Nonenzymatic Glycosylation of Human Serum Albumin Alters Its Conformation and Function*

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Approximately 10% of the albumin in normal human serum is modified by nonenzymatic glycosylation, primarily at the ε-amino group of lysine residue 525. Incubation of albumin with glucose under physiological conditions in vitro resulted in glycosylation of the same residue. After separation of glycosylated human serum albumin from the nonglycosylated form by boronate affinity chromatography, the fluorescence emission characteristics of the sole tryptophan residue (Trp 214) were monitored. The quantum yield of tryptophan fluorescence for both in vivo and in vitro glycosylated albumin was reduced 30% relative to nonglycosylated albumin, and the maximal wavelength of the fluorescence emission band was shifted to shorter wavelengths. These observations show that nonenzymatic glycosylation induces a conformational change in human serum albumin.

Ligand binding properties of glycosylated and unmodified albumin were compared. Hemin affinity was unaltered by glycosylation of albumin in vivo, whereas the affinity of bilirubin for glycosylated albumin was about 50% its value for the nonglycosylated form. The affinity of the long chain fatty acid cis-parinaric acid for albumin glycosylated in vivo and in vitro was reduced approximately 20-fold relative to nonglycosylated albumin. These differences in affinity suggest that lysine 525 plays a key role in the binding of physiologically important ligands to albumin.

Human serum albumin is a single chain polypeptide of 585 residues which comprises about 60% of the plasma protein and is the major contributor to the oncotic pressure of blood. This abundant protein has been thoroughly investigated. Its most striking property is its ability to bind an unusually broad spectrum of ligands (1, 2). These include inorganic cations, organic anions, various drugs, amino acids, and, perhaps most importantly, physiologically available hydrophobic molecules like bilirubin, hemin, and fatty acids. As a result, albumin is considered a multifunctional plasma transport protein.

There is growing evidence that under physiological conditions glucose reacts nonenzymatically with a wide variety of proteins to form stable adducts (3–5). Proteins which are exposed to glucose and have a relatively slow turnover rate are particularly susceptible to nonenzymatic glycosylation.

Although hemoglobin has been the prototype, other well studied examples of proteins which undergo nonenzymatic glycosylation include HSA, low density lipoproteins, lenses crystallin, and various forms of collagen. This post-translational modification may contribute to long term diabetic complications. It is possible that the function of certain proteins is significantly altered by modifications at specific sites. Lysine residue 525 recently has been identified in this laboratory as the principal site of nonenzymatic glycosylation of human serum albumin in vivo (6).

In this study, we demonstrate differences in conformation and ligand binding between nonglycosylated and glycosylated HSA.

EXPERIMENTAL PROCEDURES

Materials

The following materials were used: Sephacryl S-200 superfine (Pharmacia Fine Products, Piscataway, NJ), TPCK-trypsin (Worthington), sodium [1H]borohydride (New England Nuclear), Affi-Gel blue and Affi-Gel 601 (Bio-Rad), hemin and bilirubin (Porphyrin Products Inc., Logan, UT), cis-parinaric acid (Molecular Probes Inc., Eugene, OR), Durrum DC-6A cation exchange resin (Durrum Chemical Co., Palo Alto, CA), and Ultragel AcA 34 (LKB, Bromma, Sweden). GlycoGel B boronic acid affinity chromatographic gel was obtained from Pierce.

Spectrophotometric measurements were performed either on a Gilford 240 or a Cary 118 spectrophotometer. For fluorescence measurements, a Hitachi/Perkin-Elmer B44 recording spectrophuorometer was used.

Methods

Purification of Proteins—Human serum albumin from normal adult donors was purified by affinity chromatography on Affi-Gel blue (7) followed by passage through a column (2.0 × 120 cm) either of LKB Ultragel AcA 34 or Sephacryl S-200 superfine in 0.15 M NaCl, 0.01 M EDTA, 0.01 M potassium phosphate, pH 7.4. Unless otherwise stated, albumin was defatted by treatment with activated charcoal (Bio-Rad) in dilute acid (8). This defatted albumin was then subjected to gel filtration on Sephacryl or Ultragel.

Glycosylated serum albumin was separated from nonglycosylated albumin by affinity chromatography on columns of GlycoGel B boronic acid affinity gel (6, 9). Purified albumin was applied to the columns in 0.25 M ammonium acetate, pH 8.5, and the nonglycosylated albumin was eluted with this starting buffer. Glycosylated albumin adhered to the column in the starting buffer and was eluted with 0.2 M sorbitol in 0.25 M ammonium acetate, pH 8.5.

In Vitro Glycosylation of Albumin—Nonglycosylated albumin (40 mg/ml) isolated on GlycoGel B was diazoxid against Krebs-Ringer phosphate buffer, pH 7.2, and then incubated with 150 mM D-Glucose (Fisher) in a sterile test tube after passage through a 0.2-μm filter. Two drops of a penicillin/streptomycin mixture were added to retard

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The abbreviations used are: HSA, human serum albumin; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.
bacterial growth. The mixture was incubated at 37 °C for 160 h in the dark. After extensive dialysis against 0.25 M ammonium acetate, pH 8.5, the glycosylated albumin was purified by GlycoGel B boronic acid affinity chromatography.

**Structural Analysis**—Freshly purified human serum albumin was reacted with NaB3H4, as described previously (6). Albumin glycosylated in vivo was purified by GlycoGel B chromatography and reacted at pH 7.0 and 4 °C for 30 min with a 200-fold molar excess of sodium borohydride diluted with nonradioactive NaB4H4 to a final specific radioactivity of 170 mCi/mmol. Excess sodium borohydride was removed by extensive dialysis, and the albumin was subjected to digestion with trypsin as described (6), followed by lyophilization.

The tryptic peptides from B4H4-labeled albumin were dissolved in 0.05 M potassium phosphate, pH 5.0, and the soluble tryptic peptides were subjected to boronic acid affinity chromatography on Affi-Gel 601. The glycosylated tryptic peptides adhered to the boronic acid in the initial alkaline buffer and were eluted from the column with 0.05 M HCl.

The glycosylated tryptic peptides from Affi-Gel 601 chromatography were solubilized in 0.2 M pyridine acetate, pH 3.1, and then applied to a column (0.9 x 20 cm) of Durrum DC-6A cation exchange resin in the same buffer. The column was developed with a 400-ml linear gradient of 0.2 M pyridine acetate, pH 3.1, to 2.0 M pyridine acetate, pH 5.0, at 67 ml/h and 55 °C with a column pressure of 200–400 p.s.i.

**Preparation of Albumin and Ligand Solutions for Binding Experiments**—Albumin solutions were dialyzed against the desired buffer solutions and passed through a Millipore 0.2-μ filter to remove any insoluble light-scattering material. Absorption in the aromatic UV absorbance band was measured. For calculation of the albumin concentration, E280= 5.5 was used as the extinction coefficient (10).

Hemin was solubilized in 10 mM NaOH at a concentration of approximately 1 mM. This solution was then diluted 1:10 and centrifuged for 10 min at 50,000 x g. Any precipitating material was discarded. This solution was further diluted to the desired concentration for titration experiments. To estimate hemin concentration, an aliquot was diluted into 5 mM NaOH, and the hemin concentration was calculated using E280 = 58.4 (10). Hemin solutions were used immediately after preparation.

Bilirubin was solubilized in NaOH and prepared in the same manner as hemin solutions. Bilirubin solutions were kept in the dark.

The extinction coefficient used for bilirubin in 5 mM NaOH was E280 = 60.6 (11).

cis-Parinaric acid was dissolved in ethanol and further diluted with 50 mM phosphate, pH 7.5. The stock solution was flushed with nitrogen to avoid oxidation and passed through a 0.2-μ Millipore filter. Concentrations were calculated using E280 = 41.1 in 50 mM phosphate, pH 7.5 (12).

**RESULTS**

**Identification of the Sites of Nonglycosylated Glycosylation of Serum Albumin in Vivo**—In order to determine whether lysine 525 is the major site of nonglycosylated glycosylation of human serum albumin in vitro as well as in vivo (6), we incubated nonglycosylated albumin with glucose and, after dialysis, chromatographed the sample on GlycoGel B. The glycosylated albumin was then reacted with NaB3H4. This sample and another sample of albumin from an earlier study (6) which was glycosylated in vitro and reduced with NaB3H4, were subjected to tryptic digestion. The albumin glycosylated in vitro had 94% of its radioactivity in the soluble tryptic peptides which had a specific radioactivity of 140,000 cpm/mg. Albumin glycosylated in vivo had 88% of its radioactivity in the soluble tryptic peptides which had a specific radioactivity of 42,400 cpm/mg. The soluble tryptic peptides were applied to a column of Affi-Gel 601 boronic acid affinity gel.

For the peptides from the albumin glycosylated in vitro, 100% of the radioactivity was recovered and 44% adhered to the column. For the peptides from the albumin glycosylated in vivo, 98% of the radioactivity was recovered and 69% was retained by this affinity resin. The glycosylated tryptic peptides from each sample were subjected to cation exchange chromatography on a column of Durrum DC-6A (Fig. 1). Each sample contained a major glycosylated peptide and several minor peptides. For the peptides from the albumin glycosylated in vitro, 85% of the radioactivity was recovered and 35% of the recovered radioactivity was in the major peptide fraction. For the peptides from the albumin glycosylated in vivo, 80% of the applied radioactivity was recovered and 38% of the counts were in the major peptide fraction. The amino acid composition of the major glycosylated tryptic peptide from the albumin glycosylated in vitro is shown in Ref. 6. The amino acid composition of the major glycosylated tryptic peptide from albumin glycosylated in vivo is shown in Table I. The compositions of these two peptides are nearly identical and correspond precisely to positions 525–534 in the amino acid sequence of human albumin (1). Because this is a tryptic peptide, the single lysine residue would be at the COOH terminus of the peptide, corresponding to amino acid residue 534 of human serum albumin. Trypsin will cleave at lysine when its ε-amino group is glycosylated (13). Therefore, glucitol-lysine would be at the NH2 terminus of the peptide and would correspond to amino acid residue 525 of human serum albumin. These results indicate that lysine residue 525 is the...
were closely matched (within 0.2%). Thus, albumin glycosylation was confirmed, showing that the concentrations of the solutions were matched in vivo or in vitro. In addition to the reduction in quantum yield, a distinctive shift in the emission from the glycosylated albumin was reduced. The fluorescence quenching (15) was due to some tyrosine contribution.

TABLE I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar ratio</th>
<th>Integer value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1.09</td>
<td>1</td>
</tr>
<tr>
<td>Thr</td>
<td>1.89</td>
<td>2</td>
</tr>
<tr>
<td>Ser</td>
<td>1.17</td>
<td>1</td>
</tr>
<tr>
<td>Glu</td>
<td>1.99</td>
<td>2</td>
</tr>
<tr>
<td>Pro</td>
<td>2.00</td>
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</tr>
<tr>
<td>Gly</td>
<td>2.00</td>
<td>2</td>
</tr>
<tr>
<td>Ala</td>
<td>0.87</td>
<td>1</td>
</tr>
<tr>
<td>GlcLysd</td>
<td>Present</td>
<td>1</td>
</tr>
<tr>
<td>Trp</td>
<td></td>
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</tr>
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</table>

The amino acid composition of this tryptic peptide is identical to that of the major glycosylated tryptic peptide from HSA glycosylated in vivo (see Ref. 6).

Fluorescence Spectra—Stock solutions of nonglycosylated and glycosylated human serum albumin were matched in concentration by absorbance at 279 nm. The protein concentrations of these solutions were also measured by the Lowry method, confirming that the concentrations of the solutions were closely matched (within 0.2%). Thus, albumin glycosylated in vivo or in vitro has the same extinction coefficient as nonglycosylated albumin at 279 nm. The shape of the UV absorption spectra of the two albumin forms also appeared the same. For determination of relative quantum yield, fluorescence emission spectra of solutions in the range of 0.1 μM were taken. The optical density of the solutions was exactly matched and fluorescence emission was measured under identical conditions. Fig. 2A shows the emission of nonglycosylated HSA and in vivo glycosylated HSA. The fluorescence emission from the glycosylated albumin was reduced. The relative quantum yield calculated from the areas of the emission spectra was QGHSA/QNGHSA = 0.67 (where GHSAs and NGHSA represent glycosylated and nonglycosylated HSA, respectively). In addition to the reduction in quantum yield, a distinctive shift of the emission maximal wavelength to the blue was observed: nonglycosylated HSA = 339 nm; glycosylated HSA = 331 nm. Excitation at 285 nm is demonstrated in Fig. 2. The same solutions were excited at 275 and 295 nm. The reduction in quantum yield was observed at these two wavelengths as well. Excitation at 295 nm changed the shape of the emission band somewhat, but the blue shift of the maximal wavelength was observed as in the lower wavelength excitation experiments. The shoulder observed when albumin was excited at 275 nm was due to some tyrosine contribution. This feature was not observed at 295 nm excitation. HSA has 18 tyrosines and 1 tryptophan. Therefore, in spite of tyrosine emission being almost totally quenched in tryptophan-containing proteins, there is some tyrosine contribution, as previously demonstrated (14).

The fluorescence characteristics of in vitro glycosylated HSA, shown in Fig. 2B, were similar to those of in vivo glycosylated HSA. The quantum yield ratio calculated from the areas under the fluorescence emission was QGHSA/QNGHSA = 0.74. The maximal wavelength of emission shifted from 339 nm in nonglycosylated HSA to 330 nm in glycosylated HSA.

Binding of Hemin and Bilirubin to Albumin—The binding of hemin and bilirubin to albumin was measured by fluorescence quenching (15). There is only one high affinity binding site for hemin on albumin which includes amino acid residues 180-250 of albumin (2). Dilute solutions of albumin (0.1-2.0 μM) were used for ligand binding experiments. At these protein concentrations, the hemin and bilirubin in solution are dilute enough to keep the unbound ligand in a monomeric state (17). Hemin was added to the dilute albumin until the reduction in fluorescence intensity reached a constant value. Further reduction at higher hemin concentrations is due to additional binding sites and was ignored. The total amount of fluorescence intensity quenched was considered 100%. From the fractional quenching, the amount of hemin bound to albumin was estimated considering one binding site only, the highest affinity site. Free hemin concentration was calculated from the concentrations of total and bound hemin. The results are represented in Fig. 3 as a double reciprocal plot. There was no difference in the binding of hemin to nonglycosylated HSA and HSA glycosylated in vivo. The equilibrium binding constant as calculated from the slope of the linear plot in Fig. 3 is 2.4 × 10^1 M^-1. Bilirubin binding measurements were carried out under the same conditions as those with hemin, except that the pH was 8.0 to prevent aggregation of bilirubin (17). The results again are presented as double reciprocal plots (Fig. 4). In contrast...
phosphate, pH 7.5). Under these conditions, the addition of when the albumin molarity was only double that of the fatty acid. We assume therefore that the cis-parinaric acid binding observed under these conditions is associated with the high affinity site of albumin for long chain fatty acids (19). We first compared the cis-parinaric acid titration of nonglycosylated HSA with that of HSA glycosylated in vivo. As shown in Fig. 5A, the increase in cis-parinaric acid fluorescence eventually reached the same infinity value for both nonglycosylated and glycosylated HSA, but under subsaturating conditions the fluorescence increase of cis-parinaric acid was lower when glycosylated HSA was added at the same concentrations as that of nonglycosylated HSA, indicating that glycosylated HSA had a lower affinity for the fatty acid. From the increase in cis-parinaric acid emission intensity at successive albumin additions and the value of the emission signal when all of the cis-parinaric acid is bound, the fractions of the fatty acid bound and free were calculated. The data were drawn as Hill plots from which binding constants for nonglycosylated and glycosylated HSA were calculated. The association constants calculated from the data in Fig. 5A are $K_{NGHSA} = 40 \times 10^5 \text{ M}^{-1}$ and $K_{GHSA} = 2.0 \times 10^7 \text{ M}^{-1}$. Thus, the affinity of cis-parinaric acid for HSA glycosylated in vivo is 20-fold lower than that of nonglycosylated HSA.

In addition, we examined the binding of cis-parinaric acid to albumin that had been glycosylated in vitro and compared it to nonglycosylated albumin from the same incubation (Fig. 5B). The binding constants, calculated in the same manner, were $K_{NGHSA} = 31 \times 10^5 \text{ M}^{-1}$ and $K_{GHSA} = 1.4 \times 10^7 \text{ M}^{-1}$.

The absolute values of our binding constants for the various ligands are in agreement with some reports (11) but not others (10). Discrepancies are probably due to differences in techniques as well as in the concentration ranges of albumin and ligands. All ligands considered in this study are hydrophobic and tend to form micelles. It is likely that albumin binds only...
the monomeric forms of these ligands. However, the possibility of site-specific binding of micelles or aggregates cannot be ruled out (20). In this study, our principal interest was to determine the relationship between binding affinities of nonglycosylated and glycosylated HSA. Therefore, the binding experiments were performed under identical conditions for the two albumin forms.

**DISCUSSION**

Even though albumin is a larger polypeptide than hemoglobin, it reacts with glucose with greater specificity (6, 13). The primary site of nonenzymatic glycosylation of HSA in *vivo* is Lys 525 (6). As shown in our study, this residue is equally favored when albumin undergoes glycosylation *in vitro* during incubation with glucose. Because of this specificity, the observed alterations in conformation and function are amenable to structural interpretations. The fact that these abnormalities are shared by HSA glycosylated both *in vivo* and *in vitro* strongly implies that they are caused by the site-specific glycosylation and not some associated phenomenon such as aging or differences in ligand content.

The intrinsic fluorescence of proteins is primarily due to aromatic groups on tyrosine and tryptophan residues. Their emission characteristics such as wavelength of maximal emission or quantum yields are sensitive to fluorophore environments within the three-dimensional protein structure (21, 22). A given protein usually contains several tryptophan residues, and as a result, its emission characteristics lack structural assignments. HSA is one of the rare examples in which a single tryptophan exists per 65,000-dalton protein. In this study, we took advantage of this situation and monitored tryptophan fluorescence properties in glycosylated albumin in search of possible conformational changes imposed by nonenzymatic glycosylation.

In Fig. 2, we demonstrate that the fluorescence emission characteristics of HSA have indeed changed upon glycosylation. In glycosylated albumin, the quantum yield of the tryptophan fluorescence has been reduced by about 30% accompanied by a significant blue shift of the emission band. The changes observed in the protein fluorescence emission may be due to tyrosine and/or tryptophan residues. In spite of the great number of tyrosine residues in HSA, their direct contribution to fluorescence is very small (14). Indirect contribution of tyrosines to the tryptophan emission could result from nonradiative energy transfer from excited tyrosines to the tryptophan residues (23). As stated under "Results," excitation of the protein at 275 nm, where both residues absorb light, and 295 nm, where tryptophan mainly absorbs, resulted in the same change in the glycosylated protein emission. We therefore conclude that the modified fluorescence emission seen in glycosylated human serum albumin reflects changes in the tryptophan microenvironment. The shift of maximal wavelength of the protein emission to a shorter wavelength indicates that the tryptophan microenvironment has become more hydrophobic due to glycosylation (17, 22).

In spite of the fact that albumin has been one of the most thoroughly studied proteins, its three-dimensional structure has not yet been determined. On the basis of its amino acid sequence and the distribution of the disulfide bridges in the molecule, it has been proposed that albumin is composed of three homologous domains corresponding to amino acids 1-193 (I), 194-384 (II), and 385-585 (III) (1). Each domain is further divided into three subdomains (1). Thus, tryptophan 214 is included in domain II. In a previous study (6), we showed that nonenzymatic glycosylation of HSA in *vivo* occurs primarily at lysine 525, located in domain III. The results reported here show that the tertiary structure of HSA makes it possible for the modification at residue 525 to induce changes in the environment of residue 214. This finding suggests that domains II and III are in close contact in regions including the above residues. Contacts between domains II and III have been suggested by the model of Brown and Shockley (1).

Having demonstrated conformational changes in glycosylated albumin, we then asked whether this modification affects certain functional properties of the human serum albumin. We show that binding of hemin to the primary hemin site is unaffected by glycosylation (Fig. 3). The primary binding site of hemin was shown to be located in domain II (sequence 124-208) (16). Since the single tryptophan is included in the same sequence and is affected by glycosylation, it seems likely that the key amino acids in the hemin-binding site do not include Trp 214.

The comparison of bilirubin binding shown in Fig. 4 demonstrates that the affinity of the glycosylated albumin for bilirubin has been reduced to about half the value calculated under the same conditions for the nonglycosylated albumin. The primary binding site for bilirubin is located in the region including amino acid residues 180-250 and is thought to involve the lower end of albumin subdomains IC and IIA-B (2). The bilirubin-binding site was calculated to be more than 20 Å from the tryptophan (18). It is therefore possible that the bilirubin-binding site and the tryptophan are both influenced by the conformational change induced by glycosylation without tryptophan being directly involved in the bilirubin-binding site. The fact that the bilirubin-binding site is affected by glycosylation while the hemin-binding site is unaffected shows that these two tetrapyrroles bind to separate sites in domain II. This finding is in accordance with previous studies which demonstrated that the two ligands do not compete for the same site (10, 25).

We have shown thus far that glycosylation at residue 525, located in domain III, may influence binding of ligands at domain II. One of the most important functions of albumin is its ability to bind and transport free fatty acids. The helical region in each albumin domain forms an elongated pocket which renders it a site of fatty acid binding probably by arraying the hydrophobic residues at the inner surface and positively charged residues at the opening to attach the carboxyl group of the fatty acid. The binding affinity of albumin for fatty acids increases with the fatty acid chain length (2). There is strong circumstantial evidence that the portion of serum albumin containing amino acid residues 377-582, which includes subdomain IIIC, is the primary site of high affinity binding of long chain fatty acids (18). To study the strongest binding site for long chain fatty acids, we chose cis-parinaric acid. Changes in this fluorescent fatty acid due to binding to albumin show only interactions at hydrophobic pockets on albumin. Any fatty acid bound to lower affinity sites located at the surface of the albumin molecule will be excluded, giving

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2 Analysis of the excited tryptophan lifetime in human serum albumin revealed that the kinetics of the decay of the tryptophan emission could not be analyzed in terms of one first order component only (24). The albumin solution in these studies certainly contained some glycosylated fraction. On the basis of our steady state results, it is logical to suggest that time changes in the tryptophan of the glycosylated albumin occur as well. The heterogeneity in lifetime may therefore reflect the glycosylated fraction in these samples.

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3 According to the theoretical three-dimensional structural model of albumin proposed by Brown and Shockley (1), it is highly likely that lysine 525 is situated at the surface of domain III (L. Brown, personal communication).
no fluorescence signal (12, 18). Our findings (shown in Fig. 5) demonstrate that as a result of nonenzymatic glycosylation of lysine 525, the affinity of albumin for the long chain fatty acid was considerably reduced. It is important to note here that previous studies on the rate and extent of nonenzymatic glycosylation of human serum albumin (26) pointed out differences in the ability of defatted and fatty albumin to bind glucose. This phenomenon together with the findings reported here strongly suggest that the sites of fatty acid binding and glycosylation are linked. Lysine 525 may lie in a key position to bind to the negatively charged carboxyl group of the fatty acid, and as such may have an important role in the binding of long chain fatty acids to albumin.

This study demonstrates that structural changes in glycosylated albumin lead to a reduction in affinity for fatty acid. More information is needed to better understand the correlation of this finding with events such as lipid transport and atherosclerosis, the major cause of morbidity and mortality in diabetics. Other pathways of lipid transport may be impaired due to failure of glycosylated low density lipoprotein to interact with its receptors (27).

We have shown that nonenzymatic glycosylation of a large polypeptide at a single site can have marked effects on both the conformation as well as the biological properties of the protein. These results suggest that similar far reaching effects may occur in other proteins that undergo this type of post-translational modification.

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