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Fluorometric and Mass Spectrometric Analysis of Nonenzymatic Glycosylated Albumin

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Albumin is the major transport protein in blood and intramolecular movement contributes to this function. Nonenzymatic glycosylation (NEG) of albumin occurs in diabetes and, in this study, fluorometric methods were used to determine the effect of increasing levels of NEG upon intramolecular movement in human serum albumin. Low levels of NEG significantly reduced and left-shifted Trp fluorescence, reduced quenching by acrylamide and inhibited penetration of bis-ANS, while these changes became only modestly more pronounced at higher levels of NEG. Mass spectrometry of tryptic and CNBr NEG-HSA fragments identified potential glycosylation sites and demonstrated only late glycosylation of the C- and N-terminal regions of the protein. Similar changes in diabetes may contribute to altered transport function in these patients. © 2001 Academic Press

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Nonenzymatic glycosylation (NEG) of proteins occurs in diabetes and is thought to contribute to the common vascular complications of the disease (1–3). NEG is a complex and diverse process the first step of which involves the formation of a Schiff base between glucose and ϵ-amines of Lys residues as well as the N-terminal amine group (3–6). This reversible reaction may be followed by a reversible Amadori rearrangement to form fructosamine, which is readily detected using a colorimetric assay (7). Advanced glycation end products (AGE) are highly fluorescent, complex and often cross-linking compounds which may be either formed by oxidation of fructosamine, or reaction of fructosamines with each other or adjacent free amines in Lys or Arg (3–10).

Albumin is the major plasma protein and has been demonstrated to have an important role in the physiological transport of many compounds through the body including: free fatty acids, bile acids, steroids and some metals. In addition to these physiological compounds, albumin also binds numerous drugs so that in many instances, the final concentration of free drug available for pharmacological activity is dependent upon the proportion of drug bound to albumin (11). Albumin has an exceptionally high level internal molecular movement and it is believed that this exposes some ligand binding sites, thus facilitating the role of this protein as a transport molecule (11, 12).

NEG of albumin occurs in diabetes (2–10), and this alters the binding of physiological and pharmacological ligands (13, 14). In this paper, we describe studies in which fluorometric and mass spectrometric methods were used to further characterise the effect of NEG upon the structure and intramolecular movement of HSA.

MATERIALS AND METHODS

Materials. HSA was from Calbiochem (San Diego, CA). Phosphate buffered saline (PBS) tablets were purchased from Oxoid (Bakingstoke, UK). PD-10 gel filtration columns were purchased from Pharmacia (Sweden). Reagents for the fructosamine assay (7) were purchased from Roche (Somerville, NJ) while the Bradford protein assay reagent was purchased from Biorad (Hercules, CA). All other reagents used in this study were purchased from Sigma (St. Louis, MO).

Preparation of NEG-HSA. HSA (16 μM) was coincubated with glucose (50 mM) in PBS containing EDTA (1 mM), PMSF (1 mM), and 0.02% NaN_3 (40°C for up to 115 days). NEG was monitored using a colorimetric fructosamine assay based upon the nitroblue tetrazo-

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lium reaction with ketoamines to produce formazan, detected photometrically at 550 nm (7). Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) was used to confirm the increase in molecular mass associated with increasing glycosylation. SDS–PAGE according to the method described by Laemmli (15) confirmed the absence of complex formation or unwanted proteolytic degradation during glycosylation. Material with four increasing levels of glycosylation was prepared and identified as NEG-HSA 1 to 4, respectively. NEG-HSA preparations were stored at −20°C after exhaustive dialysis against PBS.

Fluorometry. Fluorometric studies of HSA, NEG-HSA 2, and NEG-HSA 4 were performed using protein dialysed into PBS, sterile filtered, and then adjusted to an equivalent protein concentration. Protein concentrations were adjusted by dilution with PBS to achieve superimposition of 280 nm absorption peaks on spectrophotometric analysis using a DU 640 spectrophotometer (Beckman Instruments, USA) and a 100 μl quartz cuvette with a 10 mm light path. A final OD of 0.862 at 280 nm was achieved in all samples used for fluorometric measurements. Fluorometry was performed using a Perkin Elmer LS 50B luminescence spectrometer (Perkin Elmer, USA) at 25°C with a 10 × 10 mm, 3 ml quartz cuvette. A magnetic stirring bead was used to ensure proper mixing during readings. The slit width for both emission and excitation beams was set at 5 nm while the excitation beam was centered on the middle of the cuvette. A 2 ml volume of solution was used in all fluorometric measurements. A minimum of 5 fluorometric scans were averaged to obtain data using PBS as a blank.

Fluorometric demonstration of AGE in HSA, NEG-HSA 2, and NEG-HSA 4. One millilitre quantities of HSA, NEG-HSA 2 or NEG-HSA 4 prepared as described above were added to 1 ml of PBS in a 3 ml quartz cuvette. Emission scans from 400 to 550 nm were with an excitation wavelength of 370 nm and these data were used to demonstrate increasing levels of AGE formation (6, 16).

Fluorometric demonstration of reduced intramolecular flexibility in NEG-HSA 2 and NEG-HSA 4. Fifty microlitre quantities of HSA, NEG-HSA 2, or NEG-HSA 4 (OD_{280} 0.862) were added to 2 ml of PBS in a 3 ml quartz cuvette. After thorough mixing, Trp fluorescence was determined in emission scans from 300 to 450 nm using an excitation wavelength of 295 nm. This excitation wavelength minimised the contribution of Phe and Tyr to readings (17).

Trp quenching was studied by adding 100 μl of protein solution (OD_{280} 0.862) to 900 μl of PBS and recording the changes in Trp fluorescence following the progressive addition of 10 μl aliquots of acrylamide (6 M in PBS). Because the maximum Trp fluorescence wavelength was left-shifted by increasing NEG, calculations of emission values were performed at 354 nm for HSA, 349 nm for NEG-HSA 2, and 346 nm for NEG-HSA 4. Internal filter and dilution effects become significant at the protein and acrylamide concentrations used. To adjust for this, correction factors were calculated based on the optical density at excitation and maximum emission wavelengths, as well as upon dilution by added acrylamide (18). Multiplication of unquenched and quenched fluorescence values by the relevant correction factor resulted in data which were used to construct Stern-Volmer plots in which the ratio of fluorescence in the absence of acrylamide relative to fluorescence in the presence of acrylamide was plotted against the acrylamide concentration (19).

4,4′-bis (1-anilinonaphthalene-8-sulfonate) (bis-ANS) is a small organic compound which is fluorescent only when associated with hydrophobic materials and as such can be used to probe for the accessibility of small molecules to hydrophobic domains in proteins (20). In this study, 50 μl of protein solution (OD_{280} 0.862) was added to 2 ml of PBS with 1 μl of bis-ANS (2.5 mM in Tris 0.01 M, pH 7.5). Emission scans were then obtained from 400 nm to 550 nm using an excitation wavelength of 385 nm.

Preparation of trypsin and CNBr fragments for mass spectrometry. Tryptic fragments of HSA and NEG-HSA 4 were prepared for MALDI-TOF MS. Before proteolysis, samples were subjected to reduction and alkylation. Briefly, 2 mg of HSA and NEG-HSA 4 in 400 μl of urea (0.5 M) were mixed with 100 μl of DTT (10 mM) in Tris–HCl (1 M, pH 8.5) and incubated at 37°C for 2 h. Iodoacetic acid (50 μl, 0.35 M) in Tris–HCl (1 M, pH 8.5) was then added and the samples incubated in the dark at RT for at least 15 min. DTT and iodoacetic acid were removed by passage through preequilibrated PD-10 gel permeation columns and eluted with urea (1 M) in Tris–HCl (25 mM, pH 8.5). Eluted protein (0.5 ml fractions) was detected by absorption spectrophotometry at 280 nm. Pooled fractions were then concentrated to a final volume of 500 μl by centrifugation dialysis (10-kDa molecular weight cut-off). MALDI-TOF MS was performed to confirm complete reduction and alkylation and to determine whether these steps had any effect upon glycosylated sites. Aliquots (μl) of these samples were used to dissolve 50 μg of lyophilized sequencing grade trypsin before incubation overnight at 37°C. Samples were desalted by solid phase extraction (Sep-Pak C18 cartridge) by washing with deionized water and eluting with acetonitrile. Peptides were mixed with a large molar excess of α-cyano-4-hydroxycinamic acid applied directly to the target and allowed to dry at room temperature and pressure.

CNBr fragments of HSA, NEG-HSA 2, and NEG-HSA 4 were also prepared for analysis by MALDI-TOF MS. Briefly, protein was dialysed into NaCl (145 mM) to a final concentration of 10 mg/ml. Protein preparations (100 μl) were added to 90% formic acid (450 μl) with CNBr (2 mg/ml). Samples were sealed under N_2 gas and then incubated at ambient temperature overnight. Samples were then mixed with 500 μl of 90% saturated ammonium sulphate and chilled on ice for 30 min. Precipitated protein was pelleted by centrifugation (13,000 RPM, 15 min, RT) and washed 4 times with 1 ml of 90% saturated ammonium sulphate. Pellets were resuspended in urea (400 μl, 0.5 M) before addition of DTT (50 μl, 10 mM) in Tris–HCl (1 M, pH 8.5). After 2 h at 37°C, iodoacetic acid (50 μl, 0.35 M) was added to each preparation and samples incubated at RT in the dark for at least 15 min. The resulting protein fragments were precipitated with 10 volumes of ice-chilled acidic acetone prepared by mixing 1 ml of concentrated HCl with 100 ml of acetone. The material was then resuspended in acetonitrile prior to MALDI-TOF MS analysis.

MALDI-TOF MS of HSA, NEG-HSA, CNBr, and trypsin fragments. Protein and peptide samples were mixed with a large molar excess of either sinapinic acid or α-cyano-4-hydroxycinamic acid respectively and dried onto stainless steal targets at room temperature and pressure. Analyses were performed on a Voyager DE STR mass spectrometer with a nitrogen laser (337 nm; 2 ns pulse) (PE Biosystems, Framingham, MA). All analyses were in the positive ion mode and the instrument was calibrated immediately prior to each series of studies. Peaks observed by MALDI-TOF MS with a minimum signal-to-noise ratio of 3 to 1 were compared with those predicted in a theoretical digest by employing the Peptide Mass prediction protocol available at the ExPASy site (http://expasy.hcuge.ch/cgi-bin/peptide-mass.pl). A maximum error of 1 Da was permitted in mapping spectral peaks to predicted peptide masses. The published sequence (21, 22) and the "A" configuration of the X-ray crystallographic structure (23, 24) for HSA were used for structural analysis.

RESULTS

NEG of HSA Occurred upon Coincubation with Glucose

Progressive NEG of HSA was monitored by measuring fructosamine. Levels did not increase after 100 days of coincubation with glucose (Fig. 1A). Four preparations of HSA, designated NEG-HSA 1 to 4, were used to monitor increasing levels of NEG by SDS–PAGE and MALDI-TOF MS and this correlated with

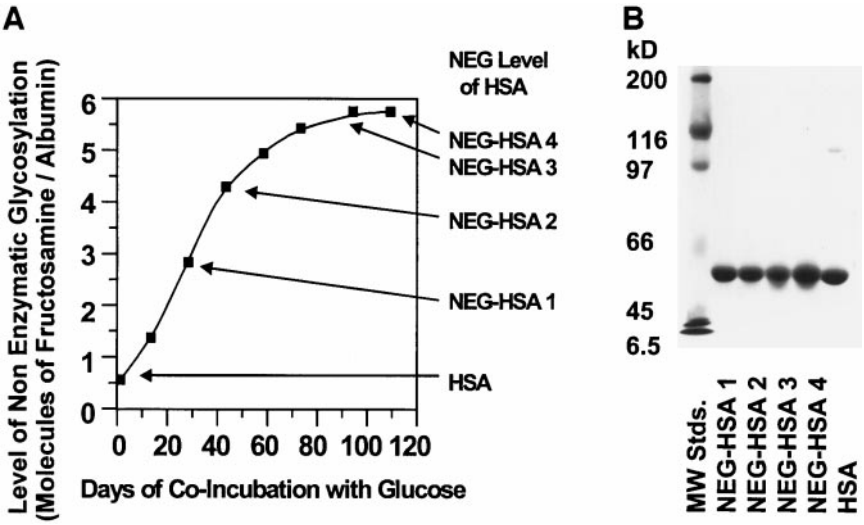

FIG. 1. Fructosamine levels (A) as well as SDS–PAGE (B) of HSA coincubated with glucose for up to 115 days. (A) Fructosamine levels appeared to be maximal by 100 days of co-incubation. HSA preparations with 4 increasing levels of NEG (NEG-HSA 1 to 4) were used to determine the effect of NEG upon the antiapoptotic activity of HSA. This correlated with increasing molecular mass as determined by MALDI-TOF MS. (B) SDS–PAGE of these preparations revealed the absence of either fragmentation or dimer formation in NEG-HSA, although a broadening of electrophoretic bands was seen as expected for glycosylated species.

increasing levels of fructosamine (Fig. 1A). Molecular mass as determined by MALDI-TOF MS of preparations of HSA and NEG-HSA were as follows: HSA, 66,434 ± 24 Da; NEG-HSA 1, 66,911 ± 33 Da; NEG-HSA 2, 67,048 ± 21 Da; NEG-HSA 3, 67,354 ± 31 Da, NEG-HSA 4, 67,582 ± 22 Da. Because NEG and AGE formation is highly diverse, it was not possible to determine the precise number of chemical modifications per molecule. However, the centroid of MW distribution indicated, on average, an increase in mass of 1144 Da in NEG-HSA 4 over that of unmodified HSA. If it is assumed that most NEG sites contain fructosamine (MW of adduct 160 Da), then this corresponds to as many as 7.15 NEG sites per NEG-HSA 4 molecule. Because AGE are formed by condensation of NEG residues it is likely that more than these 7 to 8 sites are involved. SDS–PAGE confirmed that NEG did not result in either fragmentation or polymerization of HSA although broadening of electrophoretic bands was noted with increasing levels of NEG (Fig. 1B).

Progressive NEG of HSA Resulted in Increasing AGE Formation and Reducing Intramolecular Movement

Fluorometric studies were performed to characterize changes in intra-molecular movement with increasing NEG, as well as to assess the formation of AGE. Emission fluorescence scans indicated progressive accumulation of AGE (Fig. 2A) as well as reduced and left shifted Trp fluorescence (Fig. 2B). This indicated reduced access to activating light as well as reduced access of the single Trp 214 to bulk water. Additional experiments were performed to construct Stern-Volmer plots for acrylamide quenching. Quenching was greatly reduced in NEG-HSA 2 relative to HSA, while a further but more modest reduction in acrylamide quenching was seen for NEG-HSA 4 as compared with NEG-HSA 2 (Fig. 2C). In addition, bis-ANS fluorescence decreased significantly with increasing

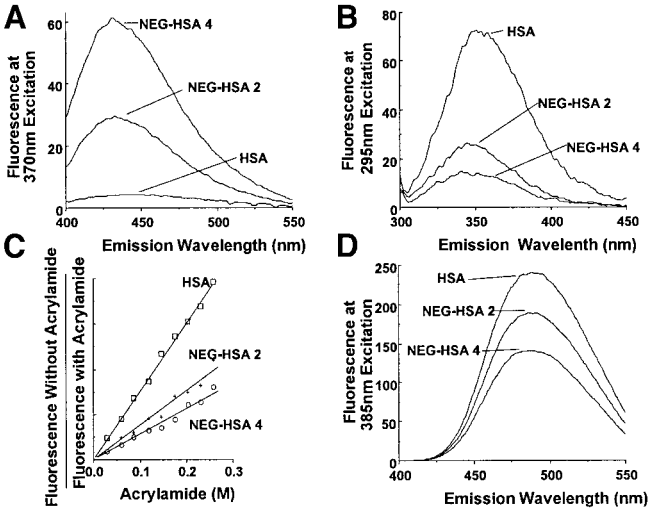

FIG. 2. Emission fluorometry of HSA, NEG-HSA 2 and NEG-HSA 4 demonstrating (A) increasing AGE levels, (B) reducing and left shifted Trp fluorescence, (C) reducing acrylamide quenching, and (D) reducing bis-ANS fluorescence with increasing levels of NEG. Increasing levels of NEG and AGE formation (A) appeared to reduce access of the single Trp 214 to light (B), acrylamide (C), and free water (B), and reduced access of bis-ANS to hydrophobic domains (D). The data indicate reduced intramolecular movement with increasing NEG.

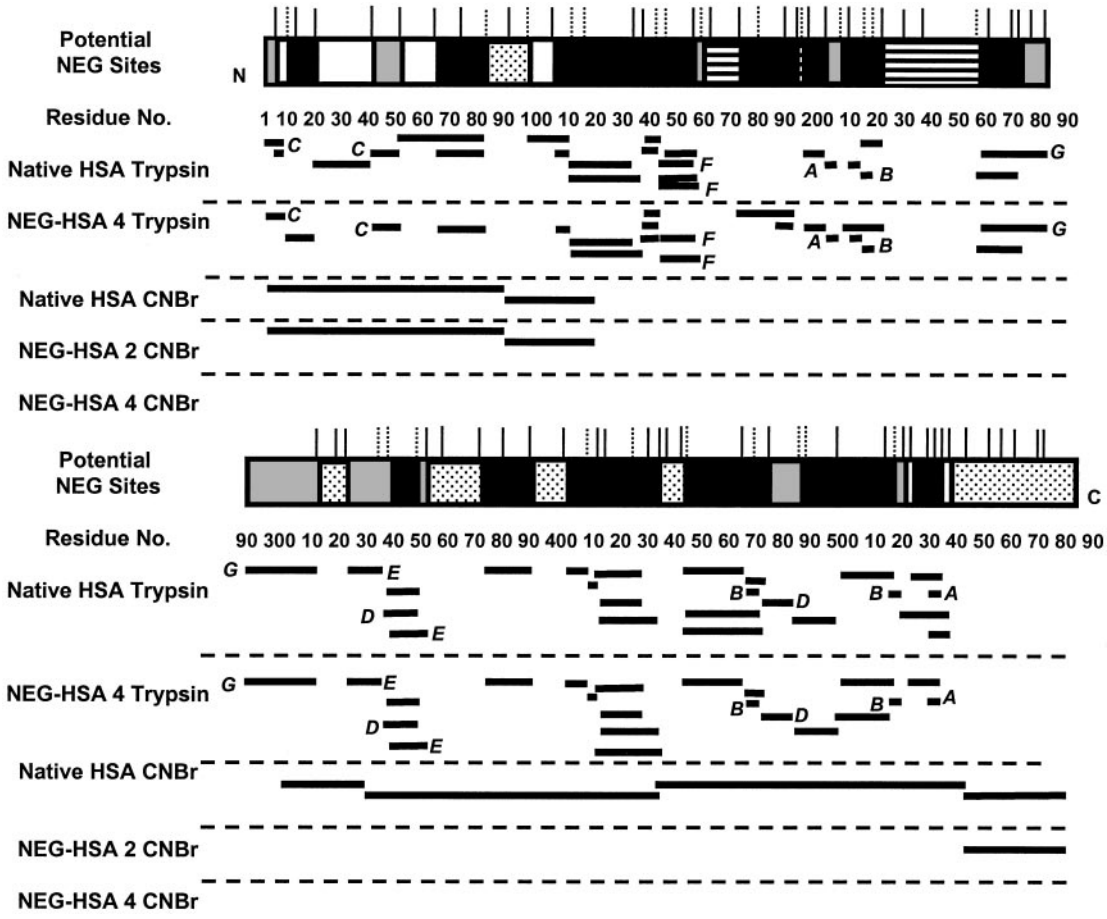

FIG. 3. Map of HSA showing the location of peptides identified by MALDI-TOF MS from CNBr digests of HSA, NEG-HSA 2 and NEG-HSA 4 as well as trypsin digests of HSA and NEG-HSA 4. CNBr fragments and tryptic peptides identified in both HSA and NEG-HSA 4 digests are indicated (—), as are tryptic peptides found only in either HSA or NEG-HSA 4 (—). Some mass spectrometric peaks corresponded to more than one peptide, and these are labelled A through to G in order of increasing size. Comparison of tryptic peptides allowed identification of areas where NEG occurred (□) while CNBr fragments suggested NEG in other areas (▨). It was not possible to exclude NEG in areas represented by peptides A to G alone, and these areas are indicated (▩). Glycosylation was excluded from several areas (■) while no data was available for some regions (▤). Lys (|) and Arg (¦) residues, the principal sites of NEG, are indicated. The C and N terminal regions appeared to be glycosylated late during NEG, correlating with loss of the antiapoptotic activity. (Please see the text for details of this analysis).

NEG (Fig. 2D), indicating reduced access of this molecule to hydrophobic domains in HSA. Collectively, these data indicate that there is reduced intramolecular movement which limits access of acrylamide and bis-ANS to internal parts of the molecule with increasing levels of NEG.

Identification of Potential Sites for Nonenzymatic Glycosylation in HSA

To identify the sites modified by NEG, both native and glycosylated preparations of HSA were cleaved with either trypsin or CNBr and the samples analysed by MALDI-TOF MS. Direct analysis gave 13 peaks from CNBr fragments of HSA and 97 peaks from tryptic digests of the native material. Of these peaks, 6 CNBr and 39 tryptic fragments of HSA mapped to specific regions while an additional 8 peaks of tryptic NEG-HSA 4 fragments corresponded to known sequence. CNBr fragments accounted for 70% of the protein sequence while tryptic fragments represented 72% of the protein. Because of overlapping CNBr and tryptic fragments, a total of 92% of the protein sequence was identified.

CNBr cleavage of NEG-HSA 2 and NEG-HSA 4 yielded 12 and 9 peaks respectively, with only 3 peaks in NEG-HSA 2 having masses identical to peptides from the theoretical digest and none of the CNBr generated peaks from NEG-HSA 4 mapping to the known sequence. New peaks were seen in CNBr cleaved glycosylated HSA, with 8 new peaks present in NEG-HSA 2 and a further 8 different peaks seen in NEG-HSA 4. Mass spectrometry of tryptic peptides from NEG-HSA

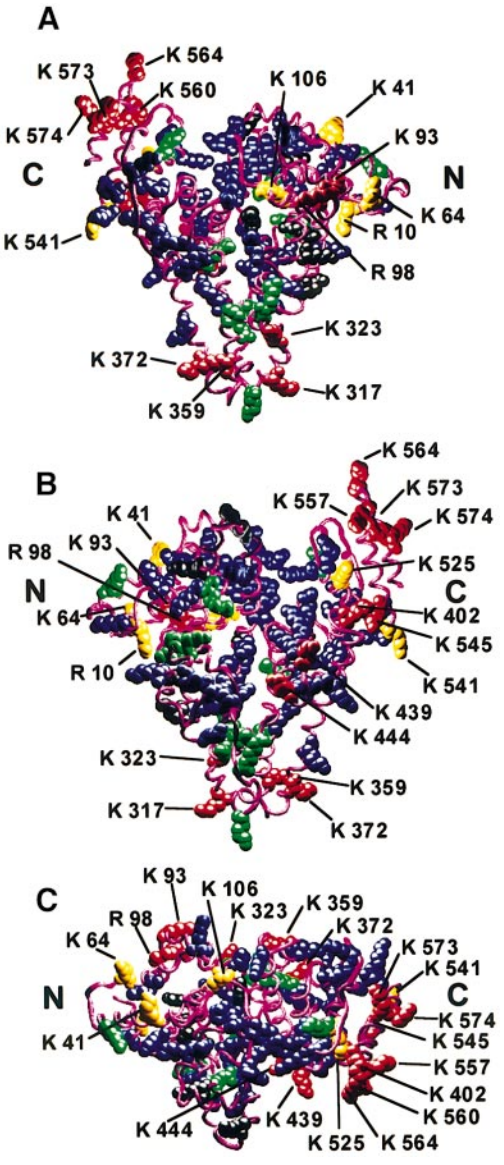

FIG. 4. Views from the front (A), back (B), and top (C) of the X-ray crystallographic structure of HSA showing Lys and Arg residues involved with and excluded from NEG. Transposition of data summarised in the legend to Fig. 3 upon the crystallographic structure of HSA reveals that NEG sites indicated by tryptic peptides (yellow) are all on the surface of the molecule as are those indicated by CNBr fragments alone (red). Lys and Arg residues in regions where individual mass spectrometric peaks correspond to more than one peptide are indicated in green. Residues which are not glycosylated on the basis of the above described analysis are coloured blue and are found to be almost exclusively in internal parts of the protein. Residues for which there is no data are coloured black. The C terminus is marked (C) while the N terminus is labelled (N). NEG is seen to involve Lys and Arg residues on the surface of the molecule rather than residues in inaccessible internal sites.

4 revealed 100 peaks, of which 35 mapped to specific protein sequences and 46 were new peaks not seen in native HSA.

Because of the extreme diversity of NEG and AGE products it was not possible to deduce the identity of new peaks in CNBr and tryptic preparations of NEG-HSA 2 and 4. However, it was possible to identify regions in the protein where NEG had occurred as well as to define other regions where NEG modification could be excluded. Figure 3 shows the location of individual CNBr and tryptic peptides in glycosylated and native HSA as well as the way in which these data were interpreted. Because NEG and AGE change the mass of peptides, glycosylated regions were identified as areas where tryptic peptides of HSA were present in the absence of tryptic peptides of NEG-HSA 4 (residues 5–10, 21–41, 52–64, 99–106, 525, 539–541; containing the following potentially glycosylated groups: Lys 41, 64, 106, 525, 541 and Arg 10). In some areas, no tryptic fragments were present but the loss of CNBr fragments upon NEG indicated glycosylation (residues 82–98, 314–323, 352–372, 390–402, 437–444, 536–585; containing the following potentially glycosylated groups: Lys 93, 317, 323, 359, 372, 402, 439, 444, 545, 557, 560, 564, 573, 574 and Arg 98). Some tryptic fragments had masses which could have represented more than one peptide and these are indicated in Fig. 3. There were some regions where, because of the ambiguous origin of these peptides, NEG may have occurred but with less certainty than the two previously described regions (residues 1–4, 42–51, 160, 206–209, 277–313; containing the following potentially glycosylated residues: Lys 4, 51, 281, 286, 313 and Arg 160, 209). Glycosylation was not seen in regions where tryptic fragments of NEG-HSA 4 were present (residues: 11–20, 65–81, 107–145, 175–195, 198–205, 210–225, 258–276, 338–348, 373–389, 403–436, 445–475, 485–521, 526–538; containing the following groups excluded from glycosylation: Lys 12, 20, 73, 136, 137, 181, 190, 195, 199, 205, 212, 225, 262, 274, 276, 378, 389, 413, 414, 432, 436, 466, 475, 500, 519, 534, 536, 538

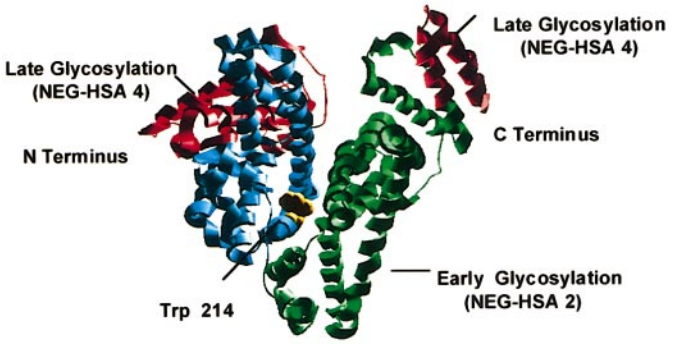

FIG. 5. Location of early (green) and late (red) glycosylation in HSA as well as the location of the single Trp 214 (yellow) in a deep molecular cleft. Location of regions where glycosylation occurs in NEG-HSA 2 (green), as well as regions where glycosylation appears to occur only in NEG-HSA 4 (red). Early NEG occurs in the body of the protein, and would be expected to limit access of Trp 214 to water, light, and acrylamide while latter glycosylation of the C- and N-terminal would have less effect upon this, consistent with the fluorometric data in Fig. 2.

and Arg 81, 114, 117, 144, 145, 186, 218, 222, 348, 410, 428, 445, 472, 485, 521). In addition to these sites, NEG was excluded from a further region where a single observed tryptic peak represented two separate possible peptides, but where these peptides were overlapping (peptides F in Fig. 3) (residues: 170–183; containing Lys 159). Finally, there were 3 regions where no data were available and from which NEG could not be excluded (residues 161–174, 196–197, 226–257; containing the following potentially glycosylated groups: Lys 162, 172, 233, 240 and Arg 197, 257). These data are summarised in Fig. 3 and it is important to note that the multiplicity of NEG sites identified is consistent with the highly diverse nature of NEG and AGE formation with numerous molecular species of NEG-HSA evident. Superimposition of these data upon the published X-ray crystallographic structure of HSA indicates that glycosylated residues are on the external surface of the molecule, while those residues excluded from glycosylation are clustered towards the centre (Fig. 4).

Figure 5 illustrates the location of regions that appear to be glycosylated early and late during NEG on the basis of MALDI-TOF MS of the CNBr fragments. The C- and N-terminal regions are unchanged in NEG-HSA 2 but are lost from CNBr digests of NEG-HSA 4 (Fig. 3). Also shown in Fig. 5 is the location of Trp 214 within a molecular cleft, becoming inaccessible with increasing NEG and AGE formation (Figs. 2A, 2B, 2C, and 5). When considered together with the fluorimetric data, the mass spectrometric data indicate that the greatest restriction in intramolecular movement occurs when the C and N terminal regions become glycosylated.

DISCUSSION

When HSA was coincubated with glucose, MALDI-TOF MS, fructosamine assays, and fluorometry for AGE indicated progressive NEG and AGE in HSA. Reduced intramolecular movement was suggested by reduced and left shifted Trp fluorescence, reduced acrylamide quenching and reduced penetration of bis-ANS into NEG-HSA. The effects of NEG upon intramolecular movement appears to be due to progressive decoration of the surface of the molecule by fructosamine residues as well as the formation of potentially cross-linking AGE while the absence of dimer formation in NEG-HSA was confirmed by SDS–PAGE.

The decreased dynamic quenching of Trp fluorescence by acrylamide indicated reduced access of Trp 214 to this small organic molecule. Since Trp 214 is located in a deep cleft of HSA (Fig. 5), it is suggested that reduced acrylamide quenching reflects reduced conformational exposure of this cleft. This is consistent with the conclusion of others that Trp 214 quenching by acrylamide is not dependent upon breaking intramolecular hydrogen bonds, but reflects periodic exposure of the residue by local unfolding (19), also consistent with the location of Trp 214 in a deep groove as seen in the crystallographic structure (Fig. 5).

The significant left shift in Trp fluorescence of NEG-HSA represents a shift in the spectral form from that thought to occur when there is contact of Trp with bulk water (spectral form III) towards that seen when Trp is exposed to water molecules with much reduced mobility (spectral form II) (17). This is consistent with the suggestion that Trp 214 is normally exposed to bulk water by intra-molecular movement and that NEG restricts this movement, also consistent with reduced acrylamide quenching.

The reduced penetration of bis-ANS into hydrophobic domains of NEG-HSA also supports the idea that NEG reduces intramolecular movement. Structurally, Alb is considered to consist of highly mobile subunits which flex and rotate with regard to each other in order to admit ligands to binding sites (11, 12). The reduced penetration of bis-ANS into the hydrophobic domains of HSA is interpreted as being due to inhibited intramolecular movement, preventing the conformational changes necessary for penetration of bis-ANS into the molecule. These observations are also consistent with reduced Trp fluorescence and fatty acid and drug binding of NEG-HSA reported by others (13, 14).

Identification of potential sites involved with NEG was performed by MALDI-TOF MS of CNBr and tryptic fragments. Although an almost equivalent number of peaks were observed in tryptic digests of HSA and NEG-HSA 4, the highly diverse nature of NEG and AGE formation made it impossible to correlate the appearance of specific peaks in NEG-HSA with the formation of specific NEG or AGE products. However, it was possible to define regions of the protein which were glycosylated as well as other sites that were not modified. New peaks in NEG-HSA preparations indicated the formation of glycosylated species, although the diversity of NEG and AGE indicates the presence of multiple molecular forms so that not all sites deduced as glycosylated will be modified in each molecule. The presence of some tryptic peptides in NEG-HSA 4 but not in native HSA suggests that the conformational changes seen in fluorometric data result in exposure of some otherwise inaccessible trypsin cleavage sites.

Several aspects of the outcome of this analysis provide confidence in the approach used. Firstly, NEG is largely dependent upon the access of Lys residues to glucose (3, 5–10) and this would be greatest at the surface of the protein. The location of glycosylated residues at the surface as compared with uninvolved residues in the more central portion of the protein is consistent with this expectation. Secondly, even though there are many Arg residues in HSA, only 2 Arg residues were present in glycosylated regions. Arg is involved with NEG primarily through reaction with

glycosylated Lys residues (8–10) so that involvement of this amino acid with NEG is expected to be less common than Lys. Finally, Lys 525 is reported as being a site of predilection for NEG of HSA (13) and in the current study, this residue was identified as both glycosylated and in a region of early NEG. The body of the protein was affected by NEG earlier than the C and N terminal regions.

Stern-Volmer plots indicated significantly reduced intra-molecular movement, even with low levels of NEG. Higher levels of NEG involving the C and N terminal domains were associated with a further, although less dramatic, reduction in intra-molecular movement.

Intramolecular movement is required for exposure of the binding sites for many drugs and natural ligands to albumin (11, 12), while this study indicates significant changes in intramolecular movement with NEG. This supports the idea that reduced intramolecular movement due to NEG contributes to altered ligand binding by this transport protein in diabetes (13, 14).

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