Chromatographic assay of glycation adducts in human serum albumin glycated *in vitro* by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate and intrinsic fluorescence

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Glycation of proteins leads to the formation of advanced glycation endproducts (AGEs) of diverse molecular structure and biological function. Serum albumin derivatives modified to minimal and high extents by methylglyoxal and glucose *in vitro* have been used in many studies as model AGE proteins. The early and advanced glycation adduct contents of these proteins were investigated using the 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC) chromatographic assay of enzymic hydrolysates. AGEs derived from methylglyoxal, glyoxal and 3-deoxyglucosone, the hydroimidazolones N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), N_{δ}-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1) and N_{δ}-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine (3DG-H1), bis(lysyl)imidazolium cross-links methylglyoxal-derived lysine dimer (MOLD), glyoxal-derived lysine dimer (GOLD), 3-deoxyglucosone-derived lysine dimer (DOLD), monolysyl adducts N_{ϵ}-(1-carboxyethyl)lysine (CEL), N_{ϵ}-carboxymethyl-lysine (CML) and pyrraline, other AGEs, N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)ornithine (THP), argpyrimidine and pentosidine, and fructosyl-lysine were determined. AGEs with intrinsic fluorescence (argpyrimidine and pentosidine) were assayed without derivatization. Human serum albumin (HSA) glycated minimally by methylglyoxal *in vitro* contained mainly MG-H1 with minor amounts of THP and argpyrimidine. Similar AGEs were found in prothrombin glycated minimally by methylglyoxal and in N_{α}-t-butyloxycarbonyl-arginine incubated with methylglyoxal. HSA glycated highly by methylglyoxal contained mainly argpyrimidine, MG-H1 and THP, with minor amounts of CEL and MOLD. HSA glycated minimally by glucose *in vitro* contained mainly fructosyl-lysine and CML, with minor amounts of THP, MG-H1, G-H1, 3DG-H1, argpyrimidine and DOLD. HSA glycated highly by glucose contained these AGEs and pyrraline, and very high amounts ($\approx$ 8 mol/mol of protein) of fructosyl-lysine. Most AGEs in albumin glycated minimally by methylglyoxal and glucose were identified. Significant proportions of arginine and lysine-derived AGEs in albumin modified highly by methylglyoxal, and lysine-derived AGEs in albumin modified highly by glucose, remain to be identified.

Key words: 3-deoxyglucosone, glucose, glycation, glyoxal, methylglyoxal.

INTRODUCTION

Glycation of proteins by glucose and other saccharide derivatives leads to the formation of fructosamines [1,2] and advanced glycation endproducts (AGEs) [3,4]. Formation of AGEs has been linked to the development of cataract [5], diabetic complications [6], uraemia [7], Alzheimer's disease [8] and other disorders [3]. The association of AGEs with disease is thought to reflect AGE-dependent changes in protein structure and function. These are (i) changes in protein structure arising from loss of lysyl, arginyl or N-terminal ionization, introduction of ionized AGE structures and changes in local hydrophobicity [9], (ii) resistance to proteolysis by AGE-mediated cross-linking [10] and (iii) binding to cell-surface receptors (AGE receptors) and other proteins (lysozyme, lactoferrin) specifically with associated cell activation or impairment of protein function (reviewed in [11]). To investigate these and other functional effects of glycation, model glycated proteins have been prepared *in vitro*.

Proteins have been glycated *in vitro* by glucose, methylglyoxal and other saccharide derivatives [12–14]. The extents of modification have been minimal (1–2 mol of glycating agent attached per mol of protein) to high (30–40 mol/mol of protein) [9,15]. Proteins have also been prepared that contain specific AGEs, such as N_{ϵ}-carboxymethyl-lysine (CML) and N_{ϵ}-(1-carboxyethyl)lysine (CEL), with minimal to high extents of modification; the N-carboxymethyl and N-carboxyethyl moieties were introduced into the protein chemically [16–18]. With glycation by saccharide derivatives, however, proteins contain two or more AGEs. AGEs are a group of arginine, lysine and N-terminal amino acid residue-derived molecules of diverse structure [3]. This structural diversity confers distinctive functional changes, typically multifunctional changes, on the glycated protein. To

Abbreviations used: AGE, advanced glycation endproduct; HSA, human serum albumin; AGE-HSA, HSA modified highly by glucose-derived glycation adducts; AGE_{min}-HSA, HSA modified minimally by glucose-derived glycation adducts; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate; CEL, N_{ϵ}-(1-carboxyethyl)lysine; CML, N_{ϵ}-carboxymethyl-lysine; 3DG-H1, N_{δ}-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine; DOLD, 3-deoxyglucosone-derived lysine dimer, 1,3-di(N^{ϵ}-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt; G-H1, N_{δ}-(5-hydro-4-imidazolon-2-yl)ornithine; G-H2, 2-amino-5-(2-amino-5-hydro-4-imidazolon-1-yl)pentanoic acid; GOLD, glyoxal-derived lysine dimer, 1,3-di(N^{ϵ}-lysino)imidazolium salt; MALDI-MS, matrix-assisted laser-desorption ionization MS; MG-H1, N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MG-H2, 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid; MG-H3, 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid; MG-HSA, HSA modified highly by methylglyoxal-derived glycation adducts; MG_{min}-HSA, HSA modified minimally by methylglyoxal-derived glycation adducts; MOLD, methylglyoxal-derived lysine dimer, 1,3-di(N^{ϵ}-lysino)-4-methyl-imidazolium salt; PR, human prothrombin; MG_{min}-PR, PR modified minimally by methylglyoxal-derived glycation adducts; THP, N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)ornithine; t-Boc, t-butyloxycarbonyl.

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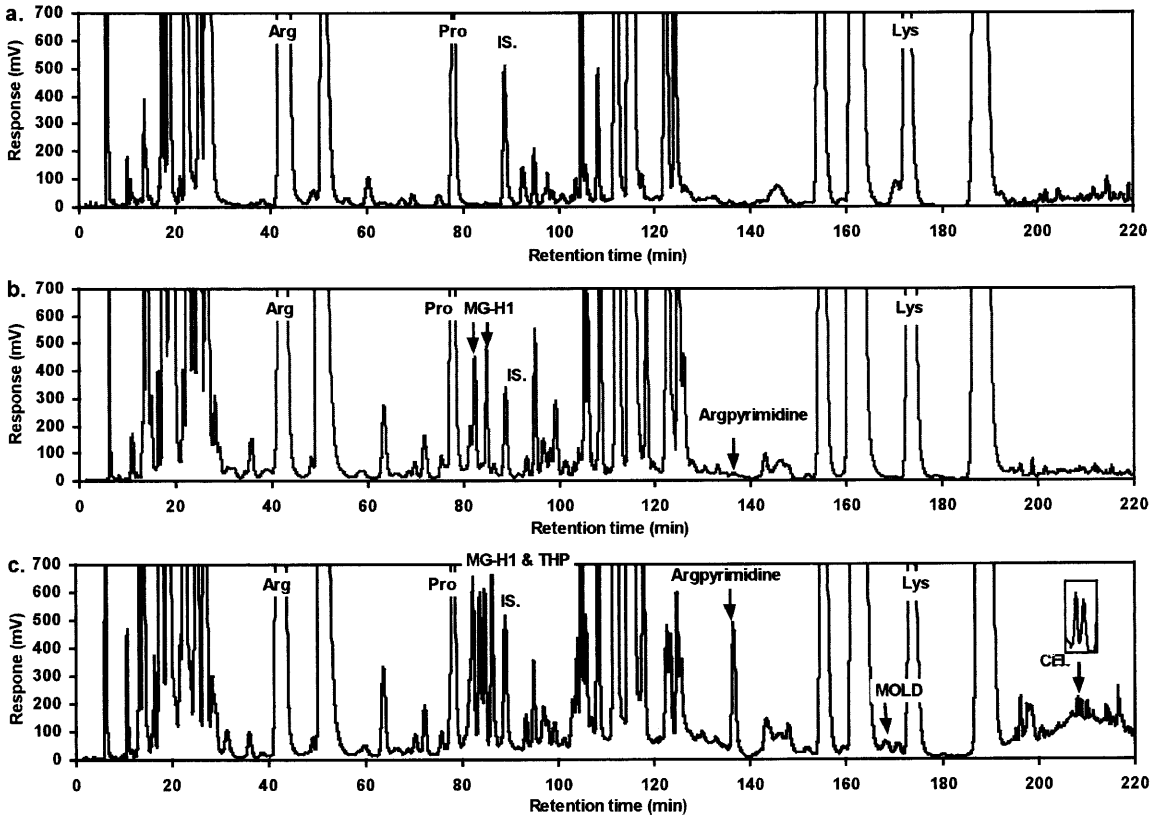

Figure 1 Chromatographic assay of AGEs

(**a**) HSA, (**b**) MG_{min}-HSA and (**c**) MG-HSA. Proteins (25 μg) were hydrolysed enzymically and hydrolysates were derivatized as described in the Materials and methods section. IS, internal standard.

correlate functional activity of glycated proteins with AGE epitopes and concentration, a survey of AGE content of glycated proteins is required.

In this report, we describe the application of the 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC) chromatographic assay of AGEs, and assay of AGEs with intrinsic fluorescence, to the measurement of AGEs in human serum albumin (HSA) glycated to minimal and high extents by methylglyoxal and glucose.

MATERIALS AND METHODS

Materials

HSA (fatty acid-free), human prothrombin (PR), pepsin (from porcine stomach mucosa; Sigma catalogue no. P6887), pronase E, prolidase, leucine aminopeptidase (type VI from porcine kidney) and N_{α}-t-Boc-arginine (where t-Boc is t-butyloxycarbonyl) were purchased from Sigma. Methylglyoxal, AQC, N_{ϵ}-(1-deoxy-D-fructos-1-yl)lysine (referred to as fructosyl-lysine) and AGEs were prepared as described in the accompanying article [17].

Preparation of proteins glycated by methylglyoxal and glucose

HSA glycated minimally by methylglyoxal-derived glycation adducts (MG_{min}-HSA) was prepared by incubation of methylglyoxal (500 μM) with HSA (6.6 mg/ml) in sodium phosphate buffer (100 mM, pH 7.4) at 37 °C for 24 h. A similar preparation of PR minimally modified by methylglyoxal-derived glycation adducts (MG_{min}-PR) was made. Unmodified protein controls were incubated without methylglyoxal and processed similarly. HSA modified highly by methylglyoxal-derived glycation adducts (MG-HSA) was prepared by incubation of HSA (7.2 mg/ml) with methylglyoxal (100 mM) in 100 mM sodium phosphate buffer, at pH 7.4 and 37 °C, for 24 h. Unmodified protein controls were incubated without methylglyoxal and processed similarly.

HSA modified minimally by glucose-derived glycation adducts (AGE_{min}-HSA) was prepared by incubation of HSA (50 mg/ml) with β-D-glucose (40 mM) in sodium phosphate buffer (150 mM, pH 7.4) at 37 °C for 5 weeks under aseptic conditions. Unmodified protein control was incubated without glucose and processed similarly. HSA modified highly by glucose-derived glycation adducts (AGE-HSA) was prepared by incubation of HSA (50 mg/ml) with β-D-glucose (1.67 M) in sodium phosphate buffer (100 mM, pH 7.4) at 37 °C for 60 days under aseptic conditions. Unmodified protein control was incubated without glucose and processed similarly.

After incubation, all proteins were dialysed against 30 mM ammonium formate, pH 7.8 and 4 °C, lyophilized to dryness and stored at −20 °C.

Model of the reaction of methylglyoxal with arginine residues in proteins

A model incubation of 500 μM methylglyoxal with 2.4 mM N_{α}-t-Boc-arginine in sodium phosphate buffer (100 mM, pH 7.4) at 37 °C for 24 h was performed, analogous to the methylglyoxal and arginine residue concentrations in the preparation of MG_{min}-HSA. An aliquot of this incubation (200 μl) was acidified to pH

1 with 2.2 M HCl (20 μl) and stirred overnight at room temperature. Of this, 1% (4.8 nmol of arginine residues) was derivatized with AQC and analysed as described for the protein AGE assay [17]. N_{α}-t-Boc-arginine and N_{α}-t-Boc-arginine-derived AGEs [N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)ornithine (THP), 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (MG-H2) and argpyrimidine] were used as calibration standards with identical acidic processing. The recoveries of these analytes were 55, 54, 54, 83 and 93%. 2-Amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (MG-H3) was not determined because of its instability during the chromatographic run.

Sample processing and assay of AGEs by the AQC and intrinsic fluorescence chromatographic methods

The protein concentration was determined by the Bradford method [19]. Protein samples were hydrolysed enzymically under nitrogen as described in [17]. The hydrolysate was then used in the assay of AGEs by the AQC and intrinsic fluorescence techniques. Aliquots of enzymic hydrolysate (50 μl, equivalent to 50 μg of protein) were derivatized by AQC and analysed by HPLC with fluorimetric detection. Argpyrimidine and pentosidine contents of the proteins were also determined without derivatization by HPLC with fluorimetric detection [17]. Only analyte concentrations higher than the statistical limit of detection are given.

RESULTS

Analysis of AGEs in HSA and PR modified minimally and highly by methylglyoxal

Analysis of AGEs by the AQC chromatographic method in unmodified HSA and PR gave evidence of low or undetectable levels of AGE analytes. A specimen chromatogram of the analysis of amino acids and glycation adducts in HSA is given in Figure 1(a), with an expansion of the section between 75 and 95 min is given in Figure 2(a). (Note that named analytes in the following description refer to AQC-analyte adducts.) Arginine eluted with threonine at $\approx$ 42 min; a repeat run of the initial 90 min with a modified gradient was required to resolve arginine and threonine, eluting at 28 and 32 min, respectively. Proline eluted at 77 min and the internal standard, α-aminobutyric acid, eluted at 89 min. Lysine eluted at 173 min. At this high sensitivity and high sample loading, many small analyte peaks can be seen between amino acid and internal-standard peaks. These many trace amino acid derivatives necessitated the extraordinary long run time for resolution. Chromatographic peaks were assigned to the following analytes under investigation: N_{δ}-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1), N_{δ}-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine (3DG-H1), MG-H1, THP, argpyrimidine and fructosyl-lysine (Tables 1–3). In HSA, there were generally low levels of MG-H1 and THP, although in one batch of HSA there was $\approx$ 0.5 mol of MG-H1/mol of HSA. Typically, however, there were higher levels of G-H1 and 3DG-H than all other AGEs (see below).

Analysis of the AGEs in MG_{min}-HSA indicated the presence of MG-H1 as the predominant AGE with minor formation of THP and argpyrimidine (Table 1 and Figures 1b and 2b). The two epimers of MG-H1 had retention times of $\approx$ 82 and 85 min, with the appearance of smaller peaks of the epimers of THP at $\approx$ 84 and 87 min. An unidentified interfering peak (retention time, 81 min) was generally found in the analysis of the proteins. This was incompletely resolved from AGE epimer $MG\text{-}H1_A$.

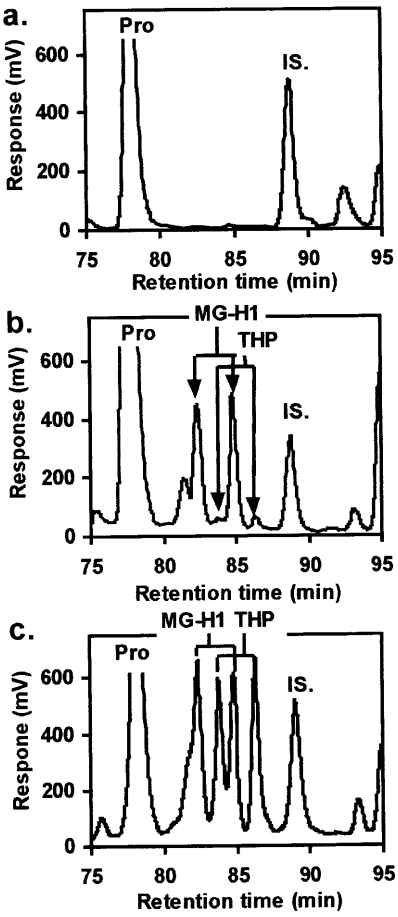

Figure 2 Enlarged sections of the chromatograms in the methylglyoxal hydroimidazolone-AQC-adducts region for the assay of AGEs in (a) HSA, (b) MG_{min}-HSA and (c) MG-HSA

Details are as described for Figure 1.

Since there was racemization of epimers of MG-H1 (the chromatographic peak areas of AGE epimers $MG\text{-}H1_A$ and $MG\text{-}H1_B$ were not significantly different), the chromatographic peak for $MG\text{-}H1_B$ was integrated for the quantification of MG-H1. Similarly, the second epimer peak of THP eluting at $\approx$ 87 min, THP_B, was integrated for the quantification of THP in AGE protein analysis. The chromatographic peaks of MG-H1 epimers were clearly evident in the chromatogram. There was no detectable formation of CEL or MOLD [methylglyoxal-derived lysine dimer, 1,3-di(N^{ϵ}-lysino)-4-methyl-imidazolium salt], nor glyoxal-derived AGEs, CML and GOLD [glyoxal-derived lysine dimer, 1,3-di(N^{ϵ}-lysino)imidazolium salt]. There was no significant decrease of lysine residues but there was a decrease of $\approx$ 2 arginine residues/mol in the formation of MG_{min}-HSA. This was accounted for mostly by the formation of MG-H1 and also the formation of THP and argpyrimidine. Similarly, the minimal modification of PR led to the formation of MG-H1, THP and argpyrimidine but there was no detectable CEL or MOLD. The formation of THP and argpyrimidine was more marked in MG_{min}-PR than in MG_{min}-HSA (Table 2).

The time course of formation of methylglyoxal-derived AGEs in MG_{min}-HSA (500 μM methylglyoxal and 100 μM HSA incubated at pH 7.4 and 37 °C for 1 day) was studied. The effect of methylglyoxal-derived AGE stability on AGE content was also

Table 1 Fructosyl-lysine, methylglyoxal-derived AGEs and related analytes in HSA glycated by methylglyoxal *in vitro*

Values for pentosidine were determined by the chromatographic assay of analytes with intrinsic fluorescence.

	HSA		MG_{min}-HSA		MG-HSA	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
Analyte (mol/mol of protein)						
MG-H1	0.04 ± 0.02	0.01–0.06	1.45 ± 0.04	1.41–1.49	1.92 ± 0.34	1.26–2.5
THP	0.03 ± 0.01	0.03–0.04	0.23 ± 0.03	0.2–0.27	1.72 ± 0.07	1.64–1.87
Argpyrimidine	0.014 ± 0.006	0.007–0.018	0.27 ± 0.03	0.11–0.3	3.19 ± 1.01	1.55–4.29
MOLD	< 0.02	–	< 0.02	–	0.57 ± 0.17	0.47–0.77
CEL	< 0.02	–	< 0.02	–	0.78 ± 0.16	0.49–1.09
Fructosyl-lysine	0.28 ± 0.17	0.09–0.43	0.16 ± 0.06	0.1–0.25	0.28 ± 0.15	0.13–0.49
Arginine	23.5 ± 2.5	20.0–26.8	22.2 ± 1.6	20.9–24.0	9.0 ± 1.3	7.1–10.8
Lysine	58.9 ± 2.1	55.3–61.6	58.6 ± 2.9	56.4–61.9	42.3 ± 1.6	40.5–44.4
Analyte (mmol/mol of protein)						
Pentosidine	0.25 ± 0.13	0.13–0.44	0.42 ± 0.06	0.35–0.48	0.77 ± 0.50	0.35–1.39

Table 2 Fructosyl-lysine, methylglyoxal-derived AGEs and related analytes in PR glycated by methylglyoxal *in vitro*

	PR		MG_{min}-PR	
Analyte (mol/mol of protein)	Mean ± S.D.	Range	Mean ± S.D.	Range
MG-H1	0.078 ± 0.044	0.05–0.14	1.54 ± 0.55	1.08–2.74
THP	0.17 ± 0.13	0.05–0.31	0.86 ± 0.19	0.65–1.01
Argpyrimidine	< 0.05	–	0.77 ± 0.3	0.5–1.1
MOLD	< 0.02	–	< 0.02	–
CEL	< 0.02	–	< 0.02	–
Fructosyl-lysine	0.29 ± 0.08	0.19–0.37	0.4 ± 0.13	0.31–0.54
Arginine	42.39 ± 3.9	37.9–45.1	32.1 ± 0.6	31.7–32.7
Lysine	29.2 ± 3.8	23.0–34.0	26.4 ± 1.2	25.1–27.2

Table 3 Fructosyl-lysine, methylglyoxal-derived AGEs and related analytes in HSA glycated by glucose *in vitro*

Values for pentosidine were determined by the chromatographic assay of analytes with intrinsic fluorescence. DOLD, 3-deoxyglucosone-derived lysine dimer, 1,3-di(N^{ε}-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt.

	HSA		AGE_{min}-HSA		AGE-HSA	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
Analyte (mol/mol of protein)						
G-H1	3.15 ± 2.2	0.74–7.65	3.88 ± 0.56	3.22–4.65	4.39 ± 1.84	2.35–6.22
3DG-H1	0.28 ± 0.1	0.22–0.39	0.75 ± 0.1	0.63–0.82	0.38 ± 0.28	0.04–0.66
MG-H1	0.04 ± 0.02	0.01–0.06	0.12 ± 0.11	0.01–0.27	0.42 ± 0.16	0.24–0.67
THP	0.03 ± 0.01	0.03–0.04	0.2 ± 0.15	0.06–0.36	0.21 ± 0.14	0.06–0.36
Argpyrimidine	0.014 ± 0.006	0.007–0.018	0.045 ± 0.03	0.016–0.085	0.076 ± 0.009	0.066–0.082
CML	< 0.03	–	1.21 ± 0.24	0.94–1.41	3.16 ± 1.66	1.7–6.39
DOLD	< 0.03	–	0.04 ± 0.02	0.02–0.07	0.25 ± 0.006	0.24–0.25
Pyrraline	< 0.02	–	< 0.01	–	0.17 ± 0.06	0.11–0.29
Fructosyl-lysine	0.28 ± 0.17	0.09–0.43	1.42 ± 0.62	0.82–2.28	7.76 ± 2.57	4.97–10.87
Arginine	23.5 ± 2.5	20.0–26.8	18.2 ± 2.3	16.8–20.8	17.1 ± 2.8	12.5–20.7
Lysine	58.9 ± 2.1	55.3–61.6	57.7 ± 3.4	54.1–61.0	16.9 ± 3.0	13.5–20.7
Analyte (mmol/mol of protein)						
Pentosidine	0.25 ± 0.13	0.13–0.44	3.45 ± 0.31	3.20–3.80	7.41 ± 0.99	6.84–8.56

studied by extending the incubation over a further 3 weeks (Figure 3a). MG-H1 formation occurred rapidly in the initial 6 h and attained a maximum value in the subsequent 18 h. Over the next 3 weeks, however, there was a further slow increase in MG-H1 concentration. THP was also formed in the initial 24 h of incubation and decreased thereafter. Argpyrimidine was formed only after 12 h of incubation and increased to a maximum after 24 h.

It was of interest to see if the same AGEs could be detected by incubation of methylglyoxal with N_{α}-t-Boc-arginine at a concentration equivalent to that of arginine residues in 100 μM HSA. Therefore, 500 μM methylglyoxal was incubated with 2.4 mM N_{α}-t-Boc-arginine under the same conditions used for the preparation of MG_{min}-HSA; the t-Boc group was removed by acid hydrolysis prior to AGE analysis. A time course of chromatograms indicating the formation of MG-H1 and THP is

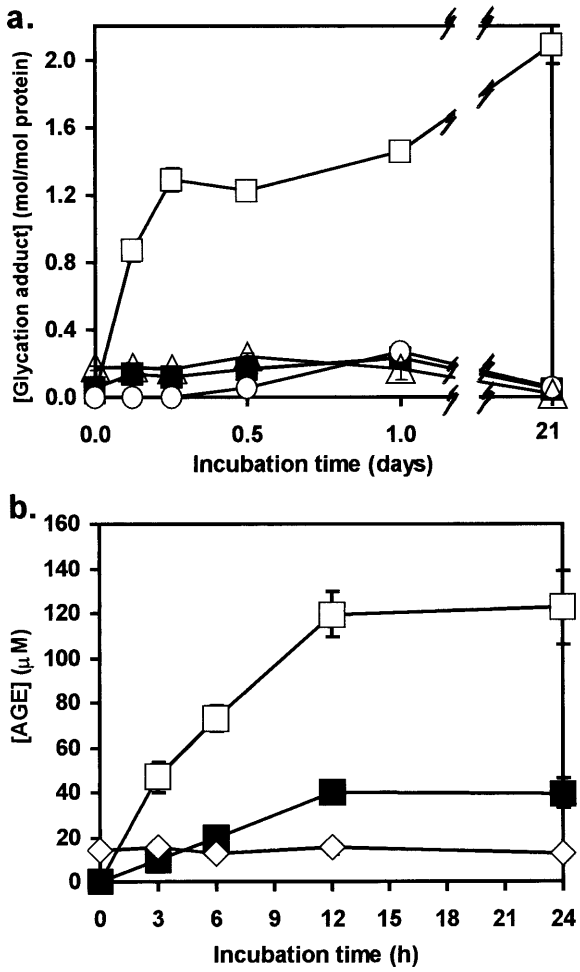

Figure 3 The time course of formation of AGEs in MG_{min}-HSA and N_{α}-t-Boc-arginine modified by methylglyoxal

(**a**) Formation of MG_{min}-HSA. The incubation contained 100 μM methylglyoxal with 500 μM HSA in 100 mM sodium phosphate buffer, pH 7.4 and 37 °C. (**b**) Glycation of N_{α}-t-Boc-arginine by methylglyoxal. The incubation contained 100 μM methylglyoxal with 2.4 mM N_{α}-t-Boc-arginine in 100 mM sodium phosphate buffer, pH 7.4 and 37 °C. At the times indicated, aliquots were removed from the incubation, washed (MG_{min}-HSA only), hydrolysed and assayed for AGEs. □, MG-H1; ■, THP; ○, argpyrimidine; ◇, MG-H2; △, fructosyl-lysine. Data are means ± S.D. from three determinations.

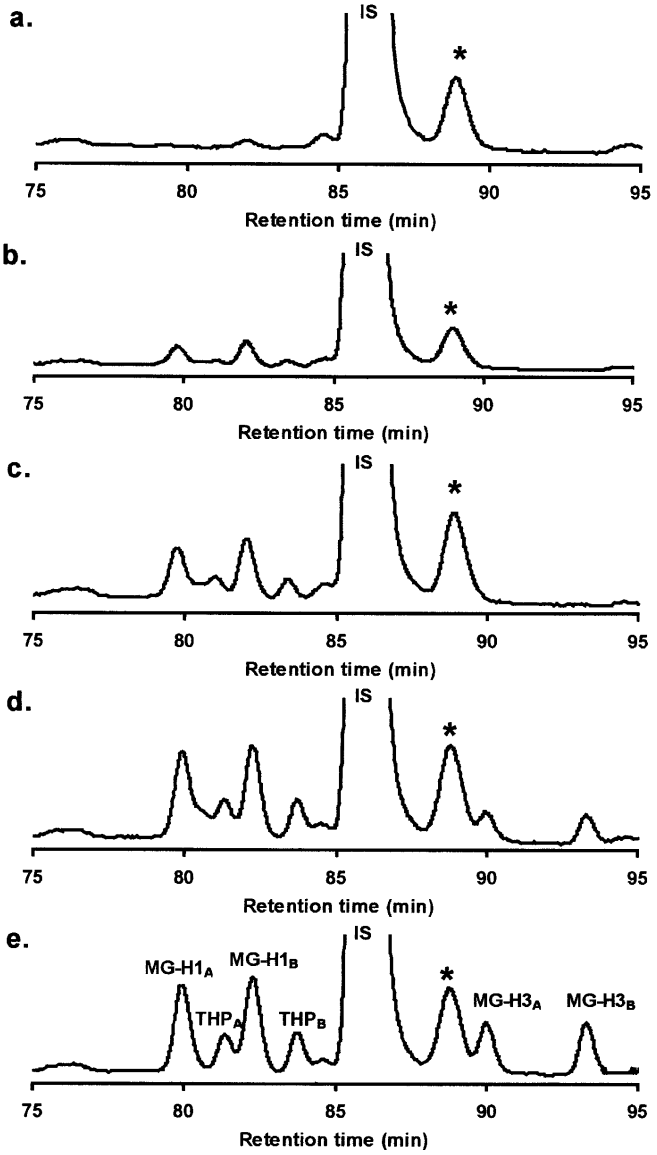

Figure 4 Sections of chromatograms of the analysis of AGEs formed by the reaction of methylglyoxal with N_{α}-t-Boc-arginine

Samples were taken at (**a**) 0 h, (**b**) 3 h, (**c**) 6 h, (**d**) 12 h and (**e**) 24 h. Methylglyoxal-derived AGE epimers are indicated in (**e**). * is an unidentified degradation product of AQC. IS, internal standard.

given in Figure 4. There was also a small, constant amount of MG-H2 present in the N_{α}-t-Boc-arginine throughout the incubation. The chromatograms shown were run immediately after AQC derivatization and also indicate the presence of MG-H3, although because this AQC adduct was unstable [17] its concentration was not deduced. Argpyrimidine was not detected. The time course of MG-H1 and THP formation over the 24 h incubation is shown in Figure 3(b). The concentrations of MG-H1 and THP reached maximum values after 12 h, at 123 ± 16 and 40 ± 7 μM, respectively. The arginine concentration decreased from 2.4 to 2.254 ± 0.088 mM, a decrease of 146 μM.

MG-HSA contained increased amounts of MG-H1 and there were also detectable levels of MOLD and CEL at retention times of 169 and 209 min. The most striking difference with the high modification of HSA by methylglyoxal, however, was the presence of high contents of THP epimers (retention times, 84 and 87 min) and argpyrimidine (retention time, 137 min; Figures 1c and 2c). The decrease of arginine residues in MG-HSA was ≈ 16 mol/mol of protein. The total methylglyoxal adducts detected (MG-H1, THP + argpyrimidine) was 6.8 mol/mol of protein. The decrease of lysine residues in MG-HSA was ≈ 17 mol/mol of protein. The total modification of lysine residues measured was 1.9 mol/mol of protein (CEL + 2 × MOLD; Table 1).

Argpyrimidine and pentosidine were assayed without derivatization by HPLC with fluorimetric detection. HSA had 6.08 mmol of argpyrimidine/mol of protein and 0.25 mmol of pentosidine/mol of protein, although this varied from batch to batch (the ranges were 2–10 mmol of argpyrimidine/mol of protein and 0.03–0.5 mmol of pentosidine/mol of protein). Glycation of HSA by methylglyoxal increased argpyrimidine content significantly ($P < 0.001$) but not pentosidine content ($P > 0.05$). In MG_{min}-HSA and MG-HSA, there was 176 ± 2 and

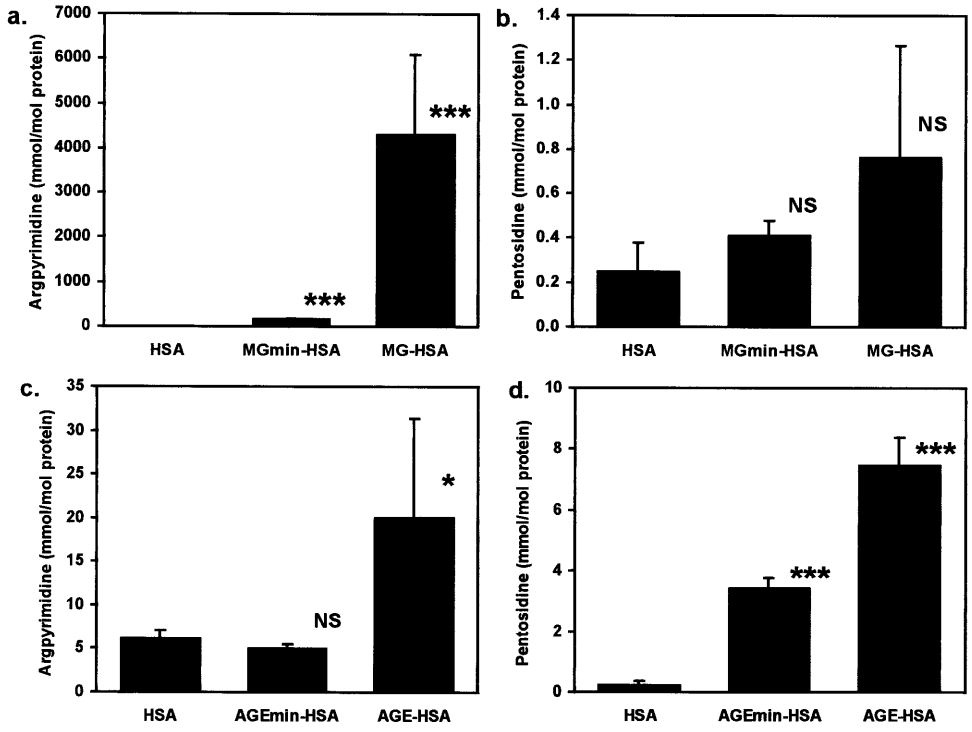

Figure 5 Chromatographic assay of AGEs with intrinsic fluorescence in HSA modified by methylglyoxal and glucose *in vitro*

(**a**, **b**) Methylglyoxal-modified HSA; (**c**, **d**) glucose-modified HSA. $^{*}P < 0.05$ and $^{***}P < 0.001$ with respect to HSA; NS, not significant with respect to HSA.

4322 ± 1765 mmol/mol of protein, respectively (Figures 5a and 5b). These estimates were in reasonable agreement with the argpyrimidine content determined by the AQC method (Table 1). Incubation of methylglyoxal (500 μM) with 100 μM HSA in the preparation of MG_{min}-HSA for 3 weeks, instead of the usual 24 h, led to a small decrease in argpyrimidine content (127 ± 37 versus 176 ± 2 mmol/mol of protein respectively, $n = 4$) which was not significant ($P > 0.05$).

HSA modified minimally and highly by glucose

Analysis of AGEs in HSA by the AQC chromatographic method gave evidence of low or undetectable levels of methylglyoxal-derived AGE analytes (described above) and higher contents of G-H1 and 3DG-H1. A specimen complete chromatogram with fluorescence detection is given in Figure 6, with an expansion of the chromatogram in the retention interval 70–120 min in Figure 7(a) (fluorescence detection) and in the retention interval 70–125 min in Figure 7(d) (absorbance detection). Absorbance detection was particularly beneficial in the resolution of CML from neighbouring peaks [see the CML peaks in Figures 7a–7c (fluorescence detection) and Figures 7d–f (absorbance detection) of the analysis of HSA, AGE_{min}-HSA and AGE-HSA, respectively]. Analysis for G-H1 and 3DG-H1 (retention times $\approx$ 56 and 59 min, respectively) indicated relatively high contents of these analytes and highly variable amounts of G-H1 (Figure 6a and Table 3). The marked variability of G-H1 content was due to differences in G-H1 content in commercial batches of HSA. This suggests that hydroimidazolones G-H1 and 3DG-H1 are major AGEs in purified proteins; they were also present at similar levels in prothrombin.

AGE_{min}-HSA contained increased levels of fructosyl-lysine and CML with smaller increases in hydroimidazolones G-H1, 3DG-H1 and MG-H1, argpyrimidine and DOLD [3-deoxyglucosone-derived lysine dimer, 1,3-di(N^{ϵ}-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt], with respect to unmodified HSA (Figures 6b, 7b and 7e, and Table 3). Similar AGEs and also pyrraline were detected in the analysis of AGE-HSA (Figures 6c, 7c and 7f, and Table 2). With the increasing extent of modification of HSA by glucose in AGE-HSA, the most marked changes in the analytes measured were (i) an increase in the fructosyl-lysine peak (retention time, $\approx$ 100 min), (ii) an increase in the CML peak ($\approx$ 116 min), (iii) the appearance of MG-H1 and THP epimer peaks in retention interval 80–84 min and (iv) a major decrease in the lysine content. In AGE-HSA, glyoxal-derived AGEs (CML and G-H1), methylglyoxal-derived AGEs (MG-H1, THP and argpyrimidine) and 3-deoxyglucosone-derived AGEs (3DG-H1, DOLD and pyrraline) were all detected.

The decrease in arginine content in AGE_{min}-HSA, relative to HSA, was $\approx$ 6 residues/mol of protein and the AGEs analysed accounted for $\approx$ 5 mol/mol of protein of this. The lysine content of AGE_{min}-HSA decreased by $\approx$ 1–2 mol/mol of protein and fructosyl-lysine and CML accounted for most of this. In AGE-HSA, the decrease in arginine content was $\approx$ 7 arginine residues/mol of protein and the AGEs analysed accounted for $\approx$ 6 mol/mol of protein. The lysine content of AGE-HSA decreased by $\approx$ 48 mol/mol of protein but the fructosyl-lysine and lysine-derived AGEs assayed only accounted for 12 mol/mol of protein.

Argpyrimidine and pentosidine in AGE_{min}-HSA and AGE-HSA were also assayed by HPLC with fluorimetric detection without derivatization. Glycation by glucose in AGE_{min}-HSA did not change argpyrimidine content significantly but increased

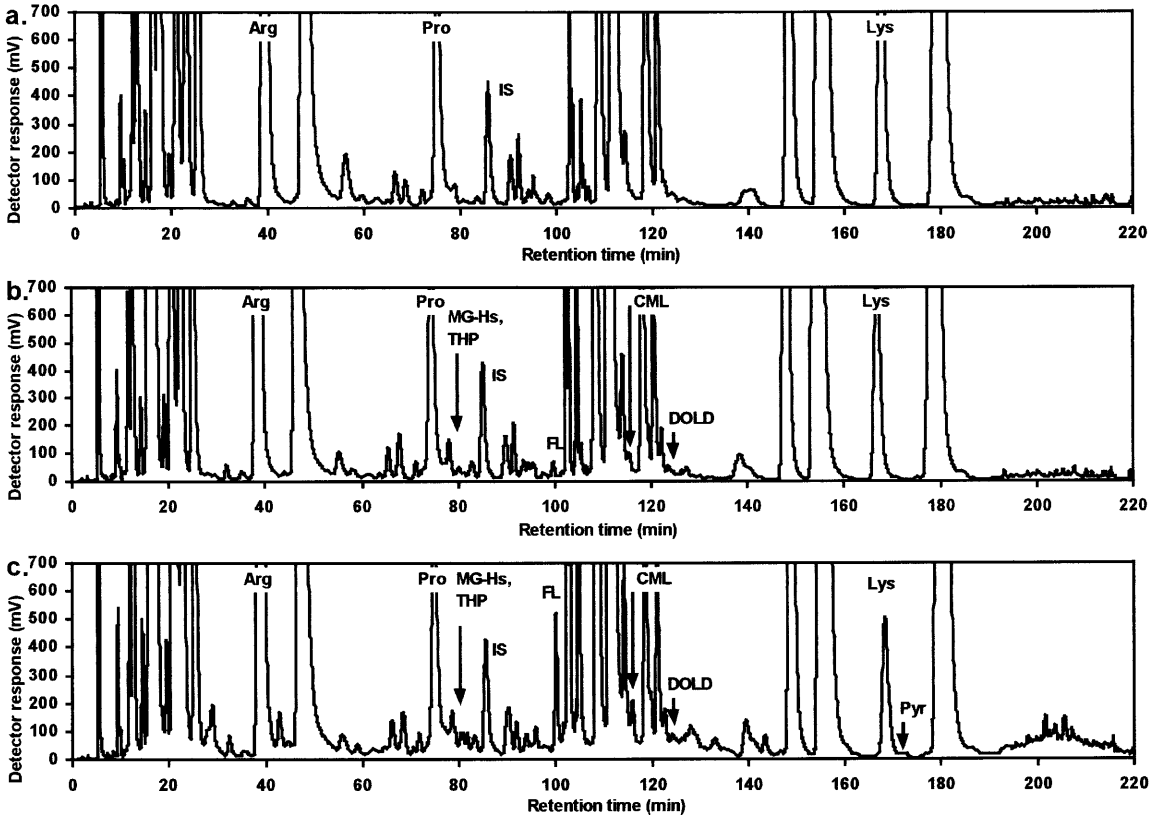

Figure 6 Chromatographic assay of AGEs

(**a**) HSA, (**b**) AGE_{min}-HSA and (**c**) AGE-HSA. Proteins (25 μg) were hydrolysed enzymically and hydrolysates derivatized as described in the Materials and methods section. IS, internal standard; DOLD, 3-deoxyglucosone-derived lysine dimer, 1,3-di(N^{ϵ}-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt; FL, fructosyl-lysine.

pentosidine content $\approx$ 13-fold. However, in AGE-HSA, argpyrimidine content increased $\approx$ 3-fold (6.2 ± 0.83 compared with 20.1 ± 11.3 mmol/mol of AGE-HSA; $P < 0.05$) and pentosidine significantly increased by $\approx$ 29-fold (0.25 ± 0.13 compared with 7.41 ± 0.99 mmol/mol of AGE-HSA; $P < 0.001$; Figures 5c and 5d).

Changes in molecular mass of proteins glycated *in vitro*

The changes in molecular mass of HSA glycated by methylglyoxal and glucose *in vitro* were investigated by matrix-assisted laser-desorption ionization MS (MALDI-MS). Some of these data were presented in a previous study of molecular-mass changes of albumin in uraemia [15]. MALDI-MS of HSA and glycated derivatives prepared *in vitro* gave two peaks with mass/charge ratios identifiable as the double- and mono-charged molecular ions. The mass range of all proteins was relatively small. There was no evidence that a small fraction of HSA derivative was glycated highly and the remainder unglycated. The mass increase of highly glycated proteins, MG-HSA and AGE-HSA, was large; 3671 and 6780 Da, respectively (Table 4).

DISCUSSION

Application of the AQC assay of AGEs to proteins glycated *in vitro*

The AQC method for assay of AGEs was deployed herein for the analysis of AGEs in HSA modified by methylglyoxal. The long analysis time (220 min) gave rise to some variation between studies in retention times for the long-retained analytes. The relative retention times were reproducible and comparison of these and other controls [17] permitted analyte identification. The advantages of this method over previous estimates of AGEs are (i) a comprehensive survey of contents of non-fluorescent and fluorescent glycation adducts and (ii) estimation of acid-labile glycation adducts. Commercial batches of HSA were found to contain a high and variable amount of G-H1. AQC derivatives of glyoxal hydroimidazolones G-H1 and 2-amino-5-(2-amino-5-hydro-4-imidazolon-1-yl)pentanoic acid (G-H2) co-eluted and so the quantification of G-H1 may be unreliable, although we expect similar sensitivities of detection for G-H1 and G-H2. Other AGEs were generally of a very low level although small amounts of 3DG-H1, MG-H1, THP and argpyrimidine could be detected, as well as some fructosyl-lysine.

HSA modified by methylglyoxal *in vitro*

The modification of HSA minimally with methylglyoxal in MG_{min}-HSA led to the formation mainly of MG-H1, and small amounts of THP and argpyrimidine. MOLD and CEL were below the limits of detection; although detected in proteins *in vivo* [20,21], they are minor products of glycation of proteins by methylglyoxal. When MG_{min}-HSA was prepared with 2-[^{14}C]methylglyoxal, 2.3 ± 0.3 mol of methylglyoxal-derived radiolabel/mol of protein were incorporated [12]. This agrees well with the decrease in arginine residues in MG_{min}-HSA found herein. The methylglyoxal residue content of MG_{min}-HSA due to MG-H1, THP and argpyrimidine was $1.41 + (2 \times 0.2) + (2 \times 0.13) = 2.07$/mol of protein (THP and argpyrimidine being formed from two methylglyoxal residues). The AGEs measured, there-

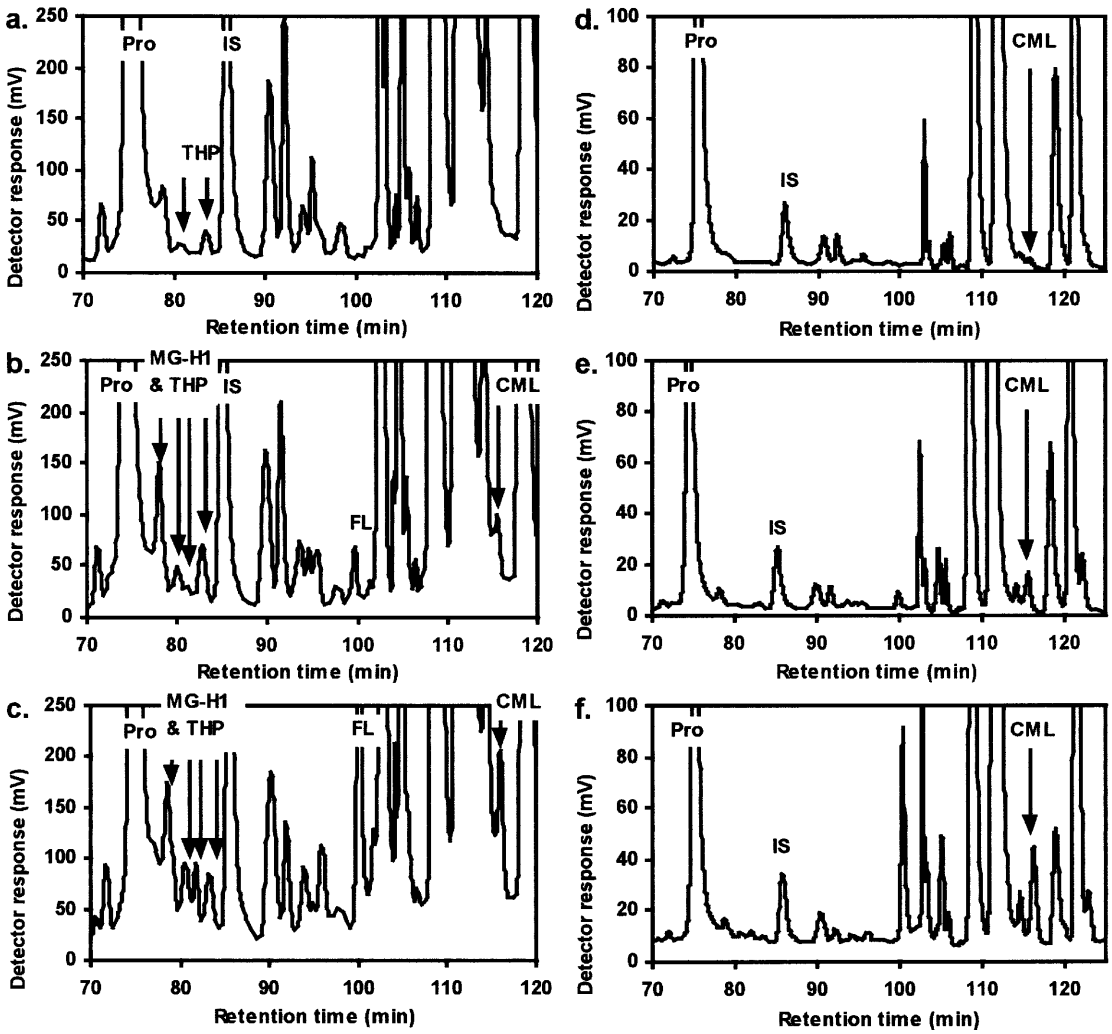

Figure 7 Sections of the chromatograms in the AQC chromatographic assay of AGEs in HSA (a, d), AGE_{min}-HSA (b, e) and AGE-HSA (c, f)

(**a**–**c**) Fluorescence detection and (**d**–**f**) absorbance detection. IS, internal standard.

Table 4 Molecular mass of HSA glycated minimally and highly by methylglyoxal and glucose

Protein	Molecular mass (Da; mean ± S.D.)	Mass increment (relative to HSA)	Significance
HSA	66446 ± 114	–	–
MG_{min}-HSA	66519 ± 121	73	Not significant, $P > 0.05$
MG-HSA	70117 ± 120	3671	$P < 0.001$
AGE_{min}-HSA	66845 ± 88	399	$P < 0.001$
AGE-HSA	73226 ± 122	6780	$P < 0.001$

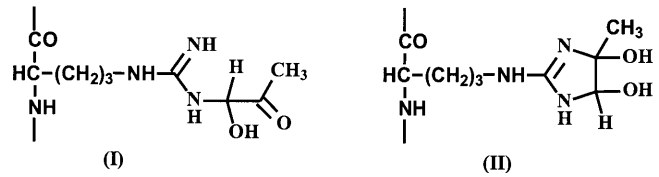

Figure 8 Structures of the glycosylamine N_{ω}-(1-hydroxy-2-oxopropyl)arginine (I) and the imidazolidine N_{δ}-(4,5-dihydroxy-4-methylimidazolidin-2-yl)ornithine (II)

fore, account for all of the modification of arginine residues in MG_{min}-HSA. This was also found for MG_{min}-PR, where total glycation adducts were 4.4 mol of methylglyoxal-derived radiolabel/mol of protein; and use of 2-[^{14}C]methylglyoxal in the preparation of MG_{min}-PR indicated an incorporation of 4.1 methylglyoxal residues/mol of protein [12]. MALDI-MS of MG_{min}-HSA showed a small increase in mass only, relative to HSA. Our previous HPLC gel filtration, SDS/PAGE and isoelectric-focusing studies of MG_{min}-HSA were consistent with this [9]. This minimal glycated HSA derivative is similar in extent of modification to HSA glycated *in vivo* [15]. The time course of formation of methylglyoxal-derived AGEs in MG_{min}-HSA indicated that MG-H1 and THP were the initial adducts formed and that argpyrimidine formation occurred later. This is consistent with the degradation of THP to argpyrimidine [17]. MG-H1 and THP formation was relatively rapid, mostly occurring within 24 h.

The reaction of methylglyoxal with HSA to form MG_{min}-HSA was modelled by the incubation of N_{α}-t-Boc-arginine with methylglyoxal. The formation of MG-H1 occurred to a similar extent to that in MG_{min}-HSA, although the initial rate of formation was approximately twice as fast with HSA as with N_{α}-t-Boc-arginine. This suggests that there are arginine residues in HSA activated for reaction with methylglyoxal. The formation of THP was increased in the N_{α}-t-Boc-arginine incubation but there was no formation of argpyrimidine; there may be catalysts for the conversion of THP into argpyrimidine in HSA (trace redox active metal ions, for example). In the N_{α}-t-Boc-arginine incubation, the reaction terminated after forming $\approx 150\ \mu$M equivalents of AGE adducts, accounting for 203 μM methylglyoxal. Most of the N_{α}-t-Boc-arginine remained unmodified. It is unlikely that this was due to the attainment of equilibrium between methylglyoxal and the glycation adducts since the half-life of MG-H1 is ≈ 12 days [17]. Rather, the incubation had become depleted of methylglyoxal because of (i) MG-H3 adduct formation, which may be similar in amount to MG-H1, (ii) spontaneous degradation of methylglyoxal, estimated to be $\approx 40\ \mu$M over 24 h in this incubation [22], and (iii) formation of reversible adducts which were lost during sample processing, the glycosylamine N_{ω}-(1-hydroxy-2-oxopropyl)arginine (structure I in Figure 8) and the imidazolidine N_{δ}-(4,5-dihydroxy-4-methylimidazolidin-2-yl)ornithine (structure II in Figure 8) [23]. Involvement of these processes in the formation of MG_{min}-HSA may explain the termination of the glycation after ≈ 6 h. Subsequent increased formation of MG-H1 over 3 weeks may arise by isomerism of the unstable MG-H3 to the more stable MG-H1. Consistent with this, the incubation of 100 μM HSA with 100 μM methylglyoxal lead to the formation of only 0.47 methylglyoxal-derived AGEs formed irreversibly/mol of protein [12].

The formation of MG-H1 and MG-H3 occurred preferentially over that of MG-H2. MG-H1 suffers the least steric hindrance from the δ-ornithyl substituent, and the initial reaction encounter to form MG-H3 involves the most nucleophilic nitrogen atom (N_{δ}) of the arginyl guanidino group reacting with the most reactive carbonyl group in methylglyoxal (the aldehyde group). MG-H2 was detected in glycated proteins in some instances; in human lens proteins *in vivo*, for example, where glycation over years achieves thermodynamic equilibrium between all three isomeric forms.

In MG-HSA, there was a further increase in the amount of MG-H1, a marked accumulation of THP and argpyrimidine, and detectable formation of MOLD and CEL. The total methylglyoxal-derived AGEs detected (≈ 7 mol of arginine-derived adducts and 2 mol of lysine-derived adducts/mol of protein) accounted for only $\approx 50\,\%$ of the arginine modification and $\approx 10\,\%$ of the lysine modification. Therefore, further methylglyoxal-derived AGEs remain to be discovered in MG-HSA. Alternatively, this protein may be resistant to enzymic hydrolysis giving a poor recovery of amino acids. The molecular mass increase of MG-HSA, relative to HSA, was 3671 Da, which corroborates well with previous estimates by SDS/PAGE [9]. Modification by MG-H1, THP, argpyrimidine, CEL and MOLD combined represents a mass increase of ≈ 540 Da. The additional mass increment is attributed to the modification of 23 arginine and lysine groups and oxidation of susceptible amino acids. Degradation of 100 mM methylglyoxal forms significant amounts of hydrogen peroxide [22]. MG-HSA is a markedly different protein to MG_{min}-HSA, with a high net negative charge [9]. It is not a good model for proteins glycated *in vivo*, although there may be similar extents of protein glycation in some foodstuffs [24].

The arginine residues modified in HSA in the formation of MG_{min}-HSA are unknown. Two reactive arginine residues have been identified in HSA: Arg-218 in ligand-binding site I (subdomain 2A) and Arg-410 in site II (subdomain 3A) [25,26]. They are highly conserved residues and are 'gatekeepers' of the ligand-binding domains, decreasing the affinity of drugs and other ligands. Arg-410 is also associated with the esterase activity of HSA. Modification of these residues by methylglyoxal is likely to lead to a loss of positive charge on the arginyl moiety and a loss of esterase activity [27]. The concentration of MG-H1 in HSA *in vivo* was below the limit of detection by the AQC chromatographic assay although it has been detected by immunoassay [28]. We estimate that $< 1\,\%$ of HSA is modified by MG-H1 *in vivo*. Hence, methylglyoxal modification of HSA *in vivo* is unlikely to have a marked effect on HSA esterase activity but it may change drug-/ligand-binding affinity of a small proportion of the available binding sites.

HSA modified by glucose *in vitro*

Preparation of AGE_{min}-HSA led to small increases in 3DG-H1, MG-H1, THP, argpyrimidine and DOLD contents and more substantial increases of CML, fructosyl-lysine and G-H1. A similar increase in fructosyl-lysine content had been seen previously by the fructosamine test [29]. AGE_{min}-HSA had a molecular mass increase of 399 Da, relative to HSA. Fructosyl-lysine and CML accounted for ≈ 220 Da of this, and for the decrease of ≈ 2 residues of lysine/mol of AGE_{min}-HSA. Pentosidine content increased ≈ 13-fold in the formation of AGE_{min}-HSA but only to a concentration of ≈ 4 mmol/mol of AGE_{min}-HSA. The arginine-derived AGEs measured herein accounted for most (80 %) of the arginine residues modified, or ≈ 5 mol/mol of protein. Hence, most of the glycation adducts in AGE_{min}-HSA were identified. Interestingly, when AGE_{min}-HSA was prepared with 1-[^{14}C]glucose only 2 mol of radiolabel/mol were incorporated [12]. Since 1 mol is due to fructosyl-lysine, most of the remaining 6 mol of AGEs in AGE_{min}-HSA must not have contained the 1-[^{14}C] label. This suggests that glucose fragmentation in the early stages of glycation [29] is a major reaction pathway in the glycation of proteins to a minimal extent.

AGE analysis of AGE-HSA showed increased contents of the same AGEs found in AGE_{min}-HSA, except for 3DG-H1, and the additional presence of pyrraline. The content of pentosidine in AGE-HSA increased by ≈ 29-fold but was still only ≈ 8 mmol/mol of AGE-HSA. The content of fructosyl-lysine was particularly high, ≈ 8 mol/mol of AGE-HSA, which suggests that AGE-HSA contains a high level of early glycation

adduct. CML, G-H1 and argpyrimidine were the major AGEs in AGE-HSA. Most of the decrease in arginine residues in AGE-HSA could be accounted for by the AGEs assayed but $\approx 75\%$ of the decrease in lysine residues (36 residues) could not. The mass increase of AGE-HSA with respect to HSA was 6780 Da: fructosyl-lysine residues accounted for ≈ 1300 Da, and AGEs assayed (MG-H1, THP, argpyrimidine, CEL, DOLD, pyrraline, CML and G-H1) for ≈ 1600 Da. The remaining mass increase unaccounted for is 3880 Da. This is on 36 modified lysine residues with unidentified modification; a mean mass increment/lysine residue of ≈ 108 Da. Furosine residues (mass increment, 108 Da) and similar structures arising from Schiff's base and fructosamine degradation are probable structures of the unidentified lysine-derived AGEs. Future work should look for the unidentified lysine-derived AGEs and investigate how these are related to the functional activities of AGE-HSA.

AGEs in HSA glycated *in vitro* to minimal and high extents by methylglyoxal and glucose

AGEs derived from methylglyoxal are present in HSA modified by methylglyoxal and modified by glucose, as we predicted [29]. There are significant amounts of fructosyl-lysine in AGE_{min}-HSA and AGE-HSA. The glycation adducts in HSA modified minimally by methylglyoxal and glucose can be accounted for by adducts measured herein, although there are small amounts of other AGEs present below the limit of detection of the AQC assay (CEL, for example, detected by CEL-specific ELISA [30]). HSA modified highly by methylglyoxal and glucose contained the same AGEs and additional unidentified AGEs compared with those in minimally modified proteins [15]. In minimally glycated HSA, CML and hydroimidazolones were quantitatively the predominant AGEs. There is a suggestion that both of these AGEs are associated with specific AGE-receptor binding and cell activation [12,18,31]. They may be important determinants of functional activity of glycated proteins in physiological systems and will be the focus of future investigations.

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