid resin, it could be stated that all protein HC is modified and only 35% in such a degree as to be retained by the affinity resin. However, in spite of these differences between methods, what seems to be unique to protein HC is its degree of basal modification (glycation). Several plasma and tissue proteins in both healthy individuals and diabetic patients have been demonstrated to be glycated and their glycation level shown to increase both with the blood glucose level and most probably also with the half-life of the protein. Interestingly, the reported proportions of glycated proteins are not higher than 20–22% in non-diabetic individuals and there is only one report of a protein (Cu,Zn- superoxide dismutase) with a glycated fraction of 40% in diabetic patients [11]. The observed protein HC basal modification (glycation) level of over 30% is particularly interesting considering the short half-life and the source (urine) of the protein and since the investigated batches of protein HC were isolated from non-diabetic individuals. In addition to the basal modification identified on protein HC, we also report here that protein HC could be further modified in a saturable fashion by in vitro glycation, although to a minor degree, by the incorporation of radiolabeled glucose. Regarding the in vitro modification, it is noteworthy that it increases the browning of the modified protein above the levels of the native protein HC, concomitant with the equivalent glycation values derived from the colorimetric assay of glycation.

The observed high level of modification (glycation) of native protein HC prompted studies on the interaction of glucose and native protein HC. Incubation with low levels of [^{14}C]glucose induced incorporation of glucose into monomeric protein HC within hours. A prolonged incubation period resulted in increased glycation of protein HC, most of the glycation occurring in the dimeric protein HC fraction. These results suggest that protein HC glycates easily and that glycation might be involved in the formation of protein HC dimers, some of which are known to form by bonds which are resistant to reduction, as is the case for the bond between protein HC and IgA in the protein HC-IgA complex [2,4,6]. Incubation of protein HC with higher concentrations of glucose resulted in precipitation of protein HC as aggregates and fibrils. Interestingly, the remaining still soluble fraction of protein HC displayed an absorption spectrum similar to that of native protein HC but with a noticeable increase in the absorption above 300 nm, characteristic of the native protein HC chromophore. These results suggest that glycation might be involved in the formation of the protein HC chromophore and, in addition, that glycation might be involved in the formation of reduction-resistant links between protein HC and several other proteins since it has been described that the chromophore of native protein HC mediates such links [6].

The present investigation demonstrates that native protein HC is highly modified (glycated), that it can be further modified by glycation in vitro and that the in vitro glycation may result in the formation of chromophores similar to those present in native protein HC. The location of the modification (glycation) sites on the polypeptide chain of protein HC is presently being addressed and at least six different galactosyltransferase-labeled [^{3}H]galactose-protein HC-derived peptides have already been located along the polypeptide chain of protein HC (manuscript in preparation). It might be of significance that the chromophores of both native and in vitro glycated protein HC display several properties similar to those described for the poorly characterized post-Amadori products. For instance, the absorption and fluorescence spectra of the protein HC chromophores are closely related to those described for AGE, in spite of the short half-life of protein HC. The protein HC chromophore has recently been shown to be involved in the linkage of protein HC to the protein partners found on natural complexes like HC-IgA in a reduction-resistant bond. This property resembles the crosslinking of glycated proteins mediated by the post-Amadori products. While generally the in vitro glycation may confer non-specific crosslinking properties to the modified protein, protein HC is highly specific regarding the protein partners crosslinked in the complexes where the chromophore acts as an intermolecular bridge involving cysteine residues. Thiols had not previously been demonstrated to be targets of glycation by glucose, let alone the massive glycation reported here. Further investigations are required to identify the chemical nature of the glycation described in this report as well as the precise sites on the polypeptide chain where this modification occurs.

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