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REACTION OF HUMAN SERUM ALBUMIN WITH ALDOSES*

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ABSTRACT

The reaction of human serum albumin (HSA) with aldoses (C_3–C_6) and acetaldehyde has been studied. U.v. and fluorescent spectra of the HSA–glyceraldehyde and HSA–GlcN adducts reveal yellow chromophores absorbing at 300–350 nm and emitting at 435 nm. However, even limited reaction of HSA with acetaldehyde induced perturbation in the Trp microenvironment. C.d. spectra of the adducts show an average 20% decrement in mean residual ellipticity [θ], which is independent of the extent of the reaction and the aldose used. It is concluded that most of the reactions with aldoses occur at the surface of the HSA molecule. With the exception of the GlcN adduct, the HSA adducts rearrange to produce pyrrole rings on the protein surface. I.e.f. analysis shows that the pI values of the modified HSA are almost linearly correlated with the chain length of the reacting aldose: from pI 4.2 for HSA–glyceraldehyde up to pI 5.0 for HSA–GlcN.

INTRODUCTION

It is now generally accepted that the most reactive groups of proteins involved in non-enzymic reactions with sugars in healthy humans and in diabetic patients are the exposed ε-amino groups of lysine (Lys) residues. In the preceding paper[1], it was shown that, under simulated physiological conditions (37°, pH 7.5), Lys, glyceraldehyde, and 2-amino-2-deoxyglucose (GlcN) were the most reactive species. We now report parallel studies with purified human serum albumin (HSA).

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EXPERIMENTAL

Purification of HSA. — The serum of normal donors was used with pseudo-ligand affinity chromatography on Affi-Gel Blue[2] (BioRad). Chromatography on a column (0.5 × 16 cm) of Concanavalin A–Sepharose[3] (Pharmacia) was used to adsorb the highly modified HSA.

Incubations. — Sterilised HSA (1 mg/mL) was incubated severally in 100mM phosphate buffer (pH 8) with 20mM D-glucose, 2-amino-2-deoxy-D-glucose, D-ribose, D-erythrose, DL-glyceraldehyde 3-phosphate, DL-glyceraldehyde, and acetaldehyde at 37° in the dark for up to 7 days in the presence of gentamycin sulphate to prevent bacterial growth. Incorporation of sugars was followed either by the thiobarbituric acid assay[4] after acid hydrolysis of the protein or by monitoring the disappearance of the Lys ε-amino groups with trinitrobenzene-sulfonate[5]. After incubation, each sample was ultrafiltered (Amicon PM 30 membranes) and, in some experiments, 100mM sodium cyanoborohydride was added simultaneously to reduce the carbohydrate–protein linkages. Each sample was then dialysed against H_2O with Thomas dialysis membranes (mol. wt. cut-off, ~2000).

Isoelectric focusing (i.e.f.). — This was performed by using ultrathin-layer (240 μm) polyacrylamide slab gels (5% T, 3% C) cast on silanised glass plates (13 × 13 cm). Gaskets were formed with Parafilm rectangles, one layer giving ~120-μm thickness. The polymerisation solution contained 12% of glycerol ammonium persulphate as catalyst, *N,N,N′,N′*-tetramethylethylenediamine accelerator, and 2.5% of LKB Ampholines (50% pH 4–6, 22% pH 5–7, and 25% pH 5–8 ranges). The samples were applied to the gel surface with Whatman 3MM paper strips. The pH gradient was evaluated[8] either with 6 pI marker proteins or with an LKB surface glass electrode at 10°. The runs were carried out at 10°, using an LKB Multiphor system for a total of 5000 Vh. The gels were prefocused for 1 h at 500 V and 13 W.

Staining was effected by the photochemical silver method[9] slightly modified for ultrathin gels[10].

Determination of pyrrole groups. — The pyrrole groups in modified HSA, dissolved either in H_2O or in 9M urea, were determined by the sulfanilic acid–$NaNO_2$ reaction[11]. A 200-μL sample was mixed with 100 μL of 29mM $NaNO_2$ and 1 mL of 32mM sulfanilic acid in 165mM HCl. After incubation at 37° for 1 h, 100 μL of 5M NaOH was added and the mixture was stored for 45 min at 37° before reading the extinction at 492 nm. Three scalar concentrations of histidine were used as standards (limit of linearity, 0.1–0.5mM). The results are given as mol of pyrrole/mol of HSA. The HSA was quantified by using the Coomassie dye-binding assay[12].

U.v., fluorimetric, and c.d. spectra. — U.v. spectra were recorded with a DU-8 Beckman spectrophotometer at 33°, and fluorimetric spectra were recorded with a Perkin–Elmer MPF-44A spectrofluorimeter (10-mm optical path) and were

uncorrected. C.d. spectra were obtained by using a Jasco J500A spectropolarimeter at room temperature (0.1- and 2-cm path-length). The spectra were expressed either as mean residue ellipticity (in the u.v. region) or as molar ellipticity (in the near-u.v. region). Ellipticities (deg.cm^2/dmol) were calculated by using 113 as the mean residue mass and 66,000 as the protein mass for HSA. The instrument was calibrated using (+)-10-camphorsulphonic acid, which shows a positive c.d. band at 290.5 nm with $\Delta\varepsilon$ + 2.36 L.mol^{-1}.cm^{-1} (the concentration was determined by using 34.5 L.mol^{-1}.cm^{-1} at 285 nm as the extinction coefficient for the anhydrous form[21]). The helix content of HSA and modified HSA was calculated by the method of Chang *et al.*[22], using 16 reference proteins of known structure by X-ray diffraction. All calculations were done on a Gould S.E.L. 32/7780 computer.

RESULTS

Table I gives the molar ratios of trioses and hexoses bound to purified HSA after 7 days of incubation at pH 8.0 and 37°. The disappearance of Lys was monitored by using the trinitrobenzenesulfonate reaction[5]. Glyceraldehyde was by far the most reactive molecule; at equilibrium, 130 mol were covalently bound to 1 mol of HSA. Also, ~6 mol of Lys/HSA molecule disappeared in the reaction with glyceraldehyde and ~10 mol of Lys/HSA for the adduct reduced with cyanoborohydride, representing 30–35% and ~60%, respectively, of the potential reactive sites, since 16 of 59 Lys residues are exposed on the surface of the HSA molecule. The ratio of aldehyde incorporated to Lys reacted was ~6:1, suggesting that it could be bound to each reacted site as a short oligomeric chain. In the yellow, oligomeric pigment formed[1] from glyceraldehyde and Lys, the ratio between the two reactants was considerably greater than 2:1. In terms of the formation of a yellow pigment absorbing at 340 nm, DL-glyceraldehyde reacted more strongly than 2-amino-2-deoxy-D-glucose and D-erythrose, D-ribose and DL-glyceraldehyde 3-phosphate reacted weakly, and D-glucose and acetaldehyde not at all. If the Amadori adduct was not allowed to rearrange spontaneously (Maillard browning[13]) in solution, but was immediately reduced with cyanoborohydride, the reactants were incorporated into the HSA molecule, but no colour developed. Fig. 1 shows that HSA has λ_{max} 278 nm, due to its aromatic residues; the HSA–GlcN adduct, in addition to a much increased peak at 283 nm, has a marked shoulder at 300–310 nm, whereas the HSA–glyceraldehyde adduct has a peak at 258 nm and a new chromophore at 297 nm, the latter corresponding to the strong c.d. peak of Fig. 3. When excited at 350 nm in the region of the yellow chromophore, the Lys–glyceraldehyde adduct strongly fluoresced (emission peak at 435 nm; Fig. 2). HSA and the cyanoborohydride-reduced species did not fluoresce. However, the lack of u.v. absorption or fluorescence does not necessarily mean the lack of formation of the adduct. The HSA–acetaldehyde reaction did not yield a coloured product but, when the product was excited at 280 nm, *i.e.*, in the region of tryptophan absorption, the fluorescence spectrum of HSA and those of HSA–acetaldehyde before

TABLE I

ANALYSIS OF TRIOSES AND HEXOSES BOUND TO MODIFIED HSA[a]

Adduct of HSA with	*SCA[b,c] (mol of HMF/mol of HSA)*		*HMF released on hydrolysis (mol/mol of HSA)*		*Carbohydrate incorporated (mol/mol of HSA)*	
	Unreduced	*+NaCNBH$_3$*	*Unreduced*	*+NaCNBH$_3$*	*Unreduced*	*+NaCNBH$_3$*
D-Glucose	0 0605	0.1624	0.48	1 38	7 9	8.5
2-Amino-2-deoxy-D-glucose	0.0955	0.0227	0.44	0.15	4 6	6 6
D-Glucitol	—	—	—	—	—	—
DL-Glyceraldehyde 3-phosphate	0 0461	0.031	2 2	1 2	47.7	38.7
DL-Glyceraldehyde	0 0420	0.0212	5.49	2.75	130.7	130
Acetaldehyde	—	—	—	—	—	—

[a]Thiobarbituric acid assay of 7-days reacted HSA with trioses and hexoses (see Experimental). [b]SCA, specific colour activity; HMF, hydroxymethylfurfural. [c]SCA was calculated from the yield of HMF from each carbohydrate in the thiobarbituric acid assay

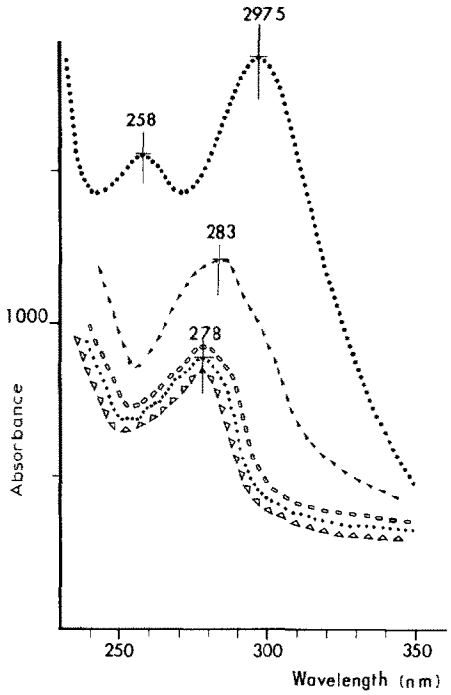

Fig. 1. U.v. spectra at 33° of HSA (■■■), HSA–glyceraldehyde (●●●), HSA–GlcN (◀◀◀), reduced ($NaCNBH_3$) HSA–glyceraldehyde (□□□), and reduced HSA–GlcN (△△△) (0.8 mg/mL in 100mM phosphate buffer, pH 8.0).

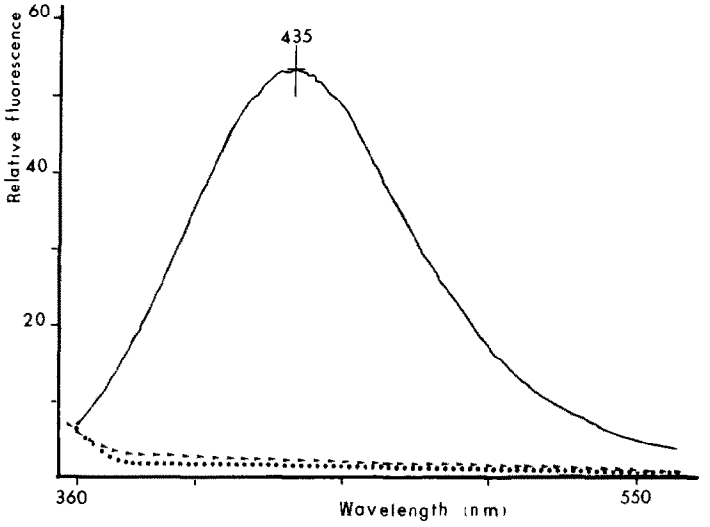

Fig. 2. Emission spectra (excitation at 340 nm) of HSA–glyceraldehyde (———), its reduced ($NaCNBH_3$) derivative (▶▶▶), and HSA (●●●).

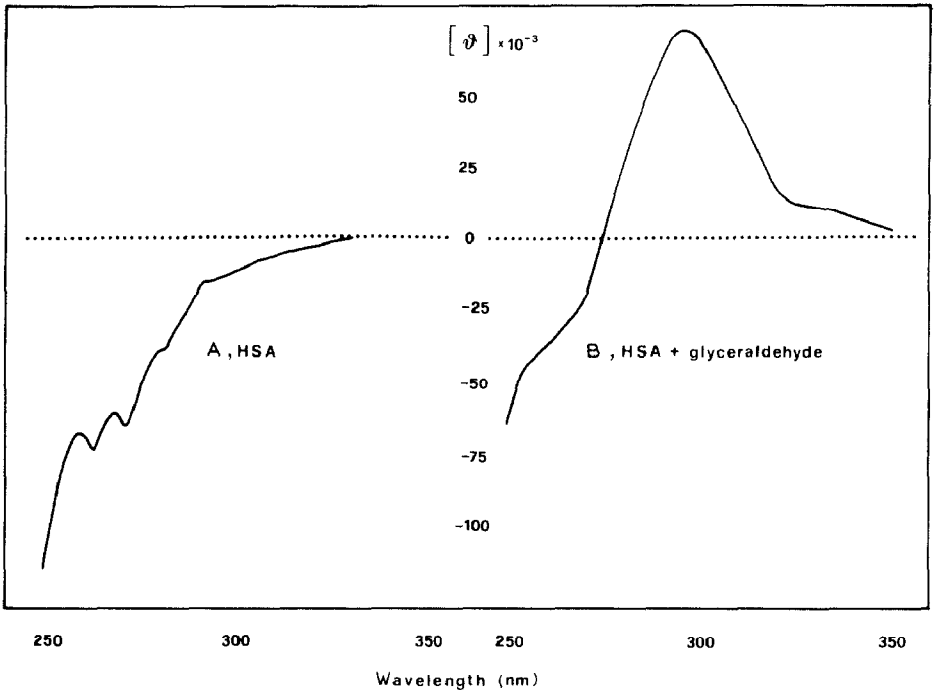

Fig. 3 C.d. spectra at room temperature of *A*, HSA (2.0 mg/mL), and *B*, HSA–glyceraldehyde (1 mg/mL); in 100mM phosphate buffer (pH 8.0)

and after reduction with cyanoborohydride are markedly different. On incubation at 37° and pH 7.4 with ^{14}C-acetaldehyde, trace labelling of HSA occurs[14] (0.05 mol/mol of HSA; 0.23 mol/mol after reduction). This small extent of labelling perturbs the tryptophan microenvironment, whose fluorescence is quenched, suggesting that conformational changes have been induced in HSA. The change in fluorescence spectrum is quite marked, when judged on the basis of the ratio of the intensities of the peaks at 330 and 305 nm, namely, 0.52, 1.31, and 1.35 for HSA and HSA–acetaldehyde before and after reduction, respectively. Similar findings have been reported[15] for the binding of glucose to Lys 525 in HSA.

The c.d. properties of the various HSA adducts have been studied. An index of conformational transitions can be obtained from the mean residual ellipticity in the 190–250-nm region and from the helix fractional content of a protein. HSA has a 53% α-helix content, and the adducts of HSA with glyceraldehyde, glyceraldehyde 3-phosphate, 2-amino-2-deoxyglucose, and acetaldehyde have α-helix contents of 35, 33, 32, and 40%, respectively. A possible explanation for these similar values is that the reaction yielding the adducts is mostly a surface phenomenon which does not greatly affect the overall protein structure. In the c.d. spectra in the near-u.v. region, the major difference among the various adducts was a large positive peak at 297 nm for the HSA–glyceraldehyde adduct (see Fig. 3). At present, we have no explanation for this finding.

The pyrrole contents of the adducts are shown in Table II. Since His and Tyr respond to the Pauly reaction, an average of 30 mol/mol found for HSA should be subtracted from the values obtained for the adducts. The reaction of HSA with the sugars generated pyrrole rings, with most being formed from glyceraldehyde and ribose (~30 mol/mol of HSA) and the least from erythrose and glucose (2–4 mol/mol of HSA in agreement with the reaction of 2–4 surface Lys). Noteworthy is the HSA–GlcN adduct which had a content of pyrrole slightly lower than that of HSA. In the preceding paper[1], 2-amino-2-deoxyglucose was shown to react strongly with lysine and to produce browning. Thus, it appears that browning can be associated with pyrrolic structures, but can also be generated by other types of presently unknown structures.

The results of i.e.f. of the adducts produced by incubation for 7 days at 37° and pH 8, on ultrathin polyacrylamide matrices in the pH range 4–7, are shown in Fig. 4. The microheterogeneous spectrum of bands (pI 4.5–5.5) for HSA–acetaldehyde (track a) can also be taken to represent control HSA, due to trace labelling, whereas glyceraldehyde and erythrose reacted with HSA to produce strongly acidic molecules, focusing between pH 4.0 and 4.5. Ribose and 2-amino-2-deoxyglucose extended the microheterogeneity of the HSA population, producing more acid as well as more basic species, with the mass of molecules still centred in the same pH interval (4.5–5.5). The reaction with glucose (track f) slightly reduced the microheterogeneity and shifted the zones slightly towards the lower pI values. Thus, when probing the net surface of HSA molecules, it can be seen that each aldose has a unique effect. There is a correlation between the pI of the modified HSA and the molecular weight of the reacting aldose, and species having the lowest pI were produced by glyceraldehyde.

TABLE II

PYRROLE CONTENT OF HSA AND THE ADDUCTS

Adducts	*Pyrrole groups (mol/mol of HSA)*	
	In H_2O	*In 9M urea*
HSA	32.1	27.8
HSA + D-glucose (20mM)	36.2	30.6
HSA + D-glucose + $NaCNBH_3$	35.8	34
HSA + 2-amino-2-deoxy-D-glucose (20mM)	23.2	24.5
HSA + 2-amino-2-deoxy-D-glucose + $NaCNBH_3$	26	26.5
HSA + D-ribose (20mM)	57.2	58.2
HSA + D-ribose + $NaCNBH_3$	62.5	61.2
HSA + D-erythrose (20mM)	34.2	23.2
HSA + D-erythrose + $NaCNBH_3$	46 2	34.5
HSA + DL-glyceraldehyde (20mM)	42.2	44.4
HSA + DL-glyceraldehyde (100mM)	53.2	60.8
HSA + DL-glyceraldehyde (200mM)	61.4	60
HSA + DL-glyceraldehyde (400mM)	61.6	62.8

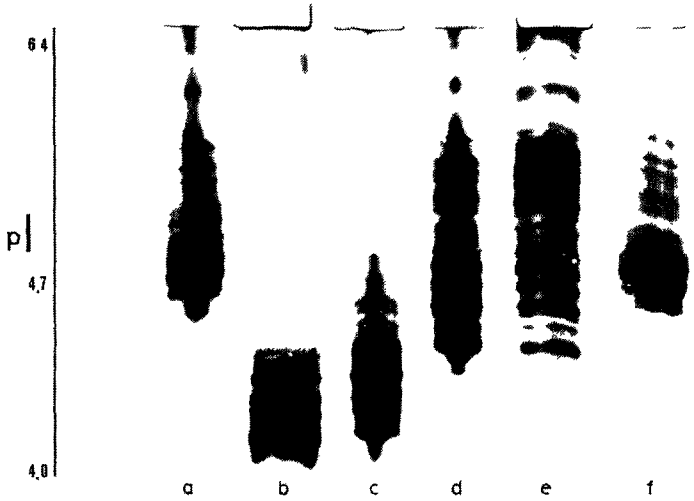

Fig 4 Ultrathin(240 μm)-layer i.e.f. in a non-linear Ampholine (pH range 4–8) of (a) HSA–acetaldehyde, (b) HSA–glyceraldehyde, (c) HSA–erythrose, (d) HSA–ribose, (e) HSA–GlcN, (f) HSA–glucose; 5000 Vh at 10°; staining with photochemical silver[9]; total sample load per track, 10 μg

DISCUSSION

The following salient points emerge. All of the aldoses from triose to hexose reacted with HSA, with glyceraldehyde being the most reactive, and even acetaldehyde effected trace labelling[14]. The ε-amino group of Lys is the most reactive group in HSA. Even trace labelling, such as with acetaldehyde (0.23 mol/mol of HSA when reduced) can induce conformational changes, as indicated by the fluorescence and c.d. spectra. The intrinsic fluorescence (emission peak and quantum yield) reflects the microenvironment of such aromatic amino acids as Tyr and Trp; HSA contains one Trp residue per 66,000 daltons which, together with the several Tyr residues, makes a small direct contribution to fluorescence[16]. Therefore, it is reasonable to assume that the modified fluorescence emission spectrum reflects changes in the microenvironment of the Trp. For the HSA–acetaldehyde adduct, the quantum yield of Trp fluorescence was reduced by ~30%, accompanied by a significant red shift of the emission peak (303 to 330 nm). The quenching was of the same order of magnitude as found[15] for the reaction of Lys-525 with glucose, but the shift in the emission peak, though similar, was in the opposite direction. This latter finding was taken to signify that the Trp microenvironment had become more hydrophobic. The α-helix content of a protein can be probed by c.d. analysis in the far u.v. The results for the modified HSA suggest a change in α-helix content which is independent of the extent of reaction. From the fluorescence and c.d. data, it is concluded that, whatever the structural change in the HSA, it occurs as soon as the first amino acid residue has been modified. Further reaction affects only the surface of the molecule, as reflected by the behaviour in isoelectric focusing.

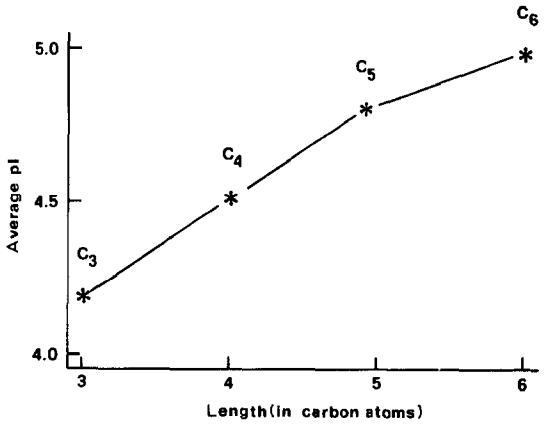

Fig. 5. Plot of average pI of HSA adducts (see Fig. 4) *vs.* the length of the carbon chain in the reacting sugar. The midpoints were obtained by integrating the total densitometric profile of all the bands in a given sample track and finding the pI value corresponding to a 50/50 distribution of absorbances along the pH axis.

Schiff's bases, formed by the reaction of free amino groups of proteins with sugars, can either undergo an Amadori rearrangement to give stable keto-amine derivatives which can then cyclise to hemiacetal structures[17,18] or can be reduced with borohydride to give stable, acyclic sugar alkylamines. In contrast to previous work[23] with hemoglobins, the u.v. and fluorescence spectra of these two types of product are different. The HSA adducts generate yellow products having marked absorption at 300–350 nm, with a corresponding strong fluorescence having λ_{max} 435 nm. The borohydride-reduced HSA adducts did not have these properties (see Figs. 1 and 2). There is also a marked difference in the overall surface charge of the two types of molecules, as detected[19,20] by gel i.e.f.

The browning of the HSA–glyceraldehyde adduct has been associated with pyrrole rings presumably connected by -CH=CH- links. The data in Table II accord with this concept, but a notable exception is 2-amino-2-deoxyglucose for which the number of pyrrole residues is slightly diminished but which can produce yellow products, presumably involving other, as yet unidentified, chromophores. This novel aspect of the browning reaction is being investigated further.

Inspection of Fig. 4 reveals a relationship between the pI at the midpoint of the spectrum of bands in each sample track and the length of the carbon chain of the aldehyde in the HSA adduct, a plot of which (Fig. 5) gives an almost linear relationship. The acidity of the HSA adduct could simply be due to the extent of reaction with the various aldehydes; glyceraldehyde, the most reactive aldehyde, produces the most acidic species. Other, secondary reactions could generate species of lower pI. Rearrangement or reduction of the product of reaction of the primary ε-amino groups with an aldehyde should produce a secondary amino group, which would have a different pK, but should be fully protonated at pH = pI. Therefore,

the acidity of the HSA adducts could either be produced by conformational transitions exposing buried carboxyl groups, or by secondary reactions occurring during the "Maillard ageing". The results of experiments involving 8M urea[19,20] tend to exclude the first hypothesis and the second one is partially corroborated by the detection of pyrrole rings in several of the adducts, which would be only partially protonated at pH = pI. Other possible reaction mechanisms are currently under investigation.

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