Non-Enzymic Glycation of Individual Plasma Proteins in Normoglycemic and Hyperglycemic Patients

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Diabetic patients in poor glycemic control show increased glycation of total plasma proteins, but little is yet known about the relative extents to which the various individual proteins are glycated. Thus, we studied the non-enzymic glycation of several major plasma proteins and plasma protein fractions in normal and diabetic patients. In vivo glycation for most plasma proteins was very low in non-diabetic patients, only gamma globulin showing more than 5% glycation. In diabetic plasmas, glycation was much greater, immunoglobulins again showing the greatest proportion, followed in descending order by albumin, complement C3, fibrinogen, transferrin, haptoglobin, and alpha-1-antitrypsin. When plasma proteins were glycated in vitro, this order was IgG > complement C3 > albumin > transferrin > haptoglobin > alpha-1-antitrypsin. In general, proteins with the longest biological half-lives, such as IgG and albumin, showed the greatest in vivo glycation. On the other hand, proteins with high intrinsic glycability, such as complement C3, showed moderate glycation, despite a short half-life. Except for albumin, more-basic proteins showed greater glycation than acidic proteins, but there was poor correlation between molar percent lysine and glycation. Evidently the relative extents of glycation of different plasma proteins are a complex function of integrated glucose concentrations over time and of the half-life and chemical characteristics of each protein.

Measurement of glycated proteins shows increasing promise in the assessment of hyperglycemia (1–4), and there is evidence to suggest that protein glycation may be involved in the long-term complications of diabetes. Non-enzymic glycation has been shown to produce crosslinking of protein molecules (5, 6), which in some cases may alter their function (7–9) or change their susceptibility to enzymatic degradation (10). Little, however, is yet known about the extent of glycation of individual plasma proteins in euglycemic individuals and diabetic patients. Thus, we investigated the extent of glycation of plasma or serum proteins in normal and diabetic states. We determined the extent of in vivo glycation of some serum proteins, using agarose gel electrophoresis and specific protein assays. We also examined the relative rates at which these proteins are glycated when they are incubated with high concentrations of glucose in vitro. Our results indicate substantial differences in the extents to which different plasma proteins are glycated in euglycemia and especially in hyperglycemic patients. These differences appear to reflect differences in intrinsic susceptibility to non-enzymic glycation as well as differences in the half-lives of the different proteins.

**Materials and Methods**

**Characterization of Glycogel columns.** Four milliliters of serum, pooled from non-diabetic patients, was glycated by incubation for five days with [14C]glucose (50 μCi, 0.5 mol/L carrier glucose in 0.1 mol/L phosphate buffer, pH 8.2), dialyzed to remove free glucose, lyophilized, and resuspended in water to the original volume. Before glycation, the [14C]glucose (Amer sham, Arlington Heights, IL 60005) was incubated for 30 min in 10 mmol/L acetic acid, pH 3.0, then dialyzed against phosphate buffer to remove labile radioactivity. Increasing amounts of glycated serum, from 25 μL to 1 mL, were applied to Glycogel B (Pierce Chemical Co., Rock ford, IL 61105); the analytical recovery of bound protein was measured with Coomassie Blue (Bio-Rad, Richmond, CA 94804) and the recovery of radiolabeled glycated protein was measured in a Packard scintillation counter. Bound fractions were also electrophoresed as described below and the recovery of labeled glucose was measured for each major protein fraction.

**Separation of glycated and non-glycated proteins.** We diluted 375 μL of serum or plasma to 750 μL with Pierce Equilibration-Wash Buffer and applied to 1.5-mL Glycogel B affinity columns, which were regenerated four times immediately before use. The columns were washed twice with 5-mL portions of Equilibration-Wash Buffer (Pierce). The pass-through and wash fractions were combined as the "unbound" (non-glycated) fraction. The "bound" (glycated) fraction was eluted with Elution Buffer (Pierce). Glycated and non-glycated protein fractions were lyophilized, redissolved in phosphate-buffered isotonic saline (pH 7.4) and dialyzed against the same buffer. Sample volumes were adjusted with saline to bring the protein concentrations to those present in the original serum or plasma specimens.

**Agarose gel electrophoresis of serum proteins.** Aliquots (4 μL) of glycated or non-glycated serum or plasma proteins were applied to commercially prepared agarose gels (Panagel; Princeton Separations, Inc., Freehold, NJ 07728) and electrophoresed in an LKB 2117 Multiphore horizontal electrophoresis apparatus (LKB, Bromma, Sweden). Electrophoresis was for 1 h at 10 °C and pH 8.6 according to the Panagel protocol. Relative densities of individual proteins were determined with an LKB 2202 Ultrascan Laser Densitometer equipped with an LKB 2220 recording integrator. Linearity was validated against a neutral-density standard (Helena Laboratories, Beaumont, TX 77704). Total protein was measured by a modified biuret method in a Dacos analyzer (Coul ter Electronics, Inc., Hialeah, FL 33010).

**Specific protein assays.** Concentrations of IgG, complement C3, haptoglobin, alpha-1-antitrypsin, and transferrin in the glycated and non-glycated serum or plasma fractions were determined by immunoturbidimetry, with use of SPQ™ test kits (Atlantic Antibodies, Scarborough, ME 07070) and a Cobas-Bio centrifugal analyzer (Roche Diagnostic Systems, Nutley, NJ 07110). Samples containing glycated and non-glycated proteins were diluted appropriately with phosphate-buffered isotonic saline and assayed.
Results

The data in Figure 1 clearly show that the analytical recovery of bound (glycated) protein from the Glycogel column is linearly related to the volume of serum applied, from 25 µL (1.6 mg protein) to at least 1 mL (38 mg of protein). Likewise, the analytical recovery of glycated protein as determined by use of protein-bound radiolabeled glucose is independent of loading to at least 600 µL of applied serum. Over 90% of the trichloroacetic acid(TCA)-precipitable counts were located in the bound fraction. About 10% of the TCA-precipitable counts were in the passage-through fraction, suggesting that some glycated proteins may not be sufficiently glycated or so configured as to be retained on borate affinity support. The 90% recovery of counts was consistent for column loads ranging from 25 µL to 600 µL.

Agarose gel electrophoresis profiles of serum proteins from a representative normoglycemic individual and four diabetic patients with hyperglycemia are presented in Figure 2. Table 1 presents the percent glycation of the major protein fractions. The serum proteins of euglycemic individuals showed little or no detectable glycation of alpha-1, alpha-2, or beta globulins. As expected, albumin showed slight glycation (about 2%). The immunoglobulin fraction, however, exhibited moderate glycation (about 10–15%). Diabetics with increased hemoglobin A1c showed much greater glycation of serum proteins than did normal individuals, and there were notable differences in the extent to which different classes of proteins were glycated. Alpha-1 and alpha-2 globulins showed little glycation, even in those patients with extremely increased hemoglobin A1c. In contrast, gamma globulins were glycated by as much as 50% in several patients. Albumin and beta globulins also showed striking increases in glycation, especially in patients with the greater increases of hemoglobin A1c. Among the beta globulins, transferrin appeared to remain mostly non-glycated, whereas complement C3 showed substantial glycation.

To determine if quantitative differences in glycation of individual proteins were attributable to differences in their intrinsic susceptibilities to non-enzymic glycation, serum specimens from euglycemic individuals were incubated at 25°C in the presence of 0.5 mol of glucose per liter (final concentration) for periods of two to 10 days, after which the glycated and non-glycated proteins were separated on Glycogel. Individual columns were loaded with 25, 50, 100, 400, or 800 µL of this treated serum. The percent glycation of the major serum fractions was determined. Representative agarose gel profiles and glycation curves derived from laser scans of them are shown in Figures 4 and 5. There were no observable differences in the patterns or recoveries obtained relative to the amount of protein applied to the columns. All classes of proteins showed at least 60% glycation after 10 days of incubation. Fibrinogen and gamma globulins showed the highest proportions of glycation, about 80% and 60%, respectively, after only two days of incubation. In contrast, the alpha-1 and alpha-2 globulins were glycated at less than half that rate. Albumin and beta globulins showed intermediate rates of glycation.

Figure 6 illustrates the time course of in vitro glycation for specific proteins, based on immunoturbidimetric data. Immunoglobulin G was most rapidly glycated, followed by complement C3. On the other hand, alpha-1-antitrypsin and haptoglobin were glycated at the slowest rates among the proteins examined. All of the proteins studied showed
Table 1. Percent Glycation of Electrophoretically Separable Serum Protein Fractions as Determined by Densitometric Scanning of Coomassie Blue-Stained Gels

<table>
<thead>
<tr>
<th>Patient</th>
<th>Glucose, g/L</th>
<th>Hemoglobin A1c (%)</th>
<th>Albumin</th>
<th>Alpha-1 globulin</th>
<th>Alpha-2 globulin</th>
<th>Beta globulin</th>
<th>Gamma globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (nondiabetic)</td>
<td>1.05</td>
<td>4.3</td>
<td>0.4</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>14.9</td>
</tr>
<tr>
<td>B (diabetic)</td>
<td>2.88</td>
<td>14.8</td>
<td>13.9</td>
<td>5.2</td>
<td>10.4</td>
<td>51.6</td>
<td>55.0</td>
</tr>
<tr>
<td>C (diabetic)</td>
<td>3.18</td>
<td>19.7</td>
<td>33.8</td>
<td>&lt;1</td>
<td>9.4</td>
<td>41.4</td>
<td>52.3</td>
</tr>
<tr>
<td>D (diabetic)</td>
<td>3.65</td>
<td>13.2</td>
<td>14.4</td>
<td>&lt;1</td>
<td>1.1</td>
<td>13.3</td>
<td>53.5</td>
</tr>
<tr>
<td>E (diabetic)</td>
<td>4.35</td>
<td>15.5</td>
<td>21.2</td>
<td>3.1</td>
<td>1.2</td>
<td>10.2</td>
<td>43.8</td>
</tr>
<tr>
<td>F (diabetic)*</td>
<td>3.55</td>
<td>12.0</td>
<td>6.6</td>
<td>&lt;1</td>
<td>1.9</td>
<td>7.4</td>
<td>24.0</td>
</tr>
</tbody>
</table>

*Not shown in Figure 2.

Fig. 3. Percent glycation of selected plasma proteins as determined by specific protein assays. Means and standard deviations are shown for groups of six nondiabetic or diabetic patients.

Fig. 4. Electrophoretic profiles on non-glycated and glycated protein fractions obtained after incubation of a non-diabetic plasma for periods from 0 to 10 days in vitro in the presence of 0.5 mol of glucose per liter at pH 7.5. Symbols as in Figure 2.

Fig. 5. In vitro glycation curves for albumin, fibrinogen, and gamma globulin obtained on densitometric scanning of the Coomassie Blue-stained agarose gels shown in Figure 4. A) Top to bottom: fibrinogen, albumin, alpha-1 globulin. B) Top to bottom: gamma globulin, beta globulin, alpha-2 globulin.

Fig. 6. Time course of glycation of selected specific proteins following incubation of serum in vitro for periods of from 0 to 10 days in buffer containing 0.5 mol of glucose per liter at pH 7.5. A) Top to bottom: albumin, haptoglobin, alpha-1 antitrypsin. B) Top to bottom: IgG, complement C3, transferrin.

There is substantial glycation after a sufficient interval of incubation in 0.5 mol/L glucose.

Discussion

The ability of boronic acid affinity columns to separate glycated proteins from non-glycated proteins is well established (11) and is the basis for their use in assays of glycated hemoglobin, albumin, and total proteins for evaluation of diabetic status (12). Because questions have been raised about the capacity of these columns (13, 14), we investigated the recovery of bound protein as a function of total protein applied. The results (Figure 1) demonstrate linearity of recovery of bound protein up to at least 1 mL of applied serum. When the data of Gould et al. (13) are analyzed in the same way, similar results are obtained. In contrast to their interpretation, however, the data are not consistent with column saturation, even at 1 mL of applied serum (65 mg of protein). Instead, the data suggest the existence of a small concentration of nonspecific binding sites, which, when very small amounts of protein are applied, lead to an overestimation of the proportion of glycated protein as compared with that obtained when larger protein loads are applied. The consistent recovery in the bound fraction of >90% of counts from labeled glycated protein across a broad range of protein loading indicates that glycated protein is in fact effectively and representatively recovered in the bound fraction and that the protein load in these experiments was well within the capacity of these columns. Because we separated the labeled proteins into glycated (bound) and unglycated (unbound) fractions, any potential nonspecific incorporation of radiolabel (15) was obviated.

Our results emphasize that the plasma proteins of non-diabetics are glycated to only a small extent. Of the proteins...
Table 2. Comparison of Percent Non-Enzymic Glycation with Half-Life, Mole Percent Lysine, and Mole Percent Carbohydrate for Selected Plasma Proteins

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Glycation in vivo (percent)*</th>
<th>Glycation in vitro (percent)*</th>
<th>Half-life (days)*</th>
<th>Lysine (mole %)†</th>
<th>Carbohydrate (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin G</td>
<td>19.8</td>
<td>55.7</td>
<td>24</td>
<td>7.0</td>
<td>3</td>
</tr>
<tr>
<td>Albumin</td>
<td>16.0</td>
<td>18.3</td>
<td>15–19</td>
<td>9.5</td>
<td>0</td>
</tr>
<tr>
<td>Complement C3</td>
<td>11.1</td>
<td>27.7</td>
<td>2–5</td>
<td>7.1</td>
<td>1</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>5.9</td>
<td>65.1</td>
<td>2.5</td>
<td>6.7†</td>
<td>3</td>
</tr>
<tr>
<td>Transferrin</td>
<td>5.2</td>
<td>13.6</td>
<td>7</td>
<td>8.7</td>
<td>6</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>2.2</td>
<td>8.7</td>
<td>2</td>
<td>10.5</td>
<td>12</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>1.4</td>
<td>1.7</td>
<td>4</td>
<td>8.9</td>
<td>12</td>
</tr>
</tbody>
</table>

*Mean of five diabetic patients (see Figure 3). †After two days of incubation in presence of 0.5 mol/L glucose (mean of five samples, see Figure 5). ‡See ref. 24.

References


Examined, only IgG shows more than 1 to 2% glycation in normal individuals. By contrast, diabetics with sustained above-normal blood glucose concentrations show substantially more glycation. Furthermore, the differences in glycation among individual serum proteins are extreme. The differences appear to depend on susceptibility to glycation and biological half-life. The dominant factor at low to moderate glucose concentrations appears to be half-life, those proteins with longer half-lives showing greater glycation. This is consistent with previous kinetic studies with hemoglobin (14, 16). At high glucose concentrations, differences in the inherent glycatability of the proteins become more important; the basis for such differences is still poorly understood.

In Table 2 we compare the relative extents of in vivo and in vitro glycation for seven serum proteins with reported values for biological half-life, lysine content, and carbohydrate content of each protein. As might be expected, there is, in general, a positive relation between protein half-life and extent of in vivo glycation. However, additional factors are clearly involved, because complement C3 shows twice as much glycation as fibrinogen, while even though both proteins have similar half-lives and fibrinogen appears to be much more susceptible to in vitro glycation.

Non-enzymatic glycation is thought to involve primarily the epsilon amino groups of lysine residues as well as the primary amino groups of N-terminal amino acids (17). However, Table 2 reveals little or no relation between mole percent lysine and in vitro or in vivo non-enzymic glycation. IgG, with only 7% lysine residues and unreactive N-terminal cyclic amino acids (18), is glycated much more readily than haptoglobin, which has more than 10% lysine residues.

Non-enzymatic glycation appears to show some degree of inverse relationship with carbohydrate content. Haptoglobin and alpha-1-antitrypsin, with about 12% carbohydrate, show little glycation; albumin and immunoglobulin G, with 0% and 3% carbohydrate, respectively, show relatively greater glycation.

One factor that appears to relate fairly well with extent of both in vivo and in vitro glycation is the overall acidity or basicity of the proteins. Agarose electrophoresis (Figures 2 and 4) clearly shows a descending gradient in percent glycation, from gamma globulins, which are the most basic and most heavily glycated, to the alpha-1 globulins, which are the most acidic of the globulins and the least glycated. Albumin, which is still more acidic, however, is an exception to this principle, being quite glycated.

The clinical consequences of non-enzymic glycation of serum proteins remain ambiguous. In non-diabetics, the effects are probably negligible. In diabetic patients, however, extensively glycated species, such as immunoglobulins, complement, and fibrinogen, perhaps could exhibit significant alterations in function. Brownlee et al. (10) have shown that non-enzymic glycation of fibrinogen in vivo decreases the susceptibility of fibrin to degradation by plasmin. In vivo glycation also inhibits heparin-catalyzed human antithrombin III activity (9). Furthermore, Dolhofer et al. (20) recently reported that the non-enzymic glycation of immunoglobulins in vitro resulted in loss of complement-fixing activity, although we have found that antigen binding by immunoglobulins is unaffected by in vitro glycation (21). Whether such effects prove to be as important as the effects of glycation of long-lived proteins such as collagen and lens crystallins (22, 23) remains an open question.