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HIGH-PERFORMANCE LIQUID CHROMATOFOCUSING AND COLUMN AFFINITY CHROMATOGRAPHY OF *IN VITRO* ^{14}C-GLYCATED HUMAN SERUM ALBUMIN

DEMONSTRATION OF A GLYCATION-INDUCED ANIONIC HETEROGENEITY

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SUMMARY

High-performance liquid chromatofocusing of human serum albumin (HSA) after *in vitro* glycation with purified [^{14}C]glucose has shown that with increasing glycation time a progressive increase in two major anionic fractions (p*I* 4.8 and 4.65) occurs, while the p*I* 4.9 fraction decreases in parallel. As early as after 5 days of glycation time, the [^{14}C]glucose content in the anionic fractions was markedly higher than in the p*I* 4.9 fraction. After 10 and 15 days of glycation, a considerable heterogeneity of 10–15 components could be demonstrated. In addition, phenylboronic acid (PBA) affinity chromatography was applied and an enrichment of the more glycated species could be obtained using this method. We conclude that, in contrast to previous reports, glycation of HSA induces anionic heterogeneity (in accordance with the theoretically expected loss of positively charged amino groups) and, although the efficiency in separating non-glycated from monoglycated HSA was found to be very low, an enrichment of these anionic species can be achieved using PBA affinity chromatography.

INTRODUCTION

In previous studies we have demonstrated a different renal clearance of glycated[a] and non-glycated albumin in diabetics with incipient nephropathy[2,3]. The reason for this difference could be a varying surface charge of glycated and non-glycated albumin

[a] According to the recomendations of the IUB (International Union of Biochemistry) and IUPAC (International Union of Pure and Applied Chemistry)[1], "glycation" is used instead of "glycosylation" or "glucosylation" to refer to the non-enzymatic reaction between glucose and free amino groups of proteins.

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or of the glomerular barrier. As a cationic charge heterogeneity of glycated albumin has been reported[4–7], which, however, is different from that theoretically expected, we have studied the electrical charge of glycated human serum albumin after 0–15 days of *in vitro* glycation with purified [^{14}C]glucose. In a previous study[8] we have demonstrated an anionic heterogeneity (using isoelectric focusing, pH 4–6.5) contradictionary to other studies[4–7], and in this paper we report the results obtained by using high-performance liquid chromatofocusing (HPLCF) before and after phenylboronic acid (PBA) affinity chromatography.

EXPERIMENTAL

Purification of [^{14}C]glucose

Fast-reacting contaminants of [U-^{14}C]glucose (Amersham International, Amersham, U.K.) were removed by incubation with human serum albumin coupled to a vinyl sulphone agarose matrix (Mini-Leak; Kem-En-Tec, Hellerup, Denmark) for 36 h at 37°C in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.02% NaN_3 as described[8].

In vitro glycation of albumin

Crystalline human serum albumin (HSA) (Behringwerke, Marburg, F.R.G.) was dissolved in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.02% NaN_3 and 50 mM [^{14}C]-D(+)-glucose (specific activity 6 μCi/mg). The solution was filtered through a 0.22-μm Millex filter (Millipore, Tåstrup, Denmark) and incubated for 0–15 days at 37°C in a shaking bath (30 cycles/min). Albumin samples incubated without glucose and with non-labelled glucose were also included. Salts, glucose and non-covalently bound glucose were separated from the HSA using a 95 × 2.5 cm I.D. Sephadex G-50 column eluted with 0.1 M ammonium hydrogen carbonate (pH 8.0) at 28 ml/h. Incubation of HSA for 0, 5, 10 and 15 days resulted in an activity of 0.9, 19.9, 47.5 and 70.3 · 10^3 dpm per mg of protein.

Affinity chromatography

Prepacked microcolumns containing 1 ml of aminophenylboronic acid immobilized to agarose (Glycogel B) (Pierce, Rockford, IL, U.S.A.) were used as described[9] for the separation of glycated and non-glycated albumin. As it has been reported that column overloading might influence this separation[10], 5 mg in addition to 0.01–0.08 mg of protein were loaded on the affinity columns. Fractions of 0.5 ml were collected and 50 μl of each fraction, mixed with 2.5 ml of Hi-Safe Optiphase scintillation cocktail (LKB, Hillerød, Denmark), were analysed for radioactivity in a Tri-Carb 460 liquid scintillation counter (Packard, Downers Grove, IL, U.S.A.). The counting efficiency was >95%. UV absorption at 280 nm was measured in 450 μl of each fraction diluted 1:1 in elution buffer using a Uvicon 810 spectrophotometer (Kontron, Zurich, Switzerland).

High-performance liquid chromatofocusing

HPLCF was performed on a 200 × 5 mm I.D. Mono P HR 5/20 column (Pharmacia, Hillerød, Denmark), equilibrated with different non-denaturing and denaturing buffers at several pH ranges between 4 and 8, followed by Polybuffer 74

(Pharmacia) in a more acidic range (above pH 4.0). The non-denaturing buffers used were 0.025 M Bis–Tris–HCl, 0.025 *M* imidazole–HCl and 0.025 *M* methylpiperazine–HCl. As denaturing buffers, 7 *M* urea, 50% ethylene glycol or 50% glycerol were used. The best separation was achieved using 12 ml of 0.025 *M* methylpiperazine–HCl (pH 5.7) followed by 27 ml of Polybuffer 74, diluted 1:10, at pH 4.39. An SP 8700 chromatograph (Spectra-Physics, Santa Clara, CA, U.S.A.) plus a U6K injector (Millipore Waters, Milford, MA, U.S.A.) were used throughout. The flow-rate was 0.5 ml/min and the absorption at 280 nm was monitored continously using a UV-VIS 200 detector (Linear Instruments, Reno, NE, U.S.A.). Fractions of 250 μl were mixed with 2.5 ml of HiSafe-Optiphase and analysed for radioactivity as described above. Before HPLCF, samples obtained from the phenylboronic acid affinity chromatography were desalted using Sephadex G-25 microcolumns (PD-10 columns from Pharmacia). Samples were eluted with 0.1 *M* ammonium hydrogencarbonate, lyophilized and dissolved in HPLCF equilibrating buffer before injection.

RESULTS

With increasing glycation time a progressive increase in two major anionic fractions of HSA (*ca.* p*I* 4.8 and 4.65) could be demonstrated using HPLCF (Figs. 1 and 2), while the p*I* 4.9 fraction decreased in parallel. Under denaturing conditions (*e.g.*, 7 M urea) similar patterns were found, but at a markedly higher pH range, approximately one pH unit higher (data not shown).

When the radioactivity corresponding to [^{14}C]glucose incorporated into HSA was analysed, the main increase in radioactivity, from 0 to 15 days of glycation, was found in the anionic components, and a considerable heterogeneity (more than ten components) could be demonstrated after 10–15 days of incubation (Fig. 2).

After PBA affinity chromatography of the same samples (from 0 to 15 days of glycation) a progressive increase in the bound fraction could be demonstrated (Fig. 3A). Further, the specific activity [^{14}C]glucose/mg HSA) of the bound fraction increased with increasing glycation time, but a parallel increase in specific activity was found in the unbound fraction (Fig. 3B). Even when minimal amounts of protein were

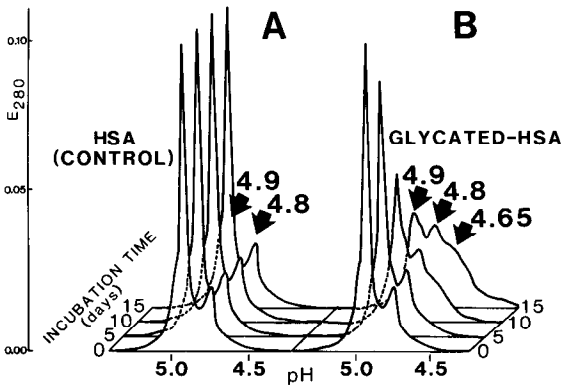

Fig. 1. HPLCF of HSA after 0–15 days of *in vitro* glycation, showing a progressive increase in two main fractions (p*I* 4.8 and 4.6) at the expenses of the first (p*I* 4.9). (A) HSA incubated without glucose; (B) HSA incubated with glucose.

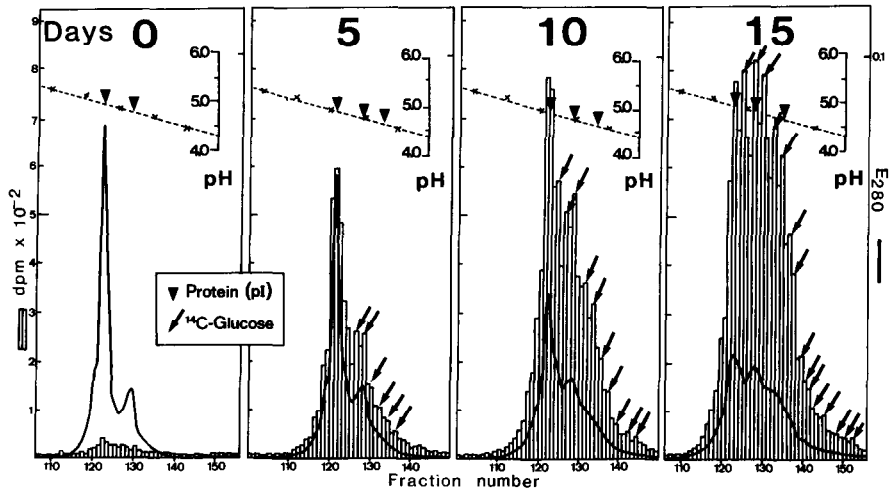

Fig. 2. UV and radioactivity HPLCF profiles of HSA after *in vitro* glycation with purified [^{14}C]glucose, showing the progressive increase in the [^{14}C]glycosyl adducts in the anionic range with, in addition, a considerable heterogeneity.

applied (in order to avoid column overloading), approximately half of the total radioactivity applied was found in the unbound fraction (Fig. 4). By adding the corresponding percentage of glycated protein present in the unbound fraction (calculated from the radioactive profiles and after correction of the column overloading) to the percentage of glycated HSA obtained from the protein profile,

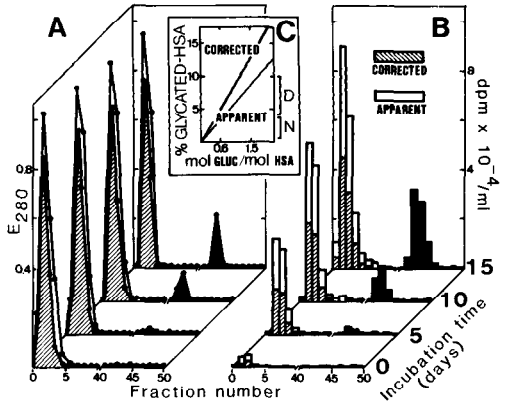

Fig. 3. Glycogel B affinity chromatography of 5 mg of *in vitro* [^{14}C]glycated HSA before and after correction for column overloading. Calculations were made using peak areas instead of peak height. (A) UV-profiles (A_{280}); (B) radioactive profiles (dpm/ml); "apparent", obtained by Glycogel B chromatography alone; "corrected", after elimination of the column overloaded protein. (C) indicates the underestimation of glycated HSA (percentage) obtainable by Glycogel B affinity chromatography used alone ("apparent") and that calculated from the radioactivity profiles after correction for column overloading ("corrected"). N and D, range of glycated HSA in normals and diabetics, respectively (as reported using the same chromatographic matrix[11–13]).

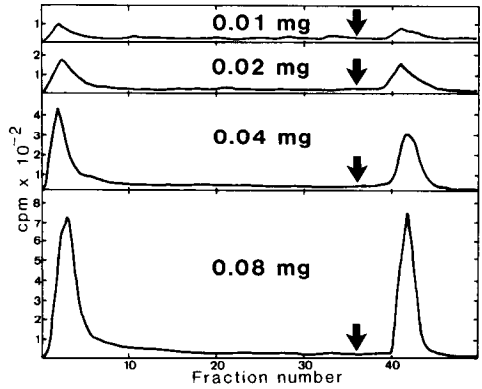

Fig. 4. Glycogel B affinity chromatography of 0.01–0.08 mg of 15-day glycated HSA. Arrows indicate buffer change.

a noticeable underestimation of the glycation extent could be demonstrated when the PBA chromatography was used alone (Fig. 3A–C).

In Fig. 5, the glucose content calculated for the PBA and HPLCF fractions is shown. Even at the pathophysiological glycation range observed in HSA (*i.e.*, an average glucose content of below 1 mol per mol HSA, according to refs. 2, 3 and 14), it is possible to demonstrate the presence of glycated HSA in the PBA-unbound fraction (Fig. 5A). Nevertheless, the glucose content in this fraction did not rise above 1 mol per mol HSA during the incubation time studied here. Parallel to this analysis, a higher glucose content in the anionic fractions (p*I* 4.8 and 4.65) could be demonstrated using HPLCF, while the p*I* 4.9 had a markedely lower glucose content (Fig. 5B).

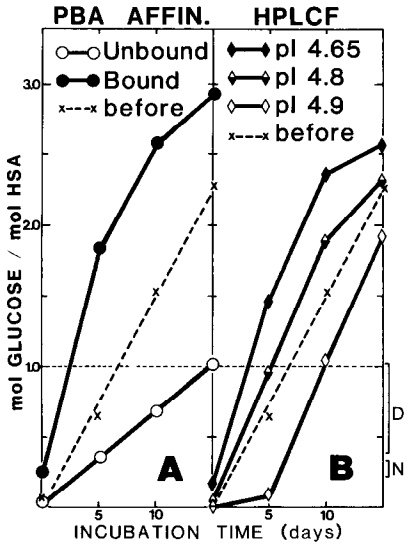

Fig. 5. Glucose content in different fractions of glycated HSA after (A) PBA affinity chromatography and (B) HPLCF. PBA-unbound values have been corrected for protein overloading. N and D indicate the range of glucose contents reported in normals and diabetic patients[2,3,14] using the furosine method.

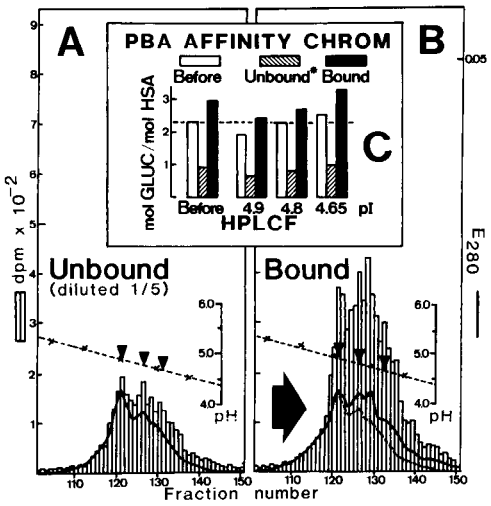

Fig. 6. Protein and radioactive HPLCF profiles of 15-day glycated HSA after PBA affinity chromatography. (A) Unbound fraction; (B) bound fraction. A dashed profile indicates the protein pattern obtained in A for comparison (arrowed). (C) indicates the glucose content of the HPLCF subfractions from PBA chromatography. * The PBA-unbound values have been corrected for column overloading.

When the bound and unbound fractions after PBA affinity chromatography were analysed separateley by HPLCF, noticeable amounts of radioactivity were also found to remain in the unbound fraction (Fig. 6A). Although radioactivity corresponding to the column overloading was included in this fraction, a larger amount of anionic species was found in the bound than in the unbound fraction (Fig. 6B). These anionic species were found to contain an increased heterogeneity (Fig. 6B) and a progressively increasing amount of glucose (Fig. 6C).

DISCUSSION

The present results confirm our previous studies using isoelectric focusing[8], but conflicts with other reports[4–7] because the observed heterogeneity is shown to be an anionic heterogeneity: more than ten anionic components were demonstrated after 15 days of glycation when the [^{14}C]glucose incorporated into the albumin was analysed. The demonstration of an increased amount of anionic fractions of glycated albumin is in accord with the theoretically expected loss of positively charged amino groups due to glycation[15].

We have previously demonstrated a reduced clearance of glycated albumin compared with the non-glycated albumin in diabetic patients with incipient nephropathy[2,3], and this reduced clearance of the glycated HSA may now be better explained by the interaction between a more heterogeneously anionic glycated albumin (as has been demonstrated above) and a less anionic glomerular barrier, owing to a loss of anionic charge in the glomerular membrane, possibly resulting from reduced incorporation of heparan sulphate[16].

An increased p*I* of albumin analysed under denaturating conditions is in accord

with previous data[17,18], and may be explained by changes in the three-dimensional structure (*i.e.*, partial or total unfolding), leading to a different surface charge distribution.

It should be noted that considerable amounts of glycated albumins (expressed as radioactivity of [^{14}C]glucose) were found in the unbound fraction after PBA chromatography, even when minimum amounts of albumin were loaded on the column. Apart from the often reported column overloading problem due to sensitivity reasons[9–13], the limitted efficiency of the PBA matrix in the separation of glycated from non-glycated HSA (see above) may explain the markedly lower estimates of glycation rates reported using this matrix[11–13] compared with other techniques (*e.g.*, the thiobarbituric acid method, CM-cellulose chromatography or the furosine method[3,19,20]). These observations agree with our previous findings[8] and a recent report[21] on the lack of specificity of PBA chromatography. A possible explanation[21] could be that the bound fraction corresponds to HSA glycated in more than one site, probably Lys-525 and Lys-199[15], and that this structure can be easily recognized by the affinity matrix due to steric accessibility.

From the present results (where calculations of glucose content in the PBA fractions are in good agreement with those reported by Johnson and Baker[21]), we find that even after 15 days of glycation the glucose content in the PBA-unbound fraction does not exceed 1 mol per mol HSA (Fig. 5A). In the PBA-bound fraction, the average glucose content increases to *ca.* 3 mol per mol HSA (Fig. 5A). The two anionic forms of HSA were found to contain more glucose than the p*I* 4.9 fraction, showing, in addition, a p*I* relationship (Fig. 5B). In similar terms, the glucose content of these two fractions, when they contained more than 2 mol glucose per mol HSA, could be noticeably enriched by using PBA chromatography, and the extent of this enrichment was also found to be p*I*-related (Fig. 6C).

All this is consistent with the postulated requirement of 2 mol glucose per mol HSA to bind PBA. However, in disagreement with Johnson and Baker[21], we believe that HSA with less than 2 mol glucose can also bind to PBA, although with a lower affinity. This is supported by the finding of the p*I* 4.9 fraction in the 15-day glycation sample after PBA chromatography (Fig. 6B) where, despite its glucose content of more than 2 mol per mol HSA (Fig. 6C), its p*I* remains at 4.9. This might indicate the presence of glucose bound to lysine groups other than Lys-199 and the (supposed) primarily involved Lys-525. Such lysine groups (*i.e.*, Lys-281 or Lys-439)[22] might be easily accesible for glycation, but not for interaction with the PBA matrix. On the other hand, a relatively low affinity for the p*I* 4.65 fraction to the PBA matrix might be explained by conformational changes in the HSA molecule (*i.e.*, disulphide breakdown, cross-linking, condensation), which could interfere with the interaction between the 2 mol glucose-containing epitopes from the HSA and the double-site recognition structures from the matrix. Conformational changes collaborating in a surface charge modification of HSA after glycation might be expected, as a lower glucose content was required to produce a p*I* variation from 4.8 to 4.65 compared with that from 4.9 to 4.8.

Consequently, we suggest that addition of 2 mol glucose per mol HSA is a sufficient but not necessary condition for PBA binding; HSA molecules with 2 mol glucose (in Lys-525 and Lys-199) will have a high-affinity binding to PBA and they might be easily enriched with a high yield by using this technique. In contrast, HSA molecules containing 1 mol glucose (in Lys-525) or more than 2 mol (in Lys-525,

Lys-199 and Lys-281 or/and Lys-439) will bind to the matrix with a lower affinity and, consequently, be enriched with a lower yield.

In vivo, including normal and diabetic ranges, the average glucose content in HSA is generally below 1 mol per mol HSA[2,3,14]. As this is a mathematical average, some HSA molecules with more than a single glucose are probably present. From the relatively small number of HSA molecules containing some glycosyl adduct (usually $< 10\%$ for humans), Lys-525 from HSA is the first glycation site involved (according to Garlick and Mazer[15]), but probably not the only one. *In vitro*, a higher extent of glycation can easily be achieved and a large number of HSA molecules with glycosyl adducts in Lys-199 can be strongly expected. This is in good agreement with the inhibitory effect on glycation of acetylsalicylic acid (ASA), involving a competitive acetylation in Lys-199[19]. The involvement of a secondary glycation-site may explain the only partial effect obtainable by using such a drug or its analogues[19,23]. Nevertheless, this effect of ASA, which was preported to be especially evident when PBA chromatography was used[23], is in accord with the above-mentioned necessity for a double recognition site for this matrix.

In addition to the effect of column overloading and (potential) conformational changes, a large number of substances may bind to the HSA and interfere with the glycation and the binding to the PBA matrix. Fatty acids, as they are present before glycation (probably in the p*I* 4.8 fraction) (Figs. 1 and 2), may interfere with glycation[24,25], although glycation does not interfere with binding of fatty acids[26], and the role of Lys-525 in this equilibrium has been particularly emphasized[24]. The effects of ASA-related compounds and fatty acids on the glycation of HSA and on its measurement are currently being investigated.

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