Proteomic Analysis of Arginine Adducts on Glyoxal-modified Ribonuclease*

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Accumulation of advanced glycation end-products (AGEs) on proteins is associated with the development of diabetic complications. Although the overall extent of modification of protein by AGEs is limited, localization of these modifications at a few critical sites might have a significant effect on protein structure and function. In the present study, we describe the sites of modification of RNase by glyoxal under physiological conditions. Arg39 and Arg85, which are closest to the active site of the enzyme, were identified as the primary sites of formation of the glyoxal-derived dihydroxyimidazolidine and hydroimidazolone adducts. Lower amounts of modification were detected at Arg10, while Arg33 appeared to be unmodified. We conclude that dihydroxyimidazolidine adducts are the primary products of modification of protein by glyoxal, that Arg39 and Arg85 are the primary sites of modification of RNase by glyoxal, and that modification of arginine residues during Maillard reactions of proteins is a highly selective process. Molecular & Cellular Proteomics 3: 1145–1153, 2004.

Glucose and its oxidative degradation products, including glyoxal (1–3), are able to modify reactive side chains of amino acids in proteins under physiological conditions to form a diverse group of protein-bound adducts known as advanced glycation end-products (AGEs).1 Such reactions, known as Maillard or browning reactions, are accelerated during hyperglycemia in diabetes, and increased chemical modification of proteins by glucose is implicated in the pathogenesis of long-term diabetic complications, including vascular and renal disease and blindness.

Glyoxal and glycolaldehyde are products of autoxidation of glucose or glucose adducts to proteins. Other carbohydrates, such as fructose, arabinose, and ascorbate, may also degrade to glyoxal, possibly through intermediate adducts to protein. Glyoxal may also be formed directly during oxidative degradation of polyunsaturated fatty acids (4) and during myeloperoxidase-mediated degradation of serine at sites of inflammation (5). Plasma glyoxal levels are much lower than those of glucose, but glyoxal is a far more reactive carbonyl compound. Normal plasma levels of glyoxal are reported to be 215–230 nM (6–8) but increase to 350–470 nM in diabetic subjects (7, 8), ~400 nM in uremia (6), and ~760 nM in end-stage renal disease (6). Because of its high reactivity, the fraction of glyoxal bound to proteins may significantly exceed the measured glyoxal concentration in plasma.

Glyoxal is able to modify the side chains of various amino acids in protein, including those of lysine and arginine, to form several products, such as N′-(carboxymethyl)lysine (CML (9)) and N′-(carboxymethyl)arginine (CMA (10)), glyoxal-derived dihydroxyimidazolidinones (G-DHs; G-DH1 and G-DH2) and N′-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1), and its isomers 5-(2-amino-5-hydro-4-imidazolon-1-yl)ornithine (G-H2) and 5-(2-amino-4-hydro-5-imidazolon-1-yl)ornithine (G-H3 (11)) (Fig. 1).

Little is known about the relative rates of formation of CML, glyoxal-derived dihydroxyimidazolines (G-DHs), glyoxal-derived hydroimidazolones (G-Hs) or CMA, or the specificity of modification of proteins by glyoxal. G-Hs and CMA would both be formed via G-DH1 or its isomer 5-(4,5-dihydroxy-2-imino-1-imidazolidinyl)ornithine (G-DH2; Fig. 1).

In previous work (11) on chemical modification of the model protein RNase by glyoxal, we concluded that CML was formed primarily by oxidation of Amadori adducts of glucose to protein and that free glyoxal, which was also formed in the reaction system, was not a significant precursor of CML. In the present study, we extend this work to analysis of the sites and products of modification of RNase by glyoxal. We show that, like the carboxymethylation of RNase, formation of G-DH and G-H in the RNase-glyoxal incubations is a site-specific process and that Arg39 and Arg85, which are the arginine residues closest to the active site of RNase, are the primary

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* The abbreviations used are: AGE, advanced glycation end-product; AG, aminoguanidine; CMA, N′-(carboxymethyl)arginine; CML, N′-(carboxymethyl)lysine; DTPA, diethylenetriaminepentaacetic acid; G-DH, glyoxal-derived dihydroxyimidazolidine; G-DH1, N′-(5-hydro-4-dihydro-5-imidazolon-1-yl)ornithine; G-DH2, 5-(4,5-dihydroxy-2-imino-1-imidazolidinyl)ornithine; G-H, glyoxal-derived hydroimidazoline; G-H1, N′-(5-hydro-4-imidazolon-2-yl)ornithine; G-H2, 5-(2-amino-5-hydro-4-imidazolon-1-yl)ornithine; G-H3, 5-(2-amino-4-hydro-5-imidazolon-1-yl)ornithine; HA, hydroxyamine; HI, hydroimidazolone; OPD, o-phenylenediamine; RA, relative amount; TIC, total ion chromatogram; UK1, Unknown 1.
sites of modification of the enzyme. Our results indicate that dicarbonyl compounds react primarily with arginine residues in protein and that there is a high degree of specificity to the modification of both arginine and lysine residues during the Maillard reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were purchased from Sigma (St. Louis, MO): d-(-)-glucose (ACS grade), bovine RNase A (RNase type II-A, P00656), glyoxal, trypsin (sequencing grade), hydroxylamine (HA, 99%), o-phenylenediamine (OPD). CMA was a gift from R. Nagai (University of Kumamoto, Kumamoto, Japan).

**Modification of Protein by Glucose or Glyoxal and Preparation of Tryptic Digests**—RNase (13.7 mg, 1 μmol) was dissolved in 1 ml of a solution of glucose (0.4 M) or glyoxal (1 or 5 mM) in phosphate buffer (0.2 M, pH 7.4) and incubated under air at 37 °C for 3, 7, and 14 days (glucose) or 1, 3, and 7 days (glyoxal). The recovered protein was reduced with DTT, derivatized with 4-vinylpyridine, and digested with trypsin (enzyme:substrate ratio of 5:100 (w/w)) at 37 °C for 5 h. All samples were prepared in triplicate. The procedures have been published in detail elsewhere (11).

**Reaction of RNase-Glyoxal Incubations with HA**—Aliquots of the 7-day RNase-1 mM glyoxal incubation (40 μl, 0.04 μmol protein) were mixed with a 10-fold excess of HA (carbonyl groups:amino groups = 1:5) in 0.2 M, pH 7.4 phosphate buffer (40 μl) containing diethylene-triaminepentaacetic acid (DTPA, final concentration 0.1 mM) and incubated under nitrogen for 1, 3, and 7 days at 37 °C. Control incubations used phosphate buffer in place of HA solution. Further aliquots of the 7-day RNase-1 mM glyoxal incubation (40 μl) were ultrafiltrated to remove any unreacted glyoxal (11), resuspended in an equivalent volume of 0.4 M, pH 7.4 phosphate buffer (40 μl) containing DTPA (final concentration 0.1 mM) and incubated for 1 and 7 days.

**Tryptic Digestion of HA Incubations**—Incubations were digested using a modification of the procedure reported by Brock et al. (11). Protein (0.5 mg) was diluted into 50 μl of 0.1 M MOPS buffer containing 6 M urea and 0.1 mM EDTA. DTT (0.1 μmol) dissolved in MOPS buffer:water (1:3, v/v, 5 μl) was added to the protein solution, which was flushed with nitrogen for 60 s prior to incubation at 37 °C for 3 h. 4-Vinylpyridine (2.5 μmol) mixed in methanol:water (5:2 v/v, 5 μl) was added and the protein was derivatized in the dark at room temperature for 1 h. DTT solution (35 μl, 3.5 μmol) was added to quench the reaction. The sample was diluted in water (355 μl). Sequencing grade trypsin (100 μg) was dissolved in 100 μl of 100 mM HCl, and 25 μl was added to the protein solution (enzyme:substrate ratio of 5:100 (w/w)), which was flushed with nitrogen and incubated at 37 °C for 5 h. Digestion was terminated by freezing at −20 °C.

**Incubations of CMA with OPD and Amino Acid Analysis**—CMA and OPD were each dissolved in 0.2 M, pH 7.4 phosphate buffer to give a final CMA concentration of 5 mM, and a CMA:OPD molar ratio of 1:5. DTPA was added to give a final concentration 0.1 mM. Aliquots were incubated under nitrogen at 37 °C for 1, 3, and 7 days. Amino acid

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**Fig. 1.** Formation of glyoxal-derived dihydroxyimidazolidines (G-DH1 and G-DH2), glyoxal-derived hydroimidazolones (G-H1, G-H2 and G-H3) and N-(carboxymethyl)arginine (CMA) adducts by reaction of glyoxal with arginine residues on protein.
analysis was conducted on a divinylbenzene cation-exchange column (3 × 250 mm) (Pickering Labs, Mountain View, CA) with a sodium citrate gradient. Amino acids were quantified by post-column fluorescence using o-phthaldehyde. CMA was quantified with reference to a standard calibration curve (0–10 nmol CMA).

### RESULTS

Lys$^{31}$ in the active site of RNase is the primary site of glycation and carboxymethylation of RNase (11). Although the extent of modification of this peptide by 0.4 mM glucose and 1 mM glyoxal was similar, CML was identified as the primary product derived from glucose. Although the glyoxal-derived modification was not characterized, we established that glyoxal was not a significant intermediate in the formation of CML. In the present study, we analyzed the products of modification of RNase by glyoxal.

#### Location of Modified Peptides in the Glyoxal Reactions

As one approach to identifying modified peptides in the RNase-glyoxal reactions, the entire TIC, obtained using the triple quadrupole mass spectrometer for each glucose and glyoxal incubation, was treated as a single chromatographic “peak,” yielding a single mass spectrum for each sample (Fig. 2, A and B). These single mass spectra were visually compared in 100 mass unit increments to locate ions that were unique to one or the other sample. Comparison of the spectra of the glucose and 5 mM glyoxal incubations at 7 days, revealed a prominent ion at m/z 982 in the glyoxal spectrum that was absent from the glucose spectrum (Fig. 2, A, B, E, and J).

Extraction of the ion chromatogram at m/z 982 from the full-scan data revealed one major peak, eluting at 27.21 min (Fig. 3A). The spectrum of this peak contained ions at m/z 1473, 982, and 736 (Fig. 3B), corresponding to the 2$^+$, 3$^+$, and 4$^+$ ions of the same unidentified peptide that was named “Unknown 1” (UK1). Q-TOF analysis established that the proposed 3$^+$ ion was triply charged.

Signals corresponding to the 2$^+$ and 4$^+$ ions, at m/z 1473 and 736, respectively, were also located in the single combined spectrum of the glyoxal incubation but not in that for the glucose incubation (Fig. 2, C, G, H, and L). The m/z ratios of these different charged forms imply a mass of ~2942 Da for UK1 that was detected only in the glyoxal, and not in the glucose, reactions.

Modification at an arginine residue in a protein would lead to the formation of a tryptic peptide with a missed cleavage,
because trypsin cannot cleave C-terminal to a modified arginine residue. Glyoxal is known to react with the guanido group of both free and peptide-bound arginine to give products including hydroimidazolones (HIs (12,13)), G-DHs (14), and CMA (10, 15). The predicted mass of UK1 corresponded to the mass of the tryptic peptide with a missed cleavage C-terminal to Arg39, peptide 38D—K 61, where Arg39 is modified to either G-DH or CMA, which are isobaric compounds.

**Confirmation of the Amino Acid Sequence of UK1 by MS/MS**—To confirm the amino acid sequence of UK1, the tryptic digest of the 7-day 5 mM glyoxal incubation was analyzed by ESI-LC-MS using the Q-TOF mass spectrometer. Initially, experiments were performed on the 2+ and 3+ ions of peptide 38D—K 61 with Arg39 modified to G-DH or CMA, but spectra were much stronger for the 3+ ion and therefore the fragmentation pattern obtained for it was analyzed in detail (Fig. 4). The data were of sufficiently high resolution for the determination of the charge state of each fragment ion and the spectrum was interpreted manually.

As shown in Fig. 4, a series of singly charged y ions (y3–y15) and the doubly charged y20 ion were observed. Some b ions were also observed but were generally less intense than the y ions. Most were doubly charged (b9, b11–b19) but some singly charged ions (b6, b9, b10, b12) were also seen. The b ions provide evidence that UK1 incorporates Arg39 and a modification accounting for 58 amu. The y ions establish that the precursor ion contains the sequence of amino acids VHESLADVQAVCS, confirming that UK1 incorporates tryptic peptide 40C—K 61. All the MS data presented in Figs. 3 and 4 establish the identity of the peptide UK1 to be 38D—K 61 with either Arg39 modified to G-DH or CMA, or Lys41 modified to CML.

We performed numerous additional MS experiments with the aim of generating additional data to confirm the identity of UK1. Both precursor ion experiments for parents of the immonium ion of G-DH or CMA at m/z 187 and multiple reaction monitoring experiments to search for the immonium ion of G-DH or CMA and the b2 ion at m/z 330, generated from peptide 38D—K 61 with Arg39 modified to G-DH or CMA, gave signals at the pertinent nominal masses but the identities were ambiguous due to the limited resolution of the triple quadrupole instrument. For example, the fragment at m/z 187 may...
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Fig. 3. An extracted ion chromatogram at m/z 982 for the 7-day RNase-5 mM glyoxal incubation contains one major peak and its spectrum contains ions that correspond to the 2+, 3+, and 4+ ions of the same peptide (UK1; m/z = 2942 Da). A, extracted ion chromatogram at m/z 982 for the tryptic digest of RNase incubated with 5 mM glyoxal for 7 days showing one major peak (at 27.21 min). B, spectrum of the peak at 27.21 min showing ions at m/z 1473, 982, and 736, corresponding to the 2+, 3+, and 4+ ions of peptide UK1 with a mass ~2942 Da. Insert is the Q-TOF survey mode data for the ion at m/z 982 showing that it is triply charged.

also represent an a-type internal ion for DV or a b-type internal ion for AD, both of which were present in the sequence of the modified peptide. Analysis by capillary LC-MS/MS on a newer generation Q-TOF API US instrument using a limited scan range (to maximize sensitivity) and different collision energies and sample concentrations also did not reveal either ion of interest. The peptide was less amenable to analysis by MALDI-TOF/TOF than ESI-MS. The immonium ion of arginine is known to be of very low intensity (16) and it is likely that the immonium ion of the G-DH or CMA adduct of Arg39 will also be weak.

Discrimination Between G-DH, CMA, and CML—The formation of G-DH is reversible, while CMA and CML are formed irreversibly (14). To investigate whether the modified peptide in the glyoxal incubations was G-DH or CMA or both adducts on Arg39 or CML on Lys41, protein from the 7-day 1 mM glyoxal reaction was incubated with a 5-fold molar excess (based on carbonyl groups) of HA. Parallel incubations were conducted without HA, both with and without prior removal of any unreacted glyoxal. As shown in Fig. 5, the amount of UK1 decreased by 75% over a 7-day period, with a half-life of ~2 days.

In contrast, authentic CMA was completely stable in a parallel incubation with OPD for up to 7 days. Because the majority (~75%) of the Arg39 adduct in the 7-day 1 mM glyoxal incubations was reversed by incubation with HA, we conclude that the peptide contained G-DH rather than the isobaric CMA.

The possibility that UK1 might be peptide 38D—K61 with Lys41 modified to CML was ruled out because CML is extremely stable, e.g. to 6 N HCl at 95 °C for 18 h (9). The identification of UK1 as peptide 38D—K61 with Lys41 modified to CML would also require that the peptide contain a missed cleavage site C-terminal to Arg39 but this unmodified peptide was not detected in tryptic digests of either the glyoxal-modified RNase incubations or of native RNase, so that cleavage of Arg39 appears to be efficient. We did detect trace levels of peptide 40C—K61 with Lys41 modified to CML but UK1 was present at >100-fold higher intensity. Thus, UK1 must be peptide 38D—K61 with Arg39 modified to G-DH, in line with earlier reports that dicarbonyls, such as methylglyoxal, modify arginine in preference to lysine residues on protein (13).

Fig. 5 also shows that the amount of the unmodified peptide 40C—K61 increases with time of incubation with HA. Peptide 38D—K61 with Arg39 modified to G-H was also located within the MS data by extracting ion chromatograms at m/z 1463, 975, and 732 for the 2+, 3+, and 4+ charged forms, respectively. This peptide also decreased on incubation with HA, but at a much slower rate than the loss of G-DH, with ~80% remaining after 7 days (compared with ~25% for G-DH). In control experiments, incubating the 1 mM glyoxal incubations, both with and without prior removal of unreacted glyoxal and in the absence of HA, resulted in no change in the amount of peptide 38D—K61 in which Arg39 was modified to G-DH. Thus, the discharge of G-DH adducts was dependent on reaction with HA.

Site Specificity and Kinetics of Formation of G-DH and G-H Adducts—We have demonstrated that although CML forms at every lysine when RNase is incubated with glucose, the distribution of CML is not uniform and Lys41 is the major site of modification (11). Therefore, we anticipated that G-DH and G-H might also form at other arginine residues in RNase, i.e. Arg10, Arg33, and Arg85, in addition to Arg39, and that their distribution among the arginine residues might be uneven. We therefore searched for ions with calculated m/z values of the different charge states of the predicted peptides, 8FERQHMDSTSAAASSNYCNQMMK31 (8F—K31), 8SRNLTK37 (8S—K37) and 67NGETNCYQSYSTIDCRETSKSYPCAYK98 (67N—K98), where Arg10, Arg33, and Arg85 were modified, respectively, to G-DH or G-H. In previous studies, we observed that trypsin produced almost no cleavage between Lys31 and Tyr32 of RNase. Therefore, modifications of peptide 67N—K98, with two missed cleavage sites, were monitored. Peptides 8F—K31 and 67N—K98, containing, respectively, Arg10 and Arg85 modified to G-DH and G-H, were located by extracting ion chromatograms from the full-scan data, thus establishing the formation of both of these adducts at Arg10 and Arg85. When the 7-day 1 mM glyoxal reaction was incu-
bated with HA, the kinetics of loss of peptides containing Arg10 and Arg85 modified to G-DH/CMA were similar to those of peptide 38D—K61 with Arg39 modified to G-DH, indicating that G-DH and not CMA was also the major or sole adduct on these arginine residues. Peptide 32S—K37 with Arg33 modified to either G-DH or G-H was not detected in the RNase-glyoxal reactions, despite a strong signal for the native peptide 34NLTK37, indicating that Arg33 was not converted to these adducts in detectable amounts in the glyoxal incubations, even after 7 days in 5 mM glyoxal.

Although G-H exists as three structural isomers, we could detect only one peak within the LC-MS data corresponding to G-H on Arg10, Arg39, and Arg85, possibly because the isomers were not resolved on the LC column or because some of them were present below the limit of detection. The 3+ ions of the G-DH adducts of peptides 8F—K31 and 67N—K58, and the G-H adduct of peptide 38D—K61 were also detected in the single combined spectrum that was extracted from the full-scan data of the glyoxal incubations, but not from the glucose incubations (Fig. 2, D, F, I, and K), and their retention times match those expected for these adducts. None of these modified peptides was present in sufficiently high concentrations for peptide sequencing experiments.

The kinetics of formation of the peptides containing arginine residues, together with the kinetics of loss of the relevant unmodified peptides in the 1 mM glyoxal incubations, are shown in Fig. 6. Similar graphs were obtained for the 5 mM glyoxal incubations, but with a 2- to 3-fold increased response. Arg39 and Arg85 were identified as the major sites of adduct formation.

**DISCUSSION**

**Location of Unpredicted Peptides**—The application of MS to the analysis of proteins is a powerful technique for identification and localization of chemical modifications of proteins. However, the technique works best when the structure of the modified amino acid is known in advance. For our studies on modification of RNase by glyoxal, we were faced with the loss of a peptide containing Lys51, but the mass of the modification was uncertain. We therefore treated the TIC as a single peak to yield a single mass spectrum for the entire run. A visual comparison of the spectra for glucose- and glyoxal-
modified protein facilitated the detection of ions that were present in only one of the samples. This allowed us to locate an unknown peptide within our RNase-glyoxal incubations that was absent from our glucose incubations, and that was subsequently identified as \(38D-K^{61}\) in which Arg\(^{39}\) was modified to G-DH. Sequencing the peptide, together with the data from the HA incubations, confirmed its identity and the site of modification. Analysis of the TIC is a simple and rapid procedure but, in general, is limited to detection of quantitatively major modifications because less abundant modified peptides are obscured by background ions. Using this approach, we identified Arg\(^{39}\) as the major site of modification of RNase by glyoxal. Weak signals for the \(3^{+}\) ions of peptides \(8F-K^{31}\) and \(67N-K^{38}\), containing Arg\(^{10}\) or Arg\(^{85}\), respectively, modified to G-DH, as well as the \(3^{+}\) ion of peptide \(38D-K^{61}\) containing Arg\(^{39}\) modified to G-H, were also observed in these analyses.

**Estimating Amounts of Modifications**—Quantitation of absolute and relative modification of proteins by LC-MS/MS is a major challenge. We have previously estimated the extent of modification of different lysine residues in RNase by calculating relative amount (RA) values (11), \(i.e.\) the area units for charged species of the modified peptide, compared with the area units of the C-terminal peptide of RNase. One advantage of this technique is that values are normalized between samples, but a disadvantage is that the mass spectrometer response to different tryptic peptides of native RNase is variable, limiting the validity of the technique for comparing amounts of different tryptic peptides. The use of RA values to estimate modifications is more meaningful when the number of amino acid residues in the unmodified and modified peptides is the same or similar; in this case the mass spectrometer response is likely to be similar. Biemel and Lederer (17) established that lysine, fructoselysine, and \(N^6\)-(2,3-dihydroxy-4-quinoxalin-2-ylbutyl)-l-lysinate gave comparable responses when analyzed by ESI-LC-MS. They also provided evidence that these modifications on an identical peptide resulted in an almost equivalent mass spectrometer response. Therefore, this approach was applied in this study to monitor the loss of...
the G-DH and G-H adducts at Arg<sup>39</sup> within peptide 38D—K<sup>61</sup> and regeneration of peptide 40C—K<sup>61</sup> in the HA reactions. These peptides differed in length by only two amino acid residues and also possessed very similar retention times on the HPLC column (Table I).

To follow the kinetics of formation of G-DH and G-H adducts at the different arginine residues within RNase, we expressed the data as a percentage of the unmodified and modified peptides within each arginine peptide group. This method eliminated variations in RA values due to inter-sample and inter-batch variations in the derivatization/digestion procedure and LC-MS response.

**Identification of Peptide 38D—K<sup>61</sup> Containing Arg<sup>39</sup> Modified to G-DH**—Based on reversibility of modification by treatment with HA, UK1 was identified as primarily (>75%) peptide 38D—K<sup>61</sup> containing the G-DH adduct at Arg<sup>39</sup>. Glomb and Lang (14) monitored the degradation of authentic G-DH in the presence of OPD or aminoguanidine (AG) at 37 °C and pH 7. These experiments yielded half-lives of 1–2 days for G-DH, consistent with the half-life we obtained following incubation of our 7-day 1 mM glyoxal incubations with HA. CMA has been identified in collagen incubated in 1 M glucose (10), and it has been demonstrated, using ESI-LC-MS, that levels of CMA on serum proteins from diabetic subjects are significantly higher than those from age-matched controls (15). G-DH was not measured in either of these studies but, based on our data and those of Glomb and Lang (14), it may be present at higher concentrations than CMA.

**Site Specificity of G-DH and G-H Formation**—As shown previously (11), the formation of CML in RNase-glucose systems is site-specific (11), occurring primarily at Lys<sup>41</sup> in the active site and derived primarily from the Amadori adduct. Based on the present work, modification of protein by glyoxal is also site-specific, occurring primarily on Arg<sup>39</sup> and Arg<sup>85</sup> in RNase, with small amounts of modifications forming on Arg<sup>10</sup>, but no detectable adducts on Arg<sup>33</sup>. In previous work, Arg<sup>39</sup> and Arg<sup>85</sup> were also identified as the main sites of modification of RNase by α-dicarbonyls (18). The earliest work involved modification of bovine RNase A by phenylglyoxal, which also yielded a reversible adduct (19). Later studies, involving the incubation of RNase with 1,2-cyclohexanedione (20, 21) and 3-ethoxy-2-ketobutanal (22), concluded that Arg<sup>39</sup> and Arg<sup>85</sup> were the most modified sites, followed by Arg<sup>10</sup>, with Arg<sup>33</sup> being unreactive. The adduct formed from 1,2-cyclohexanedione, i.e. N<sup>1</sup>·N<sup>8</sup>-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine, was characterized (20) and, like the glyoxal adduct, G-DH, its formation was reversible on incubation with HA. The lack of reactivity at Arg<sup>39</sup> was attributed to hydrogen-bonding to Asp<sup>14</sup> (19, 22).

**Relative Amounts of AGEs on Proteins**—The data presented here show that, in the glyoxal incubations, the order of abundance of the adducts was G-DH > G-H > CML. Only low levels of CML peptides were formed from glyoxal and, in the glucose incubations, no G-DH or G-H adducts could be detected. We have previously reported (11) that, in the glucose incubations, the main route to CML is by oxidation of the ARP. This is confirmed by the absence of G-DH or G-H adducts on the glycated protein, indicating that little glyoxal was formed, and the higher abundance of peptide 40C—K<sup>61</sup> carboxylated at Lys<sup>41</sup> in the glucose incubation compared with the glyoxyl system.

AGEs are potential biomarkers of various disease states, including diabetes, atherosclerosis, and neurodegenerative disease. Relative amounts of different AGEs in physiological proteins and fluids may differ according to the severity of disease or particular tissue, reflecting local differences in carbonyl formation and oxidative stress. In general, HI adducts are reported to be present at higher concentrations than CML on plasma and tissue proteins (23, 24). For example, G-H1 has been reported to be present at about twice the level of CML on plasma proteins of healthy human subjects. Levels of G-H1 on retina, nerve, and plasma proteins of streptozotocin-induced diabetic rats also increased significantly compared with those of normal controls (24), reflecting elevated levels of plasma glyoxal in diabetes. Little attention has been given to G-DH adducts on protein (14), but our studies suggest that they may be present at higher concentrations than either CMA or G-H.

**CONCLUSIONS**

In conclusion, this study has demonstrated that G-DH is the primary product formed on reaction of glyoxal with RNase under physiological conditions. Formation of both G-DH and G-H is site-specific and Arg<sup>39</sup> and Arg<sup>85</sup> are the favored sites of modification of the protein. Neither G-DH nor G-H is formed in detectable amounts when RNase is incubated with glucose. The absence of G-DH and G-H in the glucose incubations supports a conclusion from our earlier work on CML that glyoxal formation is not an important route to CML in RNase-glucose incubations. Our findings suggest that arginine residues are the favored sites of modification of proteins by dicarbonyls and that inhibition of dicarbonyl formation and entrapment of these highly reactive species may be an important means of inhibiting AGE formation in vivo.

**Acknowledgments**—We thank R. Nagai (University of Kumamoto, Japan) for the gift of CMA.

* This study was funded by the Wellcome Trust, via a Short-Term Travel Grant 06564/Z/01/Z (to J. M. A.) and by National Institutes of Health Grant DK-19971 (to J. W. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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