Amino Acids (1996) 11: 69–81

Amino
Acids
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Printed in Austria

L-Arginine inhibits in vitro nonenzymatic glycation and advanced glycosylated end product formation of human serum albumin*

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Accepted January 26, 1996

Summary. L-Arginine (Arg) has a structure similar to that of aminoguanidine (AG) and may inhibit glycation and advanced glycosylated end product (AGE) formation. Human serum albumin (HSA) (100 mg/ml) was incubated for 2 weeks with glucose (200 mM) at 37°C or with glucose and equimolar concentrations of Arg, N-α-acetyl Arg, or AG with or without 25 mM diethylenetriaminepentaacetic acid (DTPA). In the absence of DTPA, electrospray ionization mass spectrometry showed a 70% reduction of covalently bound glucose in the presence of Arg and a 30% reduction with AG. Digestibility by trypsin of HSA incubated with glucose and Arg was similar to that of HSA incubated alone. This suggests less covalent modification of HSA in the presence of Arg as compared with the absence of Arg. When incubations contained DTPA, autoradiography showed less ^{14}C labeling of HSA subunits in the presence of Arg and AG. When the α-amino group of Arg was blocked with an acetyl group, labeling was similar to that of HSA incubated with glucose, suggesting involvement of the α-amino group in the inhibition. Fluorescence of HSA at ex_{370} and em_{440} was reduced with Arg, but AG was more effective than Arg. These results suggest that Arg, like AG, can inhibit glycation and AGE formation.

Keywords: Amino Acids – Arginine – Glycation – Maillard reaction – Human serum albumin – Aminoguanidine – Electrospray

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*Presented in part at the FASEB meeting, Atlanta, GA, 1991.

Introduction

Nonenzymatic glycation, commonly known as the Maillard reaction, occurs when proteins either in vivo or in vitro are exposed to and react with reducing sugars such as glucose and fructose (Van Boekel, 1991; Wolff et al., 1991). The Maillard or browning reaction has long been recognized in industry as an indicator of the aging and deterioration process in foods and has recently been the subject of biological experimentation (Brownlee et al., 1988a). The focus of the biological research has been in developing models and mechanisms to help explain progression of diabetic complications (Van Boekel, 1991; Brownlee et al., 1984, 1988b; Dyer et al., 1991).

Glycation of long-lived structural proteins (e.g., collagen) and transport proteins (e.g., hemoglobin) can lead to loss of solubility, reduced digestibility, and subsequent deterioration of the protein (Dyer et al., 1991). Human serum albumin (HSA) is an important transport protein because of its abundance and its role in binding free fatty acids. One study reported that because of glycation of serum albumin, the half life of HSA in diabetic dogs was increased compared to its half life in normal animals (Morris and Preddy, 1986). Little is known about the mechanism of intracellular and extracellular events that lead to glycation and subsequent protein modification. Initial steps in the Maillard reaction are known; i.e., the condensation of a reducing sugar with an *alpha* or *epsilon* amino group of the protein to form a Schiff base (Brownlee et al., 1988b) and subsequent rearrangement to form an Amadori product or ketoamine. The Amadori product can undergo a complex series of reactions which eventually lead to formation of advanced glycosylated end products (AGE) (Dyer et al., 1991).

Reports in the literature suggest that aminoguanidine (AG) may impede covalent modification of proteins by glucose and/or formation of AGE (Soulis-Liparota et al., 1991; Brownlee et al., 1986; Edelstein and Brownlee, 1992). Other studies have shown competitive inhibition of lens protein glycation by amino acids such as lysine and glycine in vitro (Ramakrishnan and Sulochana, 1993) and in vivo (Sensi, M. et al., 1993). Arginine (Arg), an amino acid which has a structure similar to that of AG, may have some application as an inhibitor of protein modification by glucose and/or AGE formation. This amino acid has been reported to reduce the formation of hexosyllysines (Lubec, G. et al., 1991), AGE's such as carboxymethyllysine (Weninger, et al., 1992) and Amadori products (Menzel and Reihsner, 1991). The present study was designed to explore the possible mechanism of inhibition in an in vitro system. HSA glycation was studied under oxidative and non-oxidative conditions with or without added Arg or AG. The techniques used to characterize the degree of HSA glycation and crosslinking were electrospray ionization/mass spectrometry (ESI/MS) (Smith et al., 1990), reverse-phase high pressure liquid chromatography (HPLC) of tryptic digests of glycated and nonglycated HSA, autoradiography, and fluorescence.

Materials and methods

In vitro incubation studies

HSA (100 mg/ml) was incubated alone or with 200 mM glucose, or with 200 mM glucose plus 200 mM L-arginine-HCl, or with 200 mM glucose plus 200 mM aminoguanidine in Dulbecco's phosphate-buffered saline (pH, 7.4). All incubation tubes contained 0.1% sodium azide and were incubated in the presence of oxygen. These samples were prepared for ionization MS and reverse-phase HPLC. Another set of incubation tubes was prepared with [D-{U^{14}C}] glucose (specific activity = 332 mCi/mmol) and 25 mM diethylenetriaminepentaacetic acid (DTPA). Included in this group was an additional set of incubations with 200 mM N-α-acetyl-L-arginine substituted for Arg. Labeled glucose was purchased from ICN Biomedicals (Costa Mesa, CA) and was repurified by preincubation with 10 mg/ml HSA (Sigma Chemical Co., St. Louis, MO), essentially fatty acid-free, overnight at 37°C in Dulbecco's PBS, pH 7.40. Free glucose (repurified) was separated from protein bound contaminants by centrifugation in an Amicon MPS-1 centricon with molecular weight cutoff of 30,000 daltons (Da). Ultrafiltrate was collected and used in labeled incubations. A trace amount of repurified D-[(U)^{14}C] glucose was added to each tube containing unlabelled glucose. All other components of the labeled tubes were identical to those described above, except that tubes incubated with DTPA were purged with nitrogen and tightly sealed at the start of the incubation. All incubations were continued for 2 weeks under aseptic conditions at 37°C, and aliquots were removed at 0.5-week intervals.

Protein bound glucose was separated from unreacted glucose by gel filtration, using Sephadex G-25 columns. Total protein of the modified fraction was determined by the method of Bradford (1976), using Bio-Rad (Hercules, CA) protein reagent.

Trypsin digestions

Aliquots of equivalent amounts of HSA from the unlabeled modified fractions (incubated for 2 weeks) were digested for 2 h in 0.046 M Tris-HCl buffer (pH 8.1) at 37°C with 10 μg trypsin added at the beginning and again at 1 h after the start of incubation. Trypsin was obtained from bovine pancreas (Type III-S, activity = 13,900 BAEE units/mg protein).

Electrospray ionization/mass spectrometry (ESI/MS)

Aliquots from the unlabeled modified fraction were desalted by reverse-phase HPLC before ESI/MS. An RP-300 Aquapore 10 μm (C_8) column was used with a step gradient of 0–100% B for 30 min followed by 100% B for 30–50 min, where A was 0.1% aqueous trifluoroacetic acid (TFA), and B was 0.1% TFA in 60% acetonitrile. The molecular weights of 2-week modified fractions were determined by positive-ion mode electrospray. ESI/MS was performed by using a Vestec (Houston, TX) electrospray source fitted to a 5988A single quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA). Typically, 50 μg HSA per 100 μl 25% aqueous acetic acid was infused into the mass spectrometer at 5 μl/min by using a Harvard 22 infusion pump. The molar ratio of glucose bound/HSA was calculated by dividing the difference between the sequenced weight of HSA and the modified fraction weight by the molecular weight of glucose (180 g/mole).

Reverse-phase HPLC

Modified fractions of trypsin digested for 2 weeks were analyzed by HPLC. The instrument was equipped with an Isco 2350 pump, an Isco 22360 gradient programmer, a Hitachi L-3000 diode array detector, and a Brownlee Aquapore RP-300 column (250 × 4.6 nm). The eluant gradient used was 0–100% B in A over 2–120 min, where A = 0.1% aqueous TFA and B = 0.1% TFA in 60% aqueous acetonitrile.

Autoradiography

Aliquots from ^{14}C-labeled modified fractions incubated for 2 weeks were electrophoresed on a 5–20% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gradient gel (14 × 14 cm), using Laemmli (1970) buffers that included 0.1% SDS in the electrode buffer and the gel matrix. A 100-μg aliquot of HSA from modified fractions was loaded in each lane to provide enough radioactivity to identify glycated subunits. After electrophoresis, the gel was stained with Coomassie Blue dye, treated with Entensify (DuPont, NEN Research Products, Boston, MA), dried, and exposed for 9 months to high speed film (Kodak).

Fluorescence

Fluorescence of glycated HSA (100–200 μg/ml) was measured at an excitation wavelength of 370 nm with emission at 440 nm (pH 7.4). Data are reported as relative fluorescence/mg HSA.

Statistical analysis

Data were analyzed by a combination of analysis of covariance and least squares linear regression (Sokal and Rolf, 1981). Data were log-transformed before statistical analysis to meet the assumptions of analysis of covariance. Means within time periods were compared by the least squares difference test. Means were considered significantly different when $p < 0.05$. Trends over time were examined by linear regression analysis. Mean fluorescence was calculated from five observations per treatment group.

Results

Modified fractions that were incubated for 2 weeks without DTPA were analyzed by ESI/MS. An electrospray spectrum of HSA incubated with glucose and aminoguanidine (Fig. 1) is typical of glycosylated proteins. The broad peaks indicate a considerable degree of heterogeneity of glycosylation (Vidal et al., 1992). The molar ratios of glucose bound/HSA are reported in Table 1. When HSA is incubated with glucose in the presence of Arg or AG, there is a reduction in the moles of glucose bound to HSA. Furthermore, Arg reduces the amount of glucose bound to HSA more than does AG under aerobic conditions.

The degree of covalent modification of HSA by glucose was tested by examining its susceptibility to proteolytic digestion. Glycated HSA was digested with trypsin, and digests were analyzed by reverse-phase HPLC. The assumption was made that less susceptibility of HSA to proteolytic digestion suggested more covalent modification. Figures 2A and 2B show undigested HSA eluting as the largest peak at approximately 90 min. To compare HSA susceptibility to trypsin digestion, the areas under the undigested HSA peak were calculated (Table 2). When HSA is incubated with glucose (Fig. 2B), the area under the undigested peak is four times that of HSA incubated alone (Fig. 2A). When Arg and AG are present, both areas under the undigested HSA peak are reduced, with Arg showing the most similarity to HSA incubated alone. These findings may suggest less covalent modification of HSA by glucose in the presence of inhibitors and a relationship between the amount of glucose bound and extent of covalent modification.

Table 1. Molecular weights of human serum albumin (HSA) incubated for 2 weeks with glucose (gluc) or with glucose + inhibitors

Modified fraction[1,2]	Molecular weight (g/mole)	Molar ratio (gluc/HSA)
Sequenced HSA	66,439	
HSA + gluc	67,400 ± 200	5–7 gluc/HSA
HSA + gluc + Arg	66,570 ± 100	1–2 gluc/HSA
HSA + gluc + AG	67,000 ± 100	3–5 gluc/HSA

[1] Modified fractions incubated for 2 weeks were desalted under acidic conditions and analyzed by ESI/MS.
[2] HSA was incubated for 2 weeks at 37°C with gluc in the presence or absence of Arg or AG without DTPA using aseptic conditions.

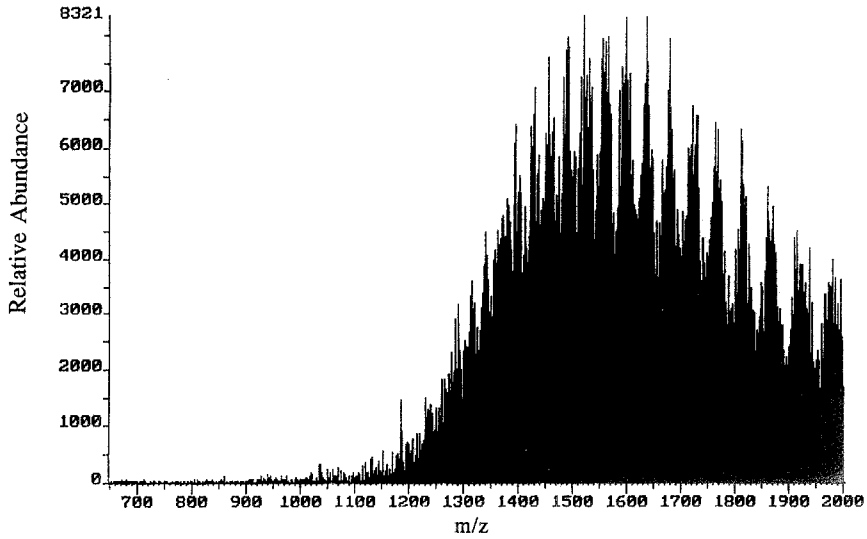

Fig. 1. Electrospray spectrum of HSA incubated with glucose and aminoguanidine (AG). HSA (100 mg/ml) was incubated with 200 mM glucose and 200 mM AG for 2 weeks at 37°C without DTPA under aseptic conditions. Glycated HSA was desalted by HPLC before analysis, and 50 μg was analyzed by electrospray. The molecular weight of the main component obtained after deconvolution of the multiple charged peaks was 67,000 ± 100 Da

Table 2. Areas under undigested HSA peak from trypsin digested samples

Modified fraction[1]	Area under peak ($Abs_{215nm} \times Time_{min}$)
HSA alone	1.0
HSA + gluc	4.5
HSA + gluc + Arg	1.4
HSA + gluc + AG	2.2

[1] HSA (500 μg) from modified fractions was removed and digested for 2 h with trypsin, and an aliquot was subjected to reverse-phase HPLC to separate peptides from undigested HSA. Area of the undigested peak was calculated.

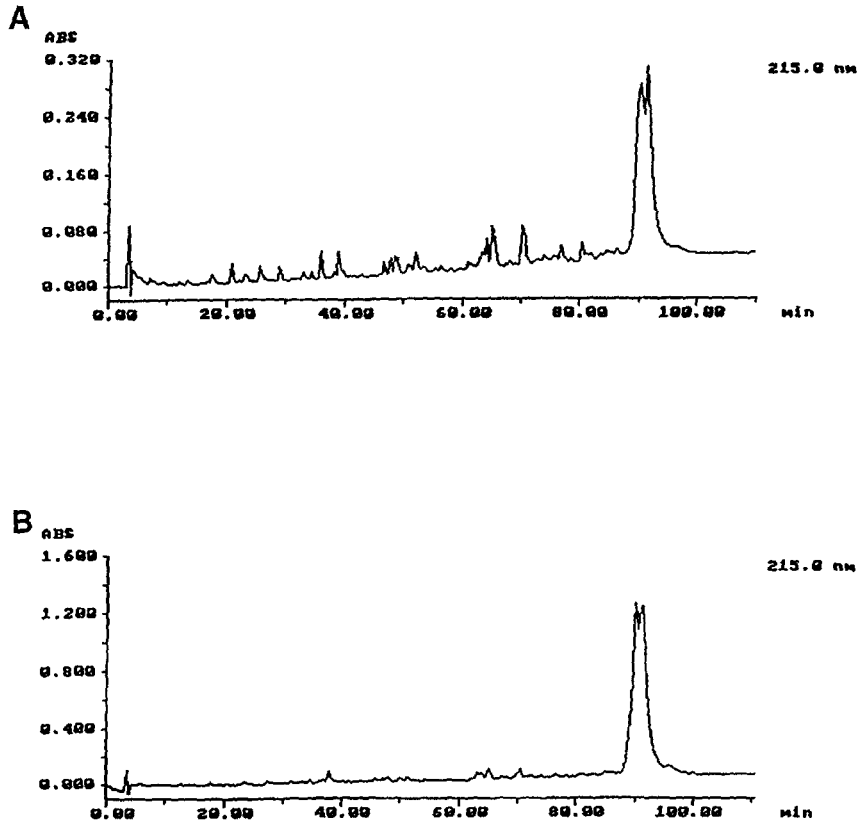

Fig. 2. Chromatogram of trypsin-digested HSA incubated alone (**A**) and with 200 mM Glucose (**B**). HSA (100 mg/ml) was incubated alone or with 200 mM glucose for 2 weeks at 37°C without DTPA under aseptic conditions. HSA (500 μg) from the modified fraction was digested with trypsin for 2 h, and an aliquot was analyzed by reverse-phase HPLC

To further characterize inhibition of glycation by Arg, HSA was incubated under nonoxidative conditions in the presence of 25 mM DTPA followed by purging incubation tubes with nitrogen. DTPA is known to inhibit autoxidation of glucose and formation of glycoxidation products (Hunt et al., 1988; Fu et al., 1992). In addition, it was of interest to know if Arg was reactive via the α-amino or the guanidinium amino group, and which amino group was important under oxidative and nonoxidative conditions. Therefore, we incubated HSA with and without DTPA in the presence of equimolar concentrations of glucose and Arg with the α-amino group blocked (N-α-acetyl-L-arginine). Radiolabeled modified fractions were electrophoresed by SDS-PAGE followed by autoradiography of the labeled gel. SDS-PAGE and autoradiography were used to determine if Arg would still inhibit covalent binding of glucose to HSA under nonoxidative conditions. Autoradiography showed less labeling in lanes 2, 4, and 5 than in lanes 1 and 3 (Fig. 3B). These data indicate (similar to ESI/MS) that Arg is inhibiting covalent glucose binding to HSA. Furthermore, blocking the α-amino group of Arg significantly increased the degree of HSA labeling (lane 3 compared to lane 2). In contrast, in the absence of DTPA (lane 5), blocking the α-NH_2 group had no

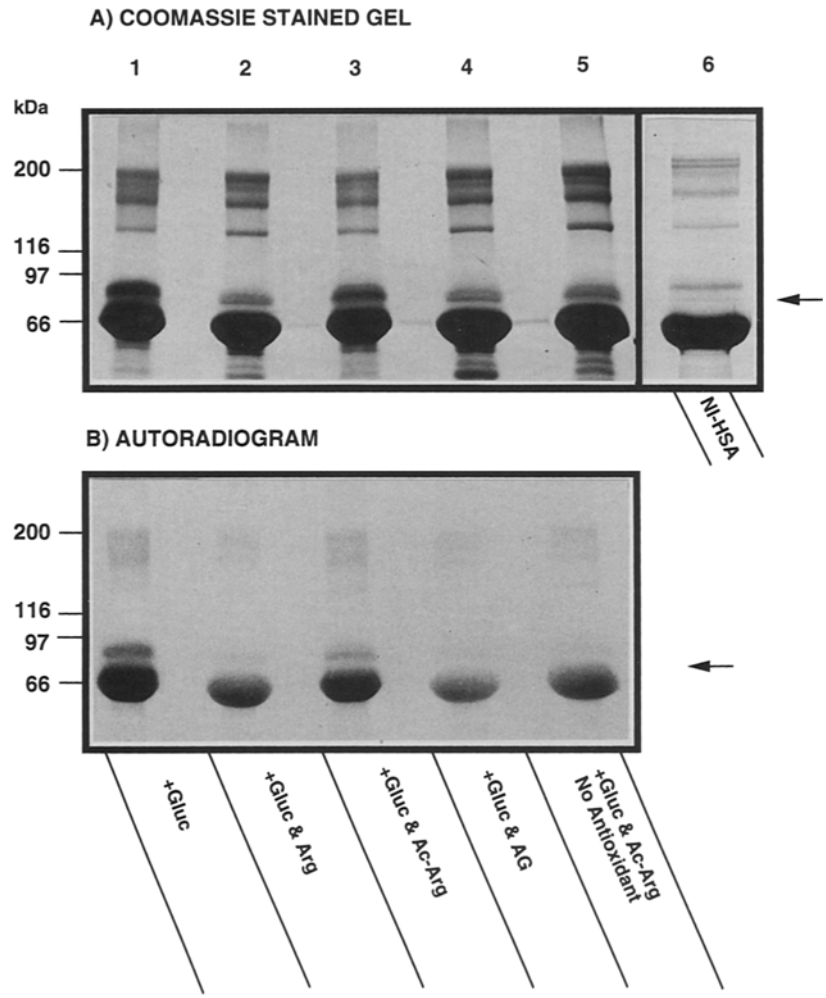

Fig. 3. SDS-PAGE and autoradiography of HSA incubated with glucose in the presence or absence of Arg or AG. Samples were incubated for 2 weeks (see Materials and methods) in the presence of 25 mM DTPA, with the exception of nonincubated HSA. Nonincubated HSA was electrophoresed separately under the same conditions as those used for modified fractions and was included in lane 6 as a control. Lane 5 shows HSA incubated with glucose and Ac-Arg in the absence of DTPA. Protein (100 μg) was loaded in each lane and electrophoresed on a 5–20% SDS gradient gel: **3A**, Coomassie Blue staining of protein bands; **3B**, autoradiography of the same gel. *NI-HSA*, nonincubated human serum albumin; *Gluc*, glucose; *Arg*, arginine; *Ac-Arg*, N-acetyl-L-arginine; *AG*, aminoguanadine

effect on glucose labeling. In fact, the labeling was reduced similar to that seen in lane 2. These data suggest that under oxidative conditions, the guanidinium group is important in reducing HSA glycation contrary to non-oxidative conditions where the α-NH_2 group appears to be more critical.

Total fluorescence of modified fractions at ex_{370} and em_{440} was measured at all time points. Because incubations were performed under anaerobic conditions, it was assumed that crosslinking of HSA was primarily due to glycation

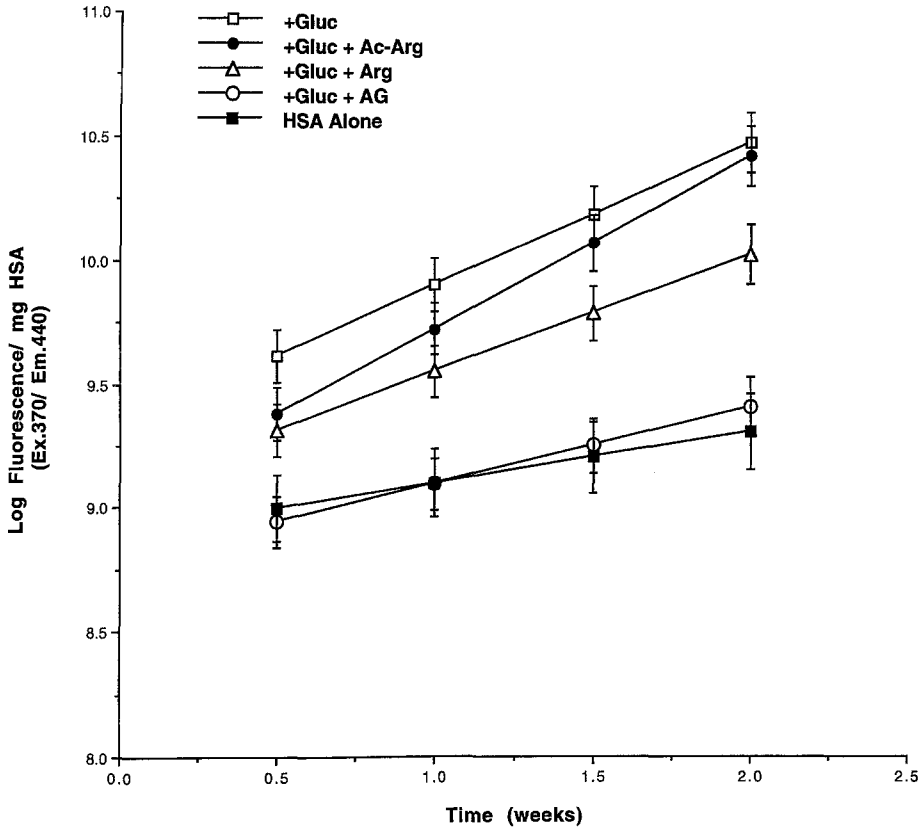

Fig. 4. Change over time in relative fluorescence of HSA incubated alone or with glucose in the presence or absence of Arg, N-acetyl Arg, or AG. Fluorescence/mg HSA at Ex_{370} Em_{440} of incubated modified fractions was measured at 0.5, 1, 1.5, and 2 weeks of incubation in the presence of 25 mM DTPA. Least square log means with the pooled SEM (n = 5) are reported. *HSA*, human serum albumin; *Gluc*, glucose; *Arg*, arginine; *Ac-Arg*, N-acetyl-L-arginine; *AG*, aminoguanidine

and formation of dicarbonyl compounds such as 1- and 3-deoxyglucosones rather than glycation and oxidation. Statistical modeling was used to examine the trend over time. HSA incubated with glucose showed a threefold increase in fluorescence compared to HSA incubated alone (Fig. 4). In the presence of Arg, there was a modest decrease in the fluorescence compared to the absence of Arg. Arg did not appear to affect fluorescence until 1.5 ($p < 0.02$) and 2.0 ($p < 0.01$) weeks. Blocking the α-amino group had no effect until 2 weeks, when the fluorescence began to approach that of HSA incubated with glucose. In contrast to Arg, AG consistently inhibited increases in HSA fluorescence at all time points ($p < 0.0001$) (Fig. 4).

Discussion

These studies were undertaken to test the viability of Arg as an inhibitor of glycation and AGE product formation as compared to an established inhibitor such as AG. High concentrations of fatty acid-free HSA (100 mg/ml) and

glucose (200 mM) were used to accelerate the rate of Amadori product formation during a relatively short-term incubation of 2 weeks (Baynes, 1984). Under these conditions, approximately 5–7 moles of glucose bound to 1 mole of HSA (Table 1). This amount of bound glucose is slightly higher than 2.35 moles glucose/mole HSA, reported by Vidal et al. (1992) and 3.3 moles glucose/mole HSA, reported by Hunt et al. (1993). This difference might be accounted for by an observation by Mereish et al. (1982), who reported that glycation of HSA in vitro was enhanced when fatty acids were removed from the HSA before reaction with glucose. In the presence of Arg, the amount of glucose bound to HSA was reduced to 1–2 moles (Table 1). This reduction in covalently bound glucose was approximately 70% compared to a 30% reduction in the presence of AG. In addition, the greater modification of HSA by glucose in the presence of AG than Arg was associated with decreased susceptibility to trypsin digestion as seen by a larger area under the undigested HSA peak (Table 2). These data suggest that under oxidative conditions (no DTPA), at 200 mM concentration, Arg is more effective than AG in inhibiting covalent modification of HSA by glucose. However, AG has been shown to be an effective inhibitor of AGE formation at 10 mM concentrations (Fu et al., 1994), and since Arg has not been tested in our system at lower concentrations, it is not known whether Arg would be equally as effective as AG at lower concentrations. In agreement with our findings of reduced covalently bound glucose by Arg are those of Menzel and Reihsner (1991) and Lubec, et al. (1991), who reported that Arg reduced incorporation of ^{14}C-glucose and Amadori product formation, respectively. Furthermore, oral administration of L-Arg to diabetic db/db mice reduced carboxymethyllysine and the thickness of GBM as discussed earlier (Weninger et al., 1992).

Because the data reported above were generated under oxidative conditions, it was not clear whether inhibition of glycation by Arg was due to its reaction with glucose or an oxidized product of glucose. It was suggested that in the presence of oxygen, glucose may undergo autoxidation to form α-ketoaldehydes (Wolff and Dean, 1987), which in turn may react with the protein itself (Fu et al., 1994) or with a primary or secondary amino acid (e.g., Arg) (Ledl, 1991). Consequently, it was of interest to know if Arg might still reduce HSA glycation under nonoxidative conditions and to determine which amino group of Arg might be involved in the inhibition. To explore these questions, we added DTPA to each incubation tube and then purged the tubes with nitrogen, using either N-alpha-acetyl-L-arginine (Ac-Arg) or Arg. Incubation tubes with Ac-Arg were also included which contained no DTPA (oxidative condition). SDS-PAGE and autoradiography of modified fractions showed less labeling of HSA with U-^{14}C-glucose in the presence of Arg and AG. This decreased labeling was associated with changes in the migration of the 66-kDa HSA subunit (Figs. 3A and B). When the α-amino group of Arg was blocked (lane 3), the 66-kDa band was more intensely labeled than that in lane 2. This finding suggests that in the presence of antioxidant, the α-amino group is involved in inhibiting HSA glycation (perhaps by reacting with glucose itself) in the early stages of the Maillard reaction. In the absence of DTPA, where glucose autoxidation occurs (Wells-Knecht et al., 1995), block-

ing the α-NH_2 group does not result in more glucose labeling of HSA (lane 5). These data suggest involvement of the guanidinium group which may react with oxidized products of glucose (e.g. dicarbonyls) and competitively inhibit binding of these products to HSA.

To determine if Arg would inhibit AGE formation, fluorescence of HSA was measured and statistical modeling was used to examine changes in the mean fluorescence over time (Fig. 4). Incubations with AG consistently decreased AGE-related fluorescence at every time point. Incubations with Arg tended to reduce the fluorescence over time, with the biggest differences occurring at 1.5 ($p < 0.02$) and 2.0 weeks ($p < 0.01$) (Fig. 4). These data provide some evidence that Arg is inhibiting formation of AGE.

Significant differences in fluorescence and glucose labeling after 2 weeks suggest that Arg is reacting with glucose itself or may be forming a nonreactive substituted Amadori product (Brownlee, et al., 1986) via the α-NH_2 group in the early stages of the Maillard reaction. In the latter stages of the Maillard reaction, Arg could be reacting with intermediate products derived from Amadori rearrangement via the guanidinium group (Ledl and Schleicher, 1990). It has been proposed that Amadori products can breakdown into dicarbonyls (Ledl and Schleicher, 1990), e.g., 3-deoxyglucosone (Shin et al., 1988 and Zyzak et al., 1995), which can interact with an α-amino group of an amino acid, e.g., Arg, via Strecker degradation (Ledl and Schleicher, 1990), or may interact with the guanidinium group of Arg (Means and Feeney, 1971). In incubations of lysozyme with 3-deoxyglucosone, Kato et al. (1987) showed that Arg residues within the protein were impaired because of interaction with 3-deoxyglucosone. Further, Igaki et al. (1990) demonstrated that both BSA and collagen formed more AGE (as measured by fluorescence) when reacting with 3-deoxyglucosone than with glucose, and AG inhibited AGE formation by interacting directly with 3-deoxyglucosone. These data support the idea that Arg may react with an Amadori breakdown product (dicarbonyls), and this interaction might result in less AGE-related fluorescence at the later time points, with little change during the earlier time points.

Thus, with or without antioxidant (DTPA), Arg inhibited covalent binding of glucose to HSA. In the early stages of the Maillard reaction, the α-NH_2 group of Arg is important in this inhibition under non-oxidative conditions, because blocking this group significantly increases HSA glycation. Under oxidative conditions, however, the guanidinium group appears to be critical. In the latter stages of the Maillard reaction, the fluorescence data suggest (under non-oxidative conditions) that the α-NH_2 group of Arg is involved in reducing AGE formation. From our data and other published studies, the following mechanism is proposed (Fig. 5). The use of Arg as an inhibitor of glycation and/or crosslinking may have clinical significance in that inclusion of dietary Arg, whether from foods or supplements, may prevent development of diabetic complications arising from glycation. Indeed, Arg administered orally to diabetic humans has been shown to inhibit fructosamine formation in skin collagen (Lubec et al., 1991). Further studies are needed to test Arg as an inhibitor of glycation and/or AGE formation.

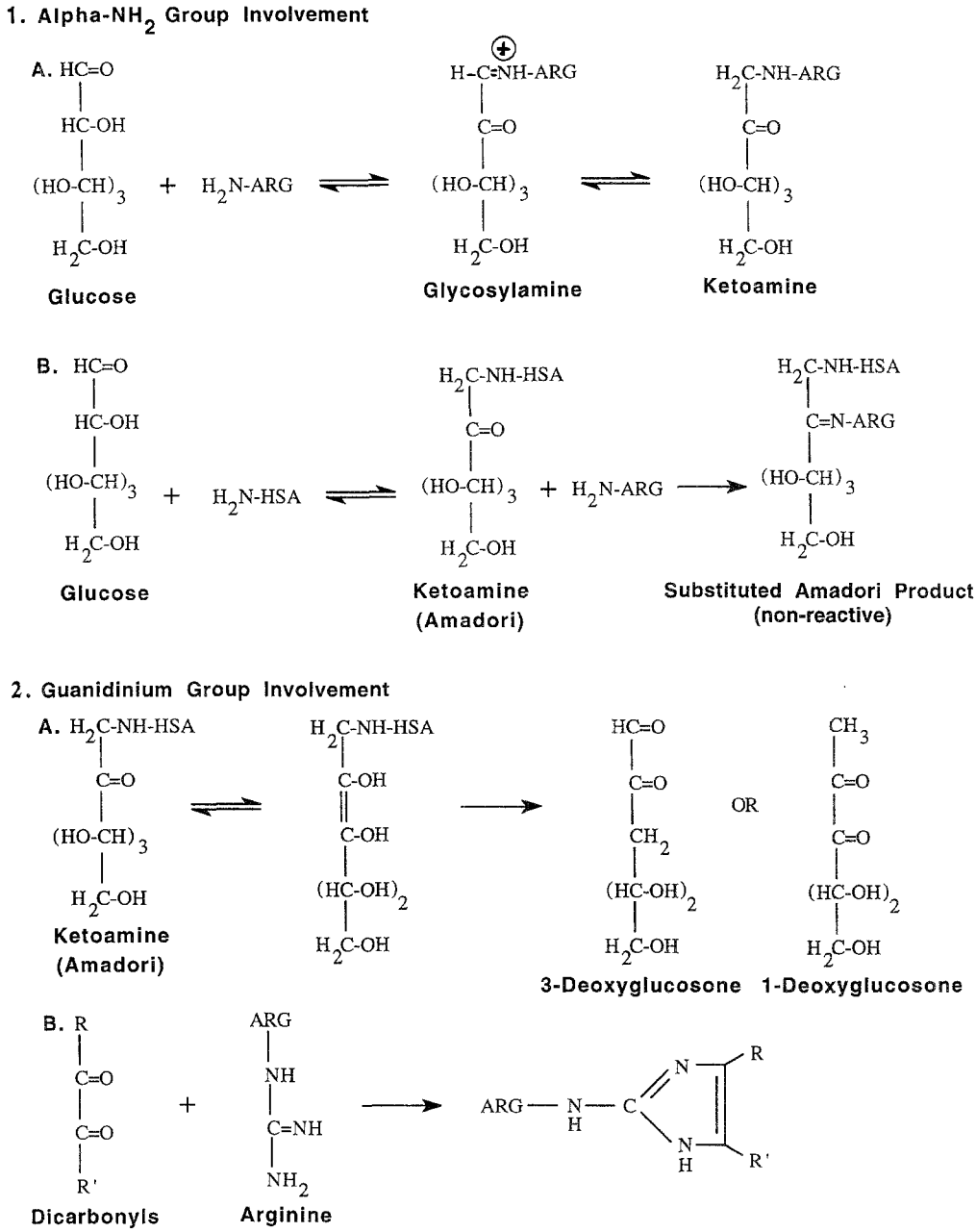

Fig. 5. Proposed mechanism of arginine inhibition of glycation and AGE formation. The chemical structures for **1** were reproduced with permission from Ledl and Schleicher (1990) Angewandte Chemie 29: 565–706 and Brownlee et al. (1986) Science 232: 1629–1632. Those from **2** were reproduced with permission from Schauenstein E, Esterbauer H, Zollner H (eds), Aldehydes in biological systems, Pion Ltd, London, pp 112–157

Acknowledgements

This work was performed and supported in part by funds from the laboratory of Dr. Otho Michaelis, IV, at the U.S. Department of Agriculture, Beltsville Human Nutrition Research Center, Beltsville, MD 20705. The authors gratefully acknowledge the support of Dr. Catherine Fenselau for providing the electrospray facilities at the Structural Bio-

chemistry Center at the University of Maryland Baltimore County, Baltimore, MD. The authors also acknowledge support from the National Science Foundation.

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