Presence of dopa and amino acid hydroperoxides in proteins modified with advanced glycation end products (AGEs): amino acid oxidation products as a possible source of oxidative stress induced by AGE proteins

Shanlin FU*, Min-Xin FU†, John W. BAYNES‡, Suzanne R. THORPE† and Roger T. DEAN*[1]

*Cell Biology Unit, Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, NSW 2050, Australia, †Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208, U.S.A., and ‡School of Medicine, University of South Carolina, Columbia, SC 29208, U.S.A.

Glycation and subsequent Maillard or browning reactions of glycated proteins, leading to the formation of advanced glycation end products (AGEs), are involved in the chemical modification of proteins during normal aging and have been implicated in the pathogenesis of diabetic complications. Oxidative conditions accelerate the browning of proteins by glucose, and AGE proteins also induce oxidative stress responses in cells bearing AGE receptors. These observations have led to the hypothesis that glycation-induced pathology results from a cycle of oxidative stress, increased chemical modification of proteins via the Maillard reaction, and further AGE-dependent oxidative stress. Here we show that the preparation of AGE-collagen by incubation with glucose under oxidative conditions *in vitro* leads not only to glycation and formation of the glycoxidation product N^{ϵ}-(carboxymethyl)lysine (CML), but also to the formation of amino acid oxidation products on protein, including *m*-tyrosine, dityrosine, dopa, and valine and leucine hydroperoxides. The formation of both CML and amino acid oxidation products was prevented by anaerobic, anti-oxidative conditions. Amino acid oxidation products were also formed when glycated collagen, prepared under anti-oxidative conditions, was allowed to incubate under aerobic conditions that led to the formation of CML. These experiments demonstrate that amino acid oxidation products are formed in proteins during glycoxidation reactions and suggest that reactive oxygen species formed by redox cycling of dopa or by the metal-catalysed decomposition of amino acid hydroperoxides, rather than by redox activity or reactive oxygen production by AGEs on protein, might contribute to the induction of oxidative stress by AGE proteins.

INTRODUCTION

The formation of advanced glycation end products (AGEs) by non-enzymic Maillard reactions is implicated in age-related pathologies such as atherosclerosis and neurodegenerative diseases and in the development of the long-term complications of diabetes [1–3]. The aerobic, non-enzymic, glycation of proteins in the presence of even minute concentrations of transition metals is accompanied by oxidative, radical-generating reactions [4]. Initially, the auto-oxidation of the free sugars, and the glycation reactions involving the oxidation products, were distinguished as 'auto-oxidative glycosylation' [5,6]. However, protein-bound sugars can also auto-oxidize [7–11]; they can also initiate the oxidation of other components in proteins, especially polyunsaturated fatty acids in lipids of lipoproteins and in cell membranes [9,12]. All these processes can be catalysed by the transition metals iron and copper, and inhibited by appropriate chelators such as diethylenetriaminepenta-acetic acid (DTPA) and phytic acid [5–13].

Several products of the auto-oxidation of glucose and of glycoxidation of proteins have been defined. The major auto-oxidation products of glucose are glyoxal and arabinose [14]. N^{ϵ}-(Carboxymethyl)lysine (CML) is quantitatively the major chemically characterized glycoxidation product formed during protein glycation *in vitro* [15] and is a major immunological determinant on the resultant glycated proteins [16,17]. In contrast with CML, N^{ϵ}-(fructose)lysine (FL), the initial adduct formed between glucose and protein, is produced even under non-oxidative conditions, for example in the absence of oxygen and in the presence of metal-ion chelators that restrict redox cycling of the metal ions, but FL can subsequently give rise to CML under metal-catalysed oxidative conditions [7]. It is not clear which products of glycoxidation are unique to that process, as CML is also produced during the peroxidation of lipoproteins or by lipid peroxidation in the presence of amino acids [18]. The formation of oxygen radicals during the auto-oxidation of free sugars in the presence of proteins was documented in early studies describing the radical-mediated hydroxylation of benzoate, protein fragmentation and other alterations to protein conformation during glycoxidation reactions [4,5,8,9,19,20].

In subsequent studies of the action of hydroxyl radicals and metal-dependent Fenton systems, we demonstrated the production of two classes of protein-bound reactive oxygen species [21]. The first were species capable of reducing transition metal ions: dopa was a major component of these [22]. The second were protein hydroperoxides, which are oxidizing species [23–25] capable of the direct generation of further radical fluxes [26] (in a manner analogous to lipid hydroperoxides) and can participate in chain reactions of protein oxidation [27]. These hydroperoxides were found particularly on aliphatic side chains, but can also occur at main chain sites [28]. Hydroxyl radicals or metal-oxo complexes are likely precursors of dopa and also of *o*-tyrosine and *m*-tyrosine, by the direct hydroxylation of tyrosine and phenyalanine respectively. Dityrosine can also form whenever pairs of tyrosyl-phenoxyl radicals occur in proximity, either as a result of hydroxyl radical attack, or by the action of hypo-

Abbreviations used: AGE, advanced glycation end product; CML, N^{ϵ}-(carboxymethyl)lysine; DTPA, diethylenetriaminepenta-acetic acid; FL, N^{ϵ}-(fructose)lysine; OPA, *o*-phthaldialdehyde.

[1] To whom correspondence should be addressed.

chlorite/myeloperoxidase systems [29]. Several of these oxidation products might also be produced by the action of reactive nitrogen intermediates, such as nitric oxide and peroxynitrite, whose complex chemistry is gradually being unravelled (reviewed in [30]).

The purpose of the present study was to assess the role of glycoxidation in the formation of such protein-bound reactive oxygen species, including products of the direct oxidation of protein, as well as the formation of the protein-bound sugar derivatives. We distinguished between the formation of protein oxidation products during glycation under oxidizing conditions in the presence of adventitious metal ions (which comprises products of reactions of both the free sugars and those of the sugars that have become protein-bound) from that occurring when proteins pre-glycated under anaerobic chelated conditions are subsequently incubated under oxidative conditions. We find that reactive protein oxidation products are generated during both stages of glycoxidation reactions, i.e. from both free and protein-bound sugar. We propose that these species contribute to the biological activities of glycoxidized proteins.

MATERIALS AND METHODS

Materials

o-Phthaldialdehyde (OPA) crystals and OPA diluent [containing 3 % (w/v) KOH and 3 % (w/v) boric acid at pH 10.4] were from Pickering Laboratories (Mountain View, CA, U.S.A.). 2-Mercaptoethanol and BSA, essentially fatty-acid-free (catalogue no. A-7511) were from Sigma Chemical Company (St. Louis, MO, U.S.A.). Mercaptoacetic acid was from Merck (Kilsyth, Victoria, Australia). CML, [$^2H_8$]CML and [$^{13}C_6$]FL were prepared as described previously [31]. Other chemicals, solvents and chromatographic materials were of AR or HPLC grade.

Glycation and glycoxidation of collagen

Rat tail collagen was isolated and extracted with 0.5 M NaCl as described previously [13]. The collagen was incubated at 37 °C in 10 ml of 0.2 M phosphate buffer, pH 7.4, with or without 250 mM glucose, under aerobic or anti-oxidative conditions (1 mM DTPA/1 mM phytic acid under a nitrogen atmosphere, as previously described [13]). Incubations were set up in triplicate in individual vials (approx. 250 mg wet weight per vial) with a few drops of toluene on top to inhibit bacterial growth. At desired times individual vials were removed and frozen at −70 °C, until all samples could be processed as a batch. The collagen was thoroughly rinsed in cold de-ionized water and, except for samples used for FL determinations, was reduced with 100 mM borohydride in 0.2 M borate buffer, pH 9.2, at 4 °C overnight. The collagen was again rinsed with deionized water and then freeze-dried.

Preglycation of collagen

Collagen (approx. 2.5 g) was pre-glycated in 0.2 M phosphate buffer, pH 7.4, containing 250 mM glucose, 1 mM DTPA and 1 mM phytic acid. The buffer was first degassed by treatment with vacuum for 10 min, then bubbled with nitrogen for an additional 10 min and finally transferred to a brown glass bottle. After addition of the collagen, the bottle was purged with nitrogen then sealed, wrapped in Parafilm and incubated at 37 °C. At the end of 4 weeks the collagen was rinsed thoroughly with deionized water. A portion (approx. 1.0 g) was reduced with $NaBH_4$ as described above. Aliquots of both reduced and unreduced pre-glycated collagen were re-incubated at 37 °C in individual vials in 0.2 M phosphate buffer, pH 7.4, without glucose. Individual vials were removed and frozen at −70 °C and processed as above.

Analysis of FL and CML

Incubations and analyses of FL and CML were conducted in Columbia; reduced, freeze-dried and coded samples were sent to Australia for blind analyses of protein oxidation products. After the addition of internal standards, [$^{13}C_6$]FL and [$^2H_8$]CML, collagen (approx. 2 mg dry weight) was hydrolysed in 6 M HCl at 110 °C for 24 h in screw-cap tubes that were purged with nitrogen. Hydrolysates were dried by centrifugal evaporation. FL and CML were converted into their *N*,*O*-trifluoroacetyl methyl ester derivatives and analysed by selected ion-monitoring GC/MS as described previously [31].

Protein hydrolysis

A gas-phase and acid-catalysed method described previously [22,25] was adopted to optimize the recovery of dopa. Briefly, each freeze-dried collagen sample (approx. 4 mg) was placed in a 1 ml brown glass autosampler vial (Activon, Thornleigh, Australia). The sample vials (maximum of eight) were placed in a Pico-Tag reaction vessel (Millipore Waters, Lane Cove, Australia), to which 1 ml of 6 M HCl containing 5 % (v/v) mercaptoacetic acid and 1 % (w/v) phenol were added. The deoxygenated reaction vessel was placed in an oven at 110 °C for 17 h. The hydrolysate was freeze-dried and dissolved in approx. 200 μl of water for the measurement of hydroxylated amino acids by HPLC. Hydroxyproline was measured as described by Stegemann and Stalder [32].

Analysis of modified amino acids in protein hydrolysates

HPLC was performed on an LC-10A HPLC system (Shimadzu), and a column oven (Waters) set at 30 °C was used for all analyses. Determination of 3-hydroxyvaline and 4-hydroxyleucine was done as described previously [25,33]. These analyses require two steps of HPLC, first on an LC-NH_2 column and second, after derivatization with OPA, on a Zorbax ODS column. Fresh OPA reagent was made before each analysis. Typically 50 mg of OPA was dissolved in 5 ml of methanol and was added to 45 ml of deoxygenated OPA diluent. Into the solution was also added 200 μl of mercaptoethanol. The OPA derivatization was automated by the autosampler. Other procedural details have been described previously [25,33,34]. The second HPLC step was also used to measure unmodified valine, leucine and phenylalanine in the hydrolysate (without any prior fractionation).

For the detection of dopa, *m*-tyrosine and dityrosine, the hydrolysate was chromatographed on another Zorbax ODS column with a Pelliguard guard column. A gradient of solvent A (100 mM sodium perchlorate in 10 mM sodium phosphate buffer, pH 2.5) and solvent B [80 % (w/v) methanol] was used with a flow rate of 1 ml/min. The gradient profile was as follows: isocratic elution with 0 % B for 12 min; then to 20 % B in 8 min; futher elution at 20 % B for 3 min before changing to 50 % B in 3 min; isocratic elution at 50 % B for a further 3 min; then re-equilibration with 100 % A for 10 min. The eluent was monitored by both UV (Shimadzu, at 280 nm) and fluorescence (Hitachi F-1080) detectors in series. The fluorescence excitation wavelength was fixed at 280 nm, with the emission wavelengths set at 320 nm for dopa and *m*-tyrosine, and 410 nm for dityrosine through the built-in time programming of the detector. In collagen incu-

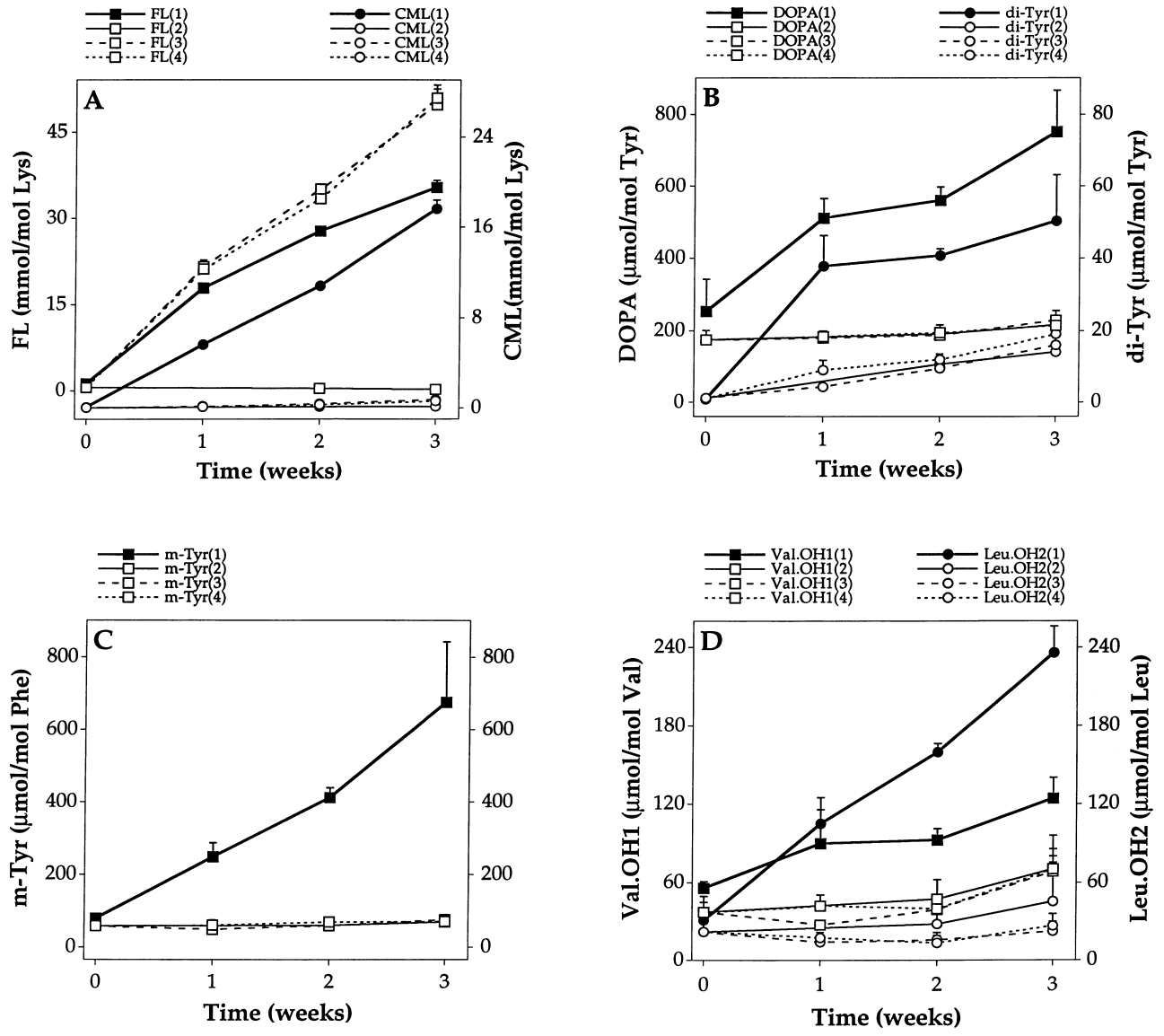

Figure 1 Glycation, glycoxidation and formation of oxidation products in collagen during exposure to glucose

Reactions were conducted as described in the Materials and methods section: (1) under aerobic, oxidative conditions (■,●); (2) under aerobic, oxidative conditions but in the absence of glucose (□,○; solid lines); (3) under air but in the presence of DTPA and phytic acid to evaluate the role of free transition metal ions in oxidative process (□,○; broken lines); (4) under anaerobic, anti-oxidative conditions (□,○; dotted lines). The absence of error bars indicates that the S.D. was within the size of the symbol. (**A**) Formation of FL and CML; (**B**) formation of dopa and dityrosine; (**C**) formation of *m*-tyrosine; (**D**) formation of 3-hydroxyvaline (Val.OH1) and 4-hydroxyleucine (Leu.OH2). The normalized concentration of modified amino acids (pmol/mg of collagen) was also determined in each experiment. Values for aerobic, oxidative conditions (condition 1) at weeks 0 and 3 (week 0/week 3) were: FL, 610/11780; CML, 30/5880; dopa, 8.38/30.88; *m*-tyrosine, 11.13/96.75; dityrosine, 0.03/2.02; Val.OH1, 10.79/24.53; Leu.OH2, 6.62/51.17.

bations, *o*-tyrosine was not measured because of interference by an unknown impurity. Because of its off-scale response in the fluorescence channel, the unmodified native *p*-tyrosine was quantified by UV measurement. Elution positions were defined on the basis of standards, and identities were confirmed by UV absorption and fluorescence spectra.

Statistics

All incubations were performed in triplicate. Results are expressed as means ± S.D. for single analyses from each time point of each incubation.

RESULTS

Generation both of AGEs and of reactive protein oxidation products during exposure of collagen to glucose

As demonstrated previously [13], exposure of collagen to glucose under air results in the time-dependent formation of FL. The rate of formation of FL, which does not involve oxidative reactions, is similar in the presence and in the absence of oxygen and chelators. The glycation reaction in air is accompanied by a metal ion-catalysed auto-oxidation of the sugar and sugar-protein intermediates, leading to the formation of CML. The accumulation of CML, which is dependent on oxidative pro-

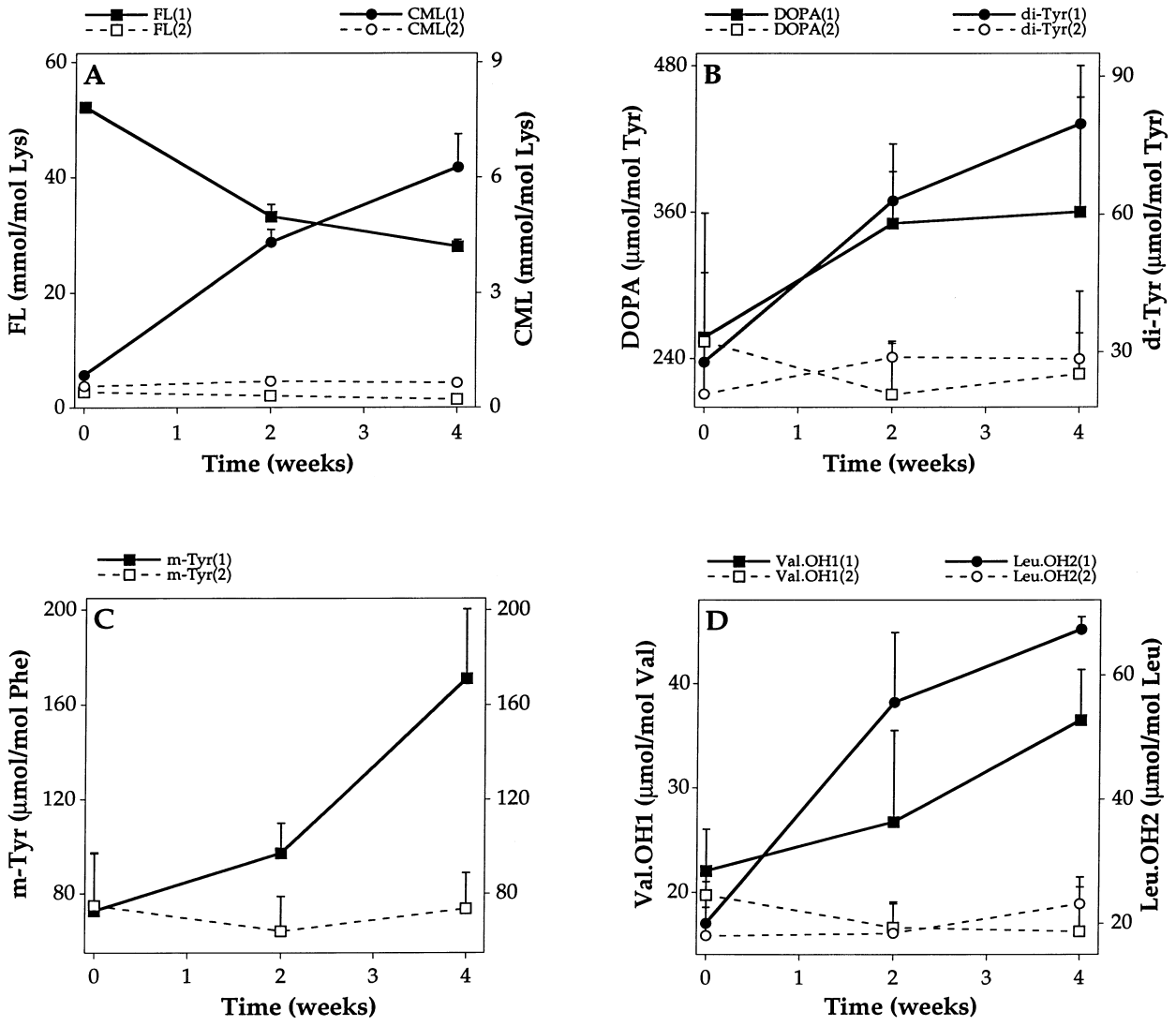

Figure 2 Measurement of glycation, glycoxidation and protein oxidation products during reincubation of pre-glycated collagen

Pre-glycated collagen was prepared as described in the Materials and methods section and reincubated in 0.2 M phosphate buffer under oxygen either before (1; ■,●), or after (2; □,○), borohydride reduction. (**A**) Changes in FL and CML; (**B**) changes in dopa and dityrosine; (**C**) changes in *m*-tyrosine; (**D**) changes in 3-hydroxyvaline (Val.OH1) and 4-hydroxyleucine (Leu.OH2).

cesses, is blocked in the absence of oxygen or on addition of the metal-ion chelators, DTPA and phytic acid, which restrict the redox cycling of metal ions (Figure 1A).

Figures 1(B) and 1(C) show that during such glycoxidation reactions the hydroxylation of aromatic residues is also significant; it too depends on the presence of oxygen and the availability of metal ions. The yields of dopa (from *p*-tyrosine; Figure 1B) and *m*-tyrosine (from phenylalanine; Figure 1C) are comparable, reaching approx. 10^{-4} of the respective parent amino acid. Although present at a concentration about an order of magnitude lower than that of CML, dopa is of particular interest because of its reducing activity [21,22]. Dityrosine, which is formed by radical–radical coupling of two tyroxyl radicals, also accumulates (Figure 1B). Its concentration is less than one-tenth that of the hydroxylated aromatics, consistent with the requirement for radical–radical coupling of tyrosine residues situated near each other in proteins. The aromatic modifications are consistent with the formation of hydroxyl radicals or a comparably reactive species.

The metal ion-dependent oxygen-radical-mediated formation of reactive amino acid hydroperoxides (measured here as their chemically reduced derivatives, the hydroxy amino acids) was also observed (Figure 1D). There was a greater rate and extent of formation of hydroxyleucine than of hydroxyvaline, as we have observed previously during attack of the relatively non-selective hydroxyl radical, generated by radiolysis, on BSA [33]. This might involve radical transfer between amino acids, as discussed earlier [33]. The hydroxyvaline and hydroxyleucine were produced at levels 2–3-fold higher than that of dityrosine, but at levels significantly lower than either dopa or *m*-tyrosine, consistent with results of experiments with radiolytically generated hydroxyl radicals.

Formation of reactive protein oxidation products during oxidative incubation of glycated collagen

These experiments were designed to determine whether collagen that had been glycated under non-oxidative conditions could

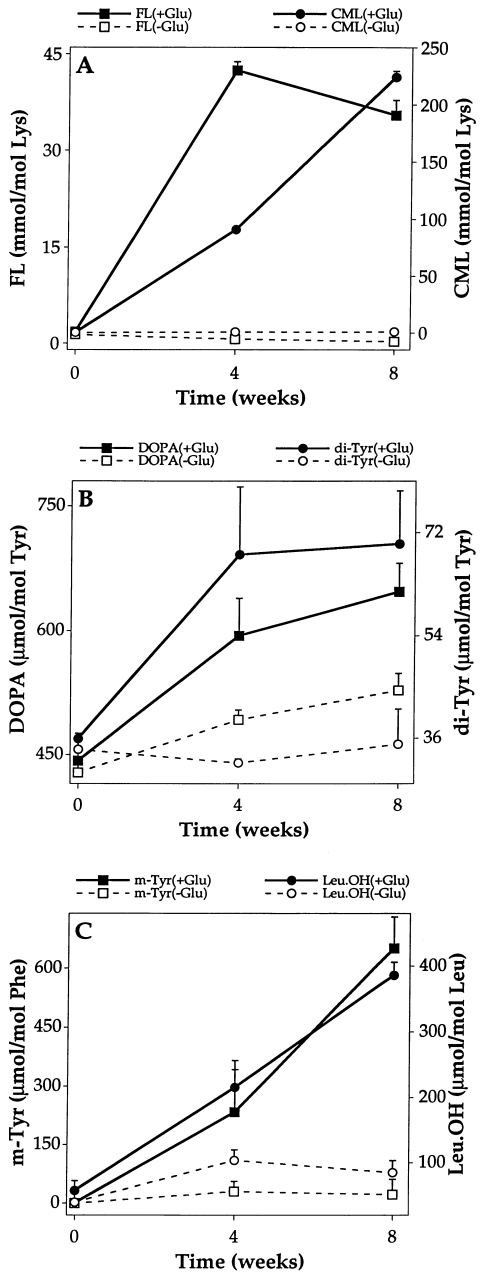

Figure 3 Measurement of glycation, glycoxidation and protein oxidation products during incubation of BSA with glucose

BSA (20 mg/ml) in 0.2 M phosphate buffer, pH 7.4, without chelators was incubated at 37 °C under air in the presence (■,●) or absence (□,○) of glucose (1.0 M). (**A**) Formation of FL and CML; (**B**) formation of dopa and dityrosine; (**C**) formation of *m*-tyrosine and 4-hydroxyleucine (Leu.OH).

generate AGEs and protein oxidation products during subsequent incubation under oxidative conditions. As expected, significant levels of FL were present after glycation, and were partly consumed during subsequent incubation in the absence of glucose and the presence of oxygen (Figure 2A). CML was not present after the non-oxidative glycation but appeared during re-incubation of the glycated protein under oxidative conditions, but not if the sample was chemically reduced before re-incubation (Figure 2A). If the glycated protein was chemically reduced before the re-incubation, less than 5% of the starting FL was detectable, confirming efficient reduction of the Amadori compound. In this case FL was converted into hexitol-lysine; this product is no longer susceptible to oxidative cleavage to CML [14]. Thus the auto-oxidation of FL in glycated proteins results in the consumption of FL and the generation of CML; only a portion (approx. 25%) of the FL consumed was recovered as CML, in agreement with previous data [7,10].

We also determined that auto-oxidation during the re-incubation of pre-glycated collagen led to the formation of protein oxidation products. As shown in Figures 2(B) and 2(C), dopa and *m*-tyrosine were formed quite efficiently; dityrosine (Figure 2B) was also formed, but again at levels roughly an order of magnitude lower. The amino acid hydroperoxide pathway was also active, as judged by the formation of hydroxyvaline and hydroxyleucine (Figure 2D) at levels similar to those of dityrosine. In all cases the chemical reduction of the glycated collagen before re-incubation prevented the subsequent generation of the oxidation products of protein amino acids, indicating the requirement for oxidizable protein-bound products of the glycation reaction. The extent of generation of both CML and the protein oxidation products in these re-incubation reactions was roughly half of that achieved in the glycoxidation experiments of Figure 1.

Generality of protein oxidation during formation of AGE proteins

To determine whether the formation of oxidized protein species, notably the reactive components dopa and amino acid hydroperoxides, is a general consequence of incubating proteins under conditions typically used to produce AGE protein *in vitro*, we chose a standard protocol for the formation of AGE protein, with BSA as the protein. We have shown previously [21,22] that during the glycoxidation of BSA, reducing moieties, now known to be substantially composed of protein-bound dopa, are formed. Here we extend this by showing that the formation of AGE–BSA was accompanied by the generation of CML (Figure 3), hydroxy aromatic amino acids and dityrosine; aliphatic amino acid hydroperoxides were also formed. The relative extents of generation of these species were consistent with those observed above for collagen. However, the basal values observed in the commercial BSA samples were significantly higher than in the collagens and higher than those in fresh plasma ([33], and S. Fu and R. T. Dean, unpublished work), suggesting some modification of the commercial samples during preparation and storage.

DISCUSSION

The results presented here show clearly that protein oxidation is a consquence of both phases of the glycoxidation reaction: glycation in the presence of auto-oxidizing sugars, and the subsequent oxidation of pre-glycated proteins. During the latter phase, FL seems to be a major substrate for oxidation, even though this component is produced initially in the absence of oxygen, and hence in the absence of protein oxidation. The kinetics of the formation of oxidized amino acids, which proceeds at maximal rates from zero time, i.e. before the formation of significant amounts of Amadori products, indicates that protein oxidation might be initiated either during auto-oxidation of the free sugars or Schiff base adducts to protein in the presence of trace metal ions and oxygen.

The ranges of protein oxidation products generated during the two stages of glycoxidation are similar. Although the second phase of the reaction, in which protein-bound sugar derivatives are oxidized, is apparently less potent, it should be noted that this

phase involves the oxidation of much smaller quantities of protein-bound moieties than are presented in the first phase as oxidizable sugars. As noted by several authors, these results indicate that the auto-oxidation of the protein-bound species can in some circumstances be more efficient than that of free sugars [11,35]. Indeed, the fructosamine assay for glycated plasma proteins depends on the greater ease of oxidation of fructosamines (Amadori adducts), than of free glucose [36]. Because a similar spectrum and ratio of protein oxidation products are generated during both stages of the reaction, similar radical species are apparently involved in both stages. The dominant generation of dopa and *m*-tyrosine, and the lower levels of the hydroperoxy amino acids, are consistent with the formation of hydroxyl radicals or metal-oxo species during glycoxidation reactions. The low levels of dityrosine are expected in a circumstance where there is a low frequency of formation of pairs of tyrosyl radicals close to each other. There are also a variety of alternative, competing pathways for formation of dityrosine from tyrosyl radicals (see, for example, [37,38]).

Our analytical procedures for the aliphatic amino acid derivatives are based on conversion of the protein hydroperoxides into hydroxides by reduction with $NaBH_4$, and measurement of the latter. We have shown that metal ions, glutathione peroxidase and intact cells detoxify protein hydroperoxides, converting them largely into hydroxides, although the intermediate chemistry involves secondary radical production in some circumstances [39]. In the present study we have not determined the quantities of aliphatic amino acid hydroperoxides directly because the presence of redox-active metal ions in our systems would ensure their rapid conversion into hydroxides [25,39]. In natural AGE proteins, however, these side products might accumulate, contributing to, if not determining, many of the biological properties of AGE proteins *in vivo* (see below). The dependence of both phases of protein oxidation on the availability of both oxygen and metal ions is consistent with a metal ion-catalysed mechanism for the production of hydroxyl radicals from primary sugar or protein-sugar derivative radicals, probably through the intermediacy of superoxide and H_2O_2. The reduction of auto-oxidizable components of the glycated protein by $NaBH_4$ prevents the protein oxidation in the second phase, again consistent with such a mechanism.

Our experiments also provide chemical evidence for two categories of reactive species produced on proteins during glycoxidation, the protein-bound Amadori compound and the amino acid oxidation products. As was already clear [3,40], the use of the term AGE proteins to describe such glycoxidized proteins is seductive but misleading, because the proteins are far from 'end products' but, rather, are reactive intermediates. Further, despite evidence that AGE proteins induce oxidative stress, there is no evidence that AGEs themselves are redox active. Indeed the known AGEs are conspicuously redox inactive (CML, pentosidine, crosslinks and dilysine imidazolium crosslinks). Some of these protein-bound redox-active intermediates or structural modifications caused in the proteins by oxidation chemistry during the formation of AGE proteins might actually serve as ligands for the plethora of cellular receptors that can bind AGE-modified proteins [41–43]. Even in those cases in which AGEs themselves are specifically recognized by receptors, the protein-bound dopa and amino acid hydroperoxides might be responsible for signal transduction, including the initiation of oxidative stress and inflammatory responses [44,45].

The presence of redox-active species on glycated and AGE proteins suggests that all of these species might initiate secondary reactions on other biomolecules. This has already been observed in terms of the initiation of lipid peroxidation [12] and of altered interaction with reactive nitrogen intermediates [30]. Indeed, the self-sustained metal ion-catalysed generation of superoxide radicals, causing superoxide dismutase-sensitive cytochrome *c* reduction, has been argued to be part of the mechanism by which AGE proteins trigger oxidative stress in certain target cells [43]. The present study suggests that protein oxidation products might be important in these reactions and, considering the greater reactivity of dopa and amino acid peroxides than of Amadori products and undefined AGEs, might be the dominant source of reactive oxygen generated by AGE protein. Because we have observed such amino acid oxidation products to be present in human plasma proteins, and in higher amounts in certain pathologies [33], these protein oxidation products might contribute importantly to the development of pathology in diabetes.

This work was supported by Research Grant DK19971 (J.W.B.), AG11472 (S.R.T.) and supported in part by the Australian Research Council (S.F.)

REFERENCES

1 Wolff, S. P., Jiang, Z. Y. and Hunt, J. V. (1991) Free Radicals Biol. Med. **10**, 339–352
2 Baynes, J. W. (1996) in The Maillard Reaction: Consequences for the Chemical and Life Sciences (Ikan, R., ed.), pp. 55–72, Wiley, New York
3 Thorpe, S. and Baynes, J. W. (1996) Drugs Aging **9**, 69–77
4 Thornalley, P. J., Wolff, S. P., Crabbe, M. J. and Stern, A. (1984) Biochem. J. **217**, 615–622
5 Wolff, S. P. and Dean, R. T. (1987) Biochem. J. **245**, 243–250
6 Wolff, S. P., Bascal, Z. A. and Hunt, J. V. (1989) Prog. Clin. Biol. Res. **304**, 259–275
7 Ahmed, M. U., Thorpe, S. R. and Baynes, J. W. (1986) J. Biol. Chem. **261**, 4889–4894
8 Dean, R. T., Thomas, S. M., Vince, G. and Wolff, S. P. (1986) Biomed. Biochim. Acta **45**, 1563–1573
9 Wolff, S. P. and Dean, R. T. (1987) Bioelectrochem. Bioenerg. **18**, 283–293
10 Ahmed, M. U., Dunn, J. A., Walla, M. D., Thorpe, S. R. and Baynes, J. W. (1988) J. Biol. Chem. **263**, 8816–8821
11 Hunt, J. V., Bottoms, M. A. and Mitchinson, M. J. (1993) Biochem. J. **291**, 529–535
12 Hicks, M., Delbridge, L., Yue, D. K. and Reeve, T. S. (1988) Biochem. Biophys. Res. Commun. **151**, 649–655
13 Fu, M.-X., Wells-Knecht, K. J., Blackledge, J. A., Lyons, T. J., Thorpe, S. R. and Baynes, J. W. (1994) Diabetes **43**, 676–683
14 Wells-Knecht, K. J., Zyzak, D. V., Litchfield, J. E., Thorpe, S. R. and Baynes, J. W. (1995) Biochemistry **34**, 3702–3709
15 Wells-Knecht, M. C., Thorpe, S. R. and Baynes, J. W. (1995) Biochemistry **34**, 15134–15141
16 Reddy, S., Bichler, J., Wells-Knecht, K. J., Thorpe, S. R. and Baynes, J. W. (1995) Biochemistry **34**, 10872–10878
17 Ikeda, K., Higashi, T., Sano, H., Jinnouchi, Y., Yoshida, M., Araki, T., Ueda, S. and Horiuchi, S. (1996) Biochemistry **35**, 8075–8083
18 Fu, M.-X., Requena, J. R., Jenkins, A. J., Lyons, T. J., Baynes, J. W. and Thorpe, S. R. (1996) J. Biol. Chem. **271**, 9982–9986
19 Hunt, J. V., Dean, R. T. and Wolff, S. P. (1988) Biochem. J. **256**, 205–212
20 Wolff, S. P. and Dean, R. T. (1988) Biochem. J. **249**, 618–619
21 Simpson, J. A., Narita, S., Gieseg, S., Gebicki, S., Gebicki, J. M. and Dean, R. T. (1992) Biochem. J. **282**, 621–624
22 Gieseg, S. P., Simpson, J. A., Charlton, T. S., Duncan, M. W. and Dean, R. T. (1993) Biochemistry **32**, 4780–4786
23 Gebicki, S. and Gebicki, J. M. (1993) Biochem. J. **289**, 743–749
24 Jessup, W., Dean, R. T. and Gebicki, J. M. (1994) Methods Enzymol. **233**, 289–303
25 Fu, S., Hick, L. A., Sheil, M. M. and Dean, R. T. (1995) Free Radicals Biol. Med. **19**, 281–292
26 Davies, M. J., Fu, S. and Dean, R. T. (1995) Biochem. J. **305**, 643–649
27 Neuzil, J., Gebicki, J. M. and Stocker, R. (1993) Biochem. J. **293**, 601–606
28 Davies, M. J. (1996) Arch. Biochem. Biophys. **336**, 163–172
29 Heinecke, J. W., Li, W., Daehnke, H. D. and Goldstein, J. A. (1993) J. Biol. Chem. **268**, 4069–4077
30 Dean, R. T., Fu, S., Stocker, R. and Davies, M. J. (1997) Biochem. J. **324**, 1–18
31 Knecht, K. J., Dunn, J. A., McFarland, K. F., McCance, D. R., Lyons, T. J., Thorpe, S. R. and Baynes, J. W. (1991) Diabetes **40**, 190–196
32 Stegemann, H. and Stalder, K. (1967) Clin. Chim. Acta **18**, 267–273
33 Fu, S. and Dean, R. T. (1997) Biochem. J. **324**, 41–48

34 Dean, R. T., Fu, S., Gieseg, S. P. and Armstrong, S. G. (1996) in Free Radicals: A Practical Approach (Punchard, N. A. and Kelly, F. J., eds.), pp. 171–183, IRL Press, Oxford
35 Zu, J., Morita, J., Nishikawa, S. and Kashimura, N. (1996) Carbohydr. Lett. **1**, 457–464
36 Johnson, R. N., Metcalf, P. A. and Baker, J. R. (1983) Clin. Chim. Acta **127**, 87–95
37 von Sonntag, C. (1987) The Chemical Basis of Radiation Biology, Taylor and Francis, London
38 Winterbourn, C. C., Pichorner, H. and Kettle, A. J. (1997) Arch. Biochem. Biophys. **338**, 15–21
39 Fu, S., Gebicki, S., Jessup, W., Gebicki, J. M. and Dean, R. T. (1995) Biochem. J. **311**, 821–827
40 Baynes, J. W. (1996) in Drugs, Diet and Disease, vol. 2, (Ioannides, C., ed.), pp. 201–240, Pergamon, London
41 Schmidt, A. M., Mora, R., Cao, R., Du Yan, S., Brett, J., Ramakrishnan, R., Tsang, T. C., Simionescu, M. and Stern, D. (1994) J. Biol. Chem. **269**, 9882–9888
42 Westwood, M. E., McLellan, A. C. and Thornalley, P. J. (1994) J. Biol. Chem. **269**, 32293–32298
43 Du Yan, S., Schmidt, A. M., Anderson, G. M., Zhang, J., Brett, J., Zou, Y. S., Pinsky, D. and Stern, D. (1994) J. Biol. Chem. **269**, 9889–9897
44 Grant, A. J., Jessup, W. and Dean, R. T. (1993) Free Radicals Res. Commun. **18**, 259–267
45 Grant, A. J., Jessup, W. and Dean, R. T. (1993) Free Radicals Res. Commun. **19**, 125–134

Received 17 July 1997/15 September 1997; accepted 1 October 1997