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Gramicidin S: a peptide model for protein glycation and reversal of glycation using nucleophilic amines

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Abstract: Nonenzymatic glycation of proteins has been implicated in various diabetic complications and age-related disorders. Proteins undergo glycation at the N-terminus or at the ε-amino group of lysine residues. Glycation of proteins proceeds through the stages of Schiff base formation, conversion to ketoamine product and advanced glycation end products. Gramicidin S, which has two ornithine residues, was used as a model system to study the various stages of glycation of proteins using electrospray ionization mass spectrometry. The proximity of two ornithine residues in the peptide favors the glycation reaction. Formation of advanced glycation end products and diglycation on ornithine residues in gramicidin S were observed. The formation of Schiff base adduct is reversible, whereas the Amadori rearrangement to the ketoamine product is irreversible. Nucleophilic amines and hydrazines can deglycate the Schiff base adduct of glucose with peptides and proteins. Hydroxylamine, isonicotinic acid hydrazide and aminoguanidine effectively removed glucose from the Schiff base adduct of gramicidin S. Hydroxylamine is more effective in deglycating the adduct compared with isonicotinic acid hydrazide and aminoguanidine. The observation that the hydrazines are effective in deglycating the Schiff base adduct even in the presence of high concentrations of glucose, may have a possible therapeutic application in preventing complications of diabetes mellitus. Hydrazines may be used to distinguish between the Schiff base and the ketoamine products formed at the initial stages of glycation.

Nonenzymatic glycosylation or glycation of proteins is a common post-translational modification of proteins, resulting from reactions between glucose and amino groups of proteins (1). The initially formed Schiff base adduct undergoes an irreversible Amadori rearrangement to a ketoamine product (2). Glycation of proteins has been linked to many pathological complications of diabetes mellitus – retinopathy, neuropathy and nephropathy (3,4), Alzheimer's disease (5) and aging (6). Several reports are available on the glycation of various proteins, notably albumin (7) hemoglobin A (8), collagen (9), RNase A (10) and several erythrocytic proteins (11). Various factors have been implicated in influencing the site and extent of glycation. The site selectivity of glycation in proteins has been suggested to be a consequence of the Amadori rearrangement ability of the glycation sites (12), which in turn depends on the microenvironment. The effect of three-dimensional structure (13) on the extent of glycation has also been reported. The importance of buffer in influencing ketoamine formation is well studied (14). At the later stages of the glycation reaction, oxidative cleavage of the sugar moiety leads to the formation of AGEs such as carboxymethyllysine (CML) (15) and pentosidine (6). CML formation is catalyzed under oxidative conditions (16). Formation of cleavage products such as glyoxal, methylglyoxal and 3-deoxyglucosone at the early stages of glycation has also been reported (17). Advanced glycation products such as carboxymethyllysine and carboxyethyllysine have been implicated as important intermediates in protein cross-linking reactions (18). The accumulation of AGEs has been correlated with aging and severity of diabetes. The CML adduct in proteins has been established *in vivo* in human tissues. A wealth of experimental evidence supports the hypothesis that AGEs have an important role in the development of diabetic complications and other diseases (19). Therefore, it is important to develop methods that can inhibit or reverse glycation at the initial stages.

In this report we describe the use of gramicidin S, a cyclic decapeptide, as a model for the study of protein glycation and its reversal in the presence of nucleophilic amines. Gramicidin S [cyclo(Leu-D-Phe-Pro-Val-Orn)$_2$] is a structurally well-defined peptide which is constrained to adopt an antiparallel αsheet structure with cyclization facilitated by two D-Phe-Pro α-turns. In this conformation, the alkylamino side chains on the two ornithine residues lie on the same phase of the α-sheet in close proximity (20,21). We show using electrospray ionization mass spectrometry (ESI-MS), multiple glycation and formation of advanced glycation products in gramicidin S and also rapid reversal of Schiff base using hydrazines such as hydroxylamine, aminoguanidine and isonicotinic acid hydrazide.

Materials and Methods

Gramicidin S hydrochloride and isonicotinic acid hydrazide (INH) were purchased from Sigma chemicals. D-Glucose, hydroxylamine hydrochloride and aminoguanidine were purchased from Ranbaxy Chemicals. The glucose solution was filtered through a 0.2-micron cellulose acetate filters (Hewlett Packard Inc.) prior to addition. Hydroxylamine hydrochloride in Milli Q water (5×10^{-3} M) was used in all experiments after adjusting the pH of the solution to 6 with sodium bicarbonate. Solutions of INH and aminoguanidine in Milli Q water (5×10^{-3} M) were used for the deglycation experiments.

Mass spectrometry

All electrospray spectra were recorded in the positive ion detection mode on a Hewlett-Packard 1100 MSD model electrospray mass spectrometer equipped with a single quadrupole and a conventional electrospray source. Pure water from a Milli Q apparatus (Millipore Inc.) with a conductance of 18.2 mΩ was mixed with equal volume of distilled methanol and used as the running solvent. The solvent flow rate was 30 μL/min. Approximately 200–300 pmol of sample was injected each time for analysis. The source temperature was kept at 300°C. Ions were extracted with an orifice potential of 4000 V applied at the nebulizer tip. The ionization was pneumatically assisted using a constant flow of pure N_2 gas at 10 L/min using a Whatman nitrogen generator. The same gas was used to maintain the nebulizer pressure at 10 bar. The nozzle skimmer potential was kept at 20 V. The mass spectrometer was calibrated using five *m/z* ions supplied by the manufacturer across the mass range 118–2500 *m/z*. The chromatographic peak width for operation was 0.18 min The cycle time was 1.90 s/cycle and the time filter was kept on. Data were acquired in the scan mode for 2 min and averaged over the complete chromatogram to obtain the mass spectrum. The charge states were determined using the isotopic distribution of the peaks and also from the difference between the peak values of the protonated and sodiated species. The major species observed is the doubly charged species in all cases.

In vitro glycation

In vitro glycation of gramicidin S was carried out by incubating the samples in glucose (analytical grade) solution at room temperature. The glycation mixture was made in a 1 : 1 mixture of methanol/water (v/v) with a final concentration of 2.5×10^{-3} M gramicidin S, 5.0×10^{-1} M glucose and 2.0×10^{-2} M sodium bicarbonate solution at pH 8.5. The rate of glycation was monitored at different intervals using electrospray ionization mass spectrometry. Deglycation of Schiff base using hydroxylamine was achieved by treating the samples with equal volumes of 5×10^{-3} M hydroxylamine solution and the samples were analyzed immediately using ESI-MS. In the cases of INH and aminoguanidine, deglycation of the Schiff base was done by treating the samples with equal volumes of 5×10^{-3} M solutions and incubating at 37°C for 4 h.

Oxime formation

Gramicidin incubated with glucose for 24 h was treated with excess hydroxylamine. The mixture was heated to 70°C in a water bath for 1 h and then analyzed using ESI-MS. A similar procedure was followed for the preparation of the aminoguanidine derivative.

Results

Glycation of gramicidin S

The ESI-MS spectrum (Fig. 1a) of gramicidin S shows the presence of two charge states $[M+H]^+$ and $[M+2H]^{2+}$ at *m/z* 1141.8 and 571.5, respectively. Upon incubation of gramicidin S with glucose at pH 8.5, glycation proceeds rapidly with ≈50% singly glycated species [**B**] being observed at 3 h. Table 1 summarizes the species detected by ESI-MS in the glycation mixture. The presence of two ornithine residues at proximal positions facilitates the glycation reaction. Multiply glycated species were observed on prolonged incubation. A significant amount of doubly glycated species [**C**] was observed after 6 h of incubation. Hydroxylamine was used to differentiate the Schiff base and ketoamine product. The former was completely converted to glucose and peptide by nucleophilic amines, whereas the later was unaffected. The sample incubated for 6 h, on treatment with hydroxylamine underwent complete deglycation indicating that the total 'glycated product' exists solely as the Schiff base adduct at this stage.

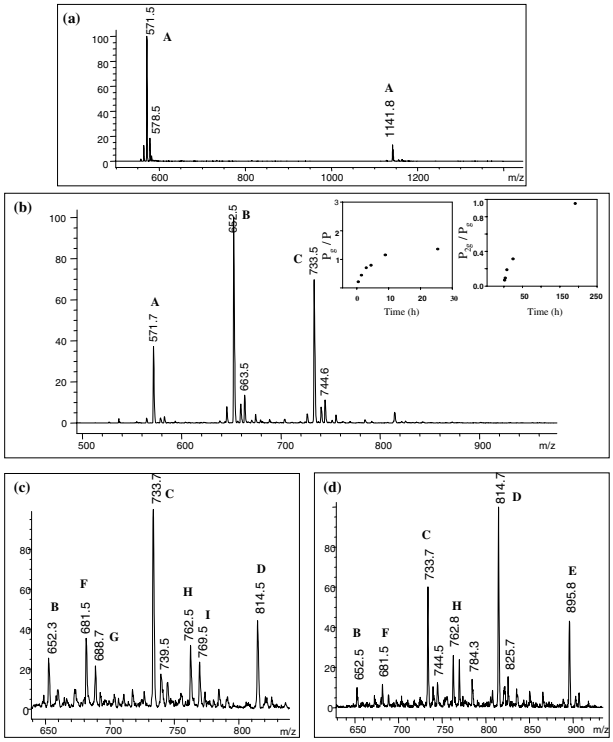

Figure 1. ESI mass spectra of (a) gramicidin S and gramicidin S incubated for (b) 1 day, (c) 100 days and (d) 140 days. The species are listed in Table 1. Insets in (b) show the ratio of singly glycated to native gramicidin (P_g/P) up to 30 h (left) and the ratio of doubly to singly glycated gramicidin (P_{2g}/P_g) up to 200 h (right).

The glycation product obtained after 24 h (Fig. 1b) of incubation with glucose, on treatment with hydroxylamine showed retention of some glycation, indicating the formation of ketoamine product. Similar results were obtained on treatment with aminoguanidine and isonicotinic acid hydrazide. Addition of excess hydroxylamine to the sample formed a significant amount of oxime on heating. In Fig. 2, the peak at *m/z* 571.6 corresponds to the doubly protonated $[M+2H]^{2+}$ gramicidin S. The peak at *m/z* 582.7 corresponds to the proton and sodium ion adduct $[M+H+Na]^{2+}$ of gramicidin S. The peaks at *m/z* 652.5 corresponds to the doubly charged state of singly glycated peptide. The peak at *m/z* 660.0 corresponds to the doubly charged state of the oxime of the singly glycated species. Similarly, the peaks at *m/z* 741.1 and 748.5 correspond to the doubly charged states of the mono- and bisoximes of the doubly glycated gramicidin S. Formation of oxime derivatives conforms that the singly and doubly glycated species are present as the ketoamine products at this stage. Formation of ketoamine product increases with time. The time course of glycation of gramicidin S is shown as inserts in Fig. 1b. The insert on the left shows the time course of the formation of singly glycated species and the insert on the right shows

Table 1. **List of species present in the glycation mixture of gramicidin S**

Code	Component	Calcd M_r	Peaks obsd *m/z*	Charged species present
A	Gramcidin S	1141	1141.8, 571.5	$[M+H]^+$, $[M+2H]^{2+}$
B	Gramicidin S+1 glucose	1303	652.5, 663.5	$[M+2H]^{2+}$, $[M+H+Na]^{2+}$
C	Gramicidin S+2 glucose	1465	733.5, 744.6	$[M+2H]^{2+}$, $[M+H+Na]^{2+}$
D	Gramicidin S+3 glucose	1627	814.5825.7	$[M+2H]^{2+}$, $[M+H+Na]^{2+}$
E	Gramicidin S+4 glucose	1789	895.5	$[M+2H]^{2+}$
F	CML of **C**	1361	681.5	$[M+2H]^{2+}$
G	CEL of **C**	1375	688.7	$[M+2H]^{2+}$
H	CML of **D**	1523	762.5, 784.5	$[M+2H]^{2+}$, $[M+H+Na]^{2+}$
I	CEL of **D**	1537	769.5	$[M+2H]^{2+}$

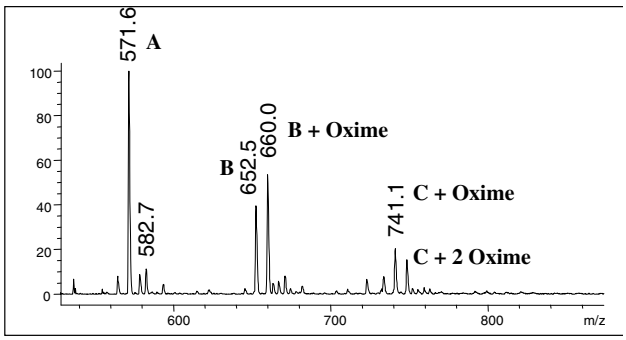

Figure 2. ESI mass spectrum of glycated gramicidin heated with hydroxylamine. Oxime derivatives of singly and doubly glycated gramicidin are observed as doubly charged species.

the time course of formation of the doubly glycated species. The rate of formation of singly glycated species is greater than that of doubly glycated species.

Continued incubation of gramicidin S with glucose resulted in the appearance of triply glycated species **D**, at 30 h of incubation. After 72 h of incubation, the spectrum shows the complete absence of native gramicidin and significant amount of triply glycated species in which there is double glycation on one of the ornithine residues (figure not shown). Treatment of this sample with hydroxylamine did not result in the deglycation of species **D**, indicating that **D** is a ketoamine product.

The mass spectrum of the sample incubated for 100 days (Fig. 1c) shows the presence of advanced glycation products CML [**F**, **H**] and CEL [**G**, **I**]. After 140 days of incubation, the major species observed (Fig. 1d) was the triply glycated species **D**. Significant amounts of species **E** (Table 1), in which there is double glycation on both the ornithine residues, were also observed. Species **D** and **E** were retained on treatment with hydroxylamine indicating that they are ketoamine adducts. At this time, singly glycated gramicidin was completely absent.

Reversal of glycation in gramicidin S

Addition of hydroxylamine to the gramicidin sample after 6 h of incubation with glucose (Fig. 3a) resulted in complete deglycation of the glucose adducts (Fig. 3b). Similarly, on passing through a reverse-phase C_{18} liquid chromatography column complete reversal of glycation was observed. Treatments with INH or aminoguanidine (Fig. 3c) also caused deglycation of the glucose adduct but hydroxylamine was found to be more effective. Prolonged incubation of the glycated peptide with INH or aminoguanidine showed that deglycation using these reagents improved with time. Partial deglycation was observed in samples incubated with sugar for more than 24 h on treatment with hydroxylamine, INH and aminoguanidine.

Discussion

Glycation of gramicidin S

Glycation of gramicidin S proceeds rapidly in glucose solution with the singly glycated species being observed within 30 min of incubation. At 3 h of incubation, the intensity of the singly glycated species is ≈50% that of the native peptide. Treatment of hydroxylamine to the sample incubated for 6 h results in the complete deglycation of the glycated species, indicating that the glycated species at this stage exists exclusively as the Schiff base. In earlier reports, the characterization and quantitation of Schiff base adducts were carried out using UV absorption or by measuring the radioactivity of the labeled derivatives of the glycated proteins (22). Here we have shown ESI-MS analysis can be used to quantify the Schiff base adduct without the need for derivatization. Mass spectrometric

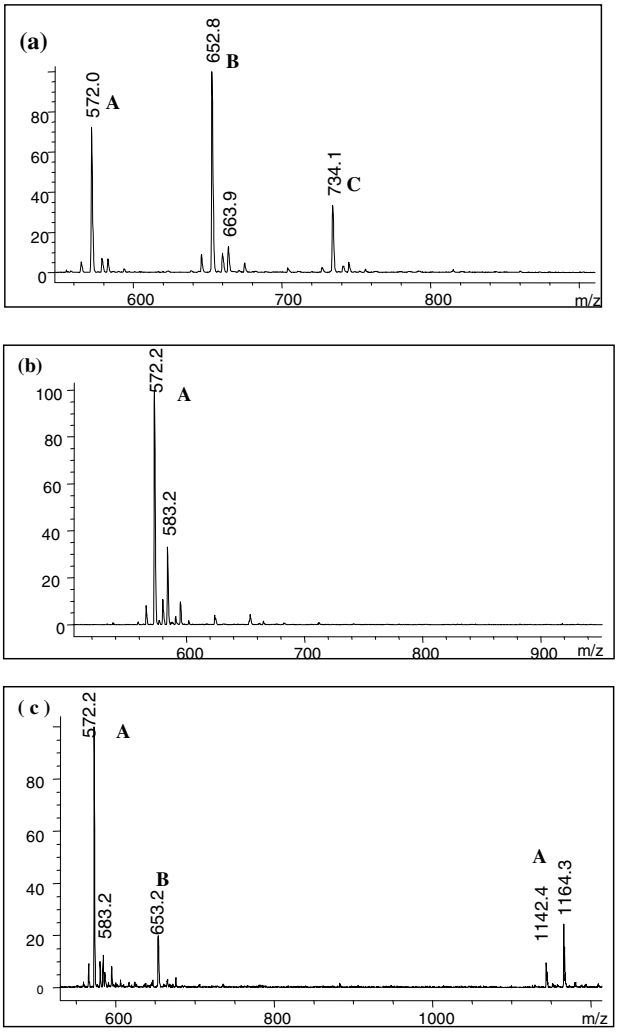

Figure 3. ESI mass spectra of glycated gramicidin: (a) incubated with glucose for 6 h, (b) after treatment with hydroxylamine and (c) after treatment with aminoguanidine. Species observed are listed in Table 1.

techniques such as ESI-MS and MALDI have been used for quantitation of total glycation, to find sites of glycation and for the characterization of AGEs. Application of ESI-MS for the quantitation of glycated hemoglobin (23,24), identification and quantitation of AGEs such as CML, pentosidine, glyoxal lysine dimer and methylglyoxal lysine dimer in human serum proteins (25) has been reported. Application of MALDI for the identification and quantitation of the various AGEs in the reaction of glucose with proteins has also been reported (26). Lapolla *et al*. (27,28) reported the application of MALDI for the characterization of glycation and glycoxidation of globins.

In this study, quantitation of ketoamine product is also shown using ESI-MS. On treatment with hydroxylamine, partial deglycation was observed in samples incubated for 12 and 24 h. This would imply that the Amadori rearrangement has taken place to some extent at this time to give the ketoamine product, which *does not* undergo deglycation with hydroxylamine. The glycated product obtained after 24 h of incubation with glucose was treated with excess hydroxylamine and warmed. The glycated adducts formed the corresponding oxime, confirming that this was indeed the ketoamine (Fig. 2). Aminoguanidine also formed the corresponding derivative showing that the hydrazines can be used to differentiate the two stages of glycation. Earlier studies used 2,4-dinitrophenylhydrazine (29) and phenylhydrazine (30) for the quantitative determination of reactive carbonyl groups in proteins and peptides. The UV absorption of phenylhydrazone derivatives at 370 nm is used for the quantitation of carbonyl groups.

The rate of formation of doubly glycated species is slow compared with that of singly glycated species. This could be because of the glycation at one of the ornithine reduces its catalytic effect on the glycation of the other ornithine. Further incubation (30 h) of the peptide with sugar resulted in the formation of glycated peptide (**D**) with three glucose molecules bound to the peptide indicating double glycation on one of the ornithine residues. The rate of formation of the triply glycated species was found to be very slow compared with the formation of singly and doubly glycated species, implying that diglycation on the same residue is a very slow process. A similar observation was made Blakytny *et al*. (31) in the diglycation of *N*-acetyl lysine. The Amadori rearrangement of the bisglucose adduct (two glucose molecules bound to the same ornitihine residue) was found to be faster than that of mono adduct. This could presumably be due to the formation of immonium ion in the Schiff base of bis-adduct (which is the rate limiting step in the Amadori rearrangement under the reaction conditions) which facilitates the formation of the enolamine and ketoamine (Fig. 4). The ESI-MS of the sample in which gramicidin S was incubated with glucose for 100 days (Fig. 1c) reveals that the major species is the triply glycated gramicidin S (**D**). Significant amounts of advanced glycation end products CML (**F**, **H**) and CEL (**G**, **I**) of the species **C** and **D** were also observed. The pH conditions used in this study (pH 8.5) facilitate the formation of CML. The rate of formation of CML from the ketoamine product of *N*-formyl-*N*-fructoselysine is reported to be higher at higher pH (15). This is analogous to the oxidative degradation of sugars in alkali. Oxidative degradation of sugars is known to proceed by a free radical mechanism (32).

The ESI-MS spectrum of the sample incubated for 140 days (Fig. 1d) shows significant amounts of species **E**,

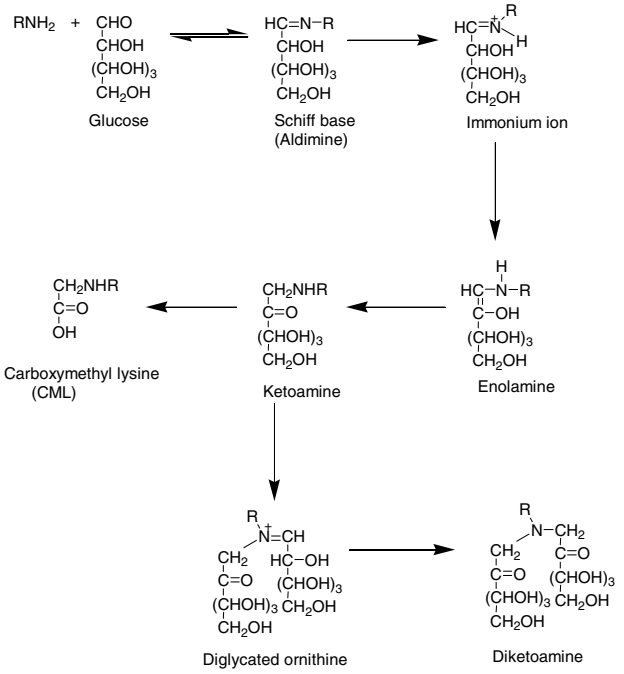

Figure 4. The mechanism of glycation reaction. R=peptide. The open chain conformation of sugar is shown for convenience.

in which four glucose molecules are bound to the peptide. A maximum of four molecules of glucose can be bound to the peptide when both the ornithines are bound to two glucose residues. Observation of species **E** implies diglycation on both the ornithine residues of the peptide, which may be facilitated by the presence of two ornithine groups in the peptide at proximal positions. The rate of formation of **E** was found to be very slow which could be due to the reduction in catalytic effect of the proximal ornithine residue, which is diglycated.

Reversal of glycation

The initial glycation product is a Schiff base adduct (aldimine) of glucose with the peptide, which undergoes Amadori rearrangement to give the ketoamine product in a slow irreversible process (12). The Schiff base adduct exists in dynamic equilibrium with the glucose in the medium. Because the Schiff base adduct formation is a reversible reaction (Fig. 5), the removal of sugar by column chromatography (or dialysis in proteins) shifts the equilibrium (33) resulting in deglycation of the peptide glucose adduct. Various agents such as aminoguanidine (34–36), D-lysine (37,38), desferrioxamine (39), D-penicillamine (40), thiamine pyrophosphate and pyridoxamine (41), and aryl ureido and aryl carboxamido phenoxy isobutyric acids (42) phenyl 4,5-dimethylthiazolium chloride (ALT 711) (43),

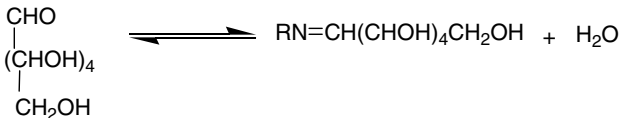

Figure 5. Formation of Schiff base adduct from glucose.

amadoriases (44), chelators, sulfhydryl compounds and antioxidants (45) have been investigated in both *in vitro* and *in vivo* to interact at various stages of glycation.

Among the various inhibitors of glycation, aminoguanidine has been studied extensively with specific focus on its possible clinical role in the treatment of chronic diabetic complications. Brownlee *et al*. (34) reported inhibition of advanced glycosylation product formation and glucose-derived collagen cross-linking *in vitro* using aminoguanidine. Interaction of aminoguanidine at the various stages of the glycation reaction has been reported. Aminoguanidine reacts with dicarbonyl compounds such as glyoxal, methylglyoxal and deoxyglucosone, which are formed from the oxidative degradation of free sugar and with the dicarbonyl compounds formed from the post Amadori glycation product of proteins (Fig. 6) (36). It has also been reported to interact with the ketoamine stage of the glycation reaction and forms substituted Amadori product and thus prevents the formation of glucose-derived cross-linked products (34). Interaction of aminoguanidine at the initial, Schiff base stage of glycation has not been studied.

In our study, hydrazines are used to cause reversal of glycation at the Schiff base stage. Hydrazines are effective in deglycating the Schiff base adduct at equimolar concentration of the glycated species. Hydroxylamine, which is a strong nucleophile, causes specific deglycation of the Schiff base adduct even in the presence of excess glucose. Nucleophilic attack of hydroxylamine (transglycosylation) on the Schiff base presumably releases the glucose as the glucose oxime. (Our attempts to characterize the glucose oxime formed in the transglycosylation reaction were unsuccessful because of the poor ionization of the glucose oxime under the ESI conditions used in the study.) Although there is a competing reaction between the free sugar in the reaction mixture and the hydroxylamine, the rate of transglycosylation is higher than the reaction of the free sugar, resulting in effective deglycation with hydroxylamine. Indeed, the rate of nucleophilic attack of semicarbazide on Schiff bases has been shown to be several orders of magnitude more rapid than the attack on the free aldehydes (Fig. 7). This large rate difference has been attributed to the greater basicity of the Schiff base compared with the aldehydes (46).

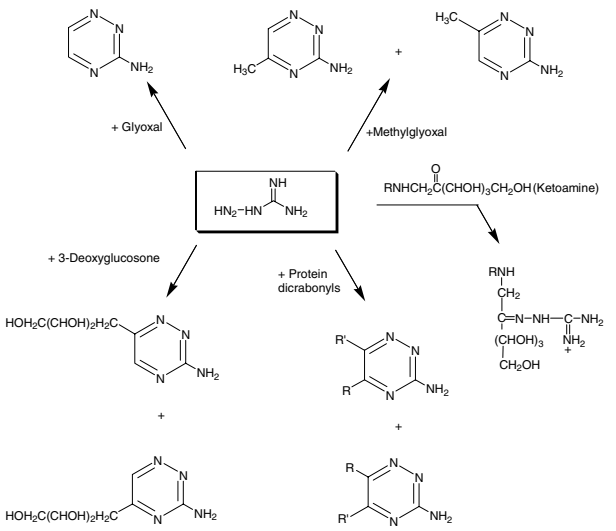

Figure 6. Reactions of aminoguanidine with the carbonyl compounds at the various stages of protein glycation (32,34).

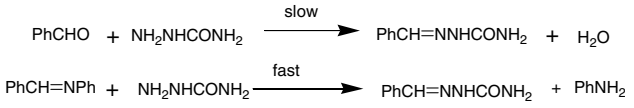

Figure 7. Nucleophilic addition of semicarbazide to aldehyde and Schiff base (46).

In order to examine the generality of the mechanism of transglycosylation, the effects of isonicotinic acid hydrazide (INH) and aminoguanidine on glycation were studied. INH is an antituberculosis drug that is known to interfere with pyridoxal phosphate (PLP) containing enzyme functions by reacting with PLP Schiff bases (47). In our studies we note that INH and aminoguanidine were also effective in deglycation of the glucose adduct at the Schiff base stage. Aminoguanidine and INH are poorer nucleophiles than hydroxylamine, resulting in slower transglycosylation rates compared with hydroxylamine. However, the transglycosylation is faster than the reaction of INH/aminoguanidine with free sugar resulting in deglycation of the peptide glucose adduct. Hence, addition of aminoguanidine or INH to the glycation reaction mixture results in significant reduction in the intensity of the glycated species indicating that these are effective deglycating agents at the Schiff base stage of the glycation reaction. Deglycation of glycated peptide at the Schiff base stage using the hydrazines restores the native peptide, a process that is not achieved by deglycation at the advanced glycation stages using the other deglycating agents such as antioxidants.

Conclusions

Gramicidin S is shown to be a good model system to study the various stages of glycation reaction in proteins. ESI-MS is a convenient tool to follow the glycation reaction. Quantitation of Schiff base and the ketoamine adduct, characterization of AGEs, diglycation of ornithine residues and reversal of glycation using nucleophilic hydrazines have been studied using ESI-MS. The mode of action of hydrazines involves deglycation of the Schiff base adduct rather than reaction at the level of the ketoamine, thus terminating the deleterious effect of glycation at a reversible stage. The effectiveness of INH, a clinically used antituberculosis drug is noteworthy. Compounds with similar functional groups can be screened in order to develop a viable therapeutic agent. Hydrazines may also be used to distinguish between the Schiff base and ketoamine adduct.

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