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New Insights Into Protein Crosslinking Via the Maillard Reaction: Structural Requirements, the Effect on Enzyme Function, and Predicted Efficacy of Crosslinking Inhibitors as Anti-ageing Therapeutics

Antonia G. Miller, Susie J. Meade and Juliet A. Gerrard*

Department of Plant and Microbial Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

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Abstract—Protein crosslinking via the Maillard reaction with α-dicarbonyl compounds has been the subject of intense literature scrutiny. We report here a systematic study of three previously-neglected aspects of the reaction. Firstly, structural requirements were probed. An arginine-free peptide that contains two lysine residues, and a lysine-free peptide that contains arginine, were reacted with glyoxal, methylglyoxal and biacetyl. Methylglyoxal was able to crosslink in the absence of arginine residues, but glyoxal and biacetyl were not. Glyoxal crosslinked the lysine-free peptide via the N-terminus, but methylglyoxal and biacetyl could not. In this study, crosslinking did not require the presence of arginine but did require a free amino group, from a lysine residue, or the N-terminus. Thus specificity in structural requirements for protein crosslinking by α-dicarbonyls has been demonstrated. Secondly, protein function following glycation was examined by treating ribonuclease A with the three α-dicarbonyls, which were shown both to crosslink the enzyme and impair enzymatic activity. Thirdly, the effects of two reported Maillard reaction inhibitors, aminoguanidine and 3,5-dimethylpyrazole-1-carboxamidine on the crosslinking reaction were assessed, with a parallel measurement of the effect on enzyme activity. The results demonstrate that preventing protein crosslinking does not necessarily preserve enzyme activity. These results cast doubt on the likely efficacy of some purported anti-ageing compounds in vivo.

Introduction

The Maillard reaction, or non-enzymatic glycation, is initiated by condensation of an amine with a carbonyl-containing compound. A multitude of products result from the later stages of this complex process, which are termed advanced glycation end products (AGEs). A subset of these AGEs are protein crosslinks. Such crosslinks have been isolated in long-lived proteins such as collagen[1] and lens crystallins,[2,3] where they are thought to modify the function of the protein via structural change. It has been further postulated that protein crosslinks have deleterious consequences throughout the body, and play an important role in the ageing process.[4,5] As such, prevention[6] or reversal[7] of protein crosslinking has been proposed as a novel strategy for anti-ageing therapies.[8]

Despite the considerable literature attention that the Maillard reaction in vivo has attracted, the details of the chemical and biochemical reactions that result in protein crosslinking remain elusive. There are several reasons for this, amongst them the sheer complexity of the processes involved and the harsh conditions—typically a 6 M HCl hydrolysis, refluxed over 24 h—that are often employed to analyse the products of crosslinking reactions, which are likely to create artefactual products.[9] Recent results, using immunochemical approaches and mass spectrometry or enzyme digests, in place of the acidic hydrolysis step, support the contention that many of the crosslinks likely to occur in vivo remain uncharacterized.[10–13] In order to control the Maillard reaction in vivo, and design more appropriate anti-ageing intervention strategies, the details of these crosslinking reactions must be unraveled. To circumvent the problems associated with analysis of a complex mixture of products, we have taken a novel approach to examining the crosslinking phenomenon. Here, we have carefully selected a range of starting materials in order to establish precise structural requirements for efficient crosslinking.

*Corresponding author. Tel.: +64-3-364-2987x7302; fax: +64-3-364-2083; e-mail: j.gerrard@botn.canterbury.ac.nz

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Traditionally, the role of glucose as the participating sugar in the crosslinking reaction has been studied, along with such sugars as ribose and ascorbate.[14–16] However, more recently, attention has been directed toward α-dicarbonyl compounds such as methylglyoxal and glyoxal (Fig. 1), as these have been found to be active crosslinkers in vitro and in vivo.[17–21] The mechanisms of glyoxal and methylglyoxal generation in vivo have been determined, in an attempt to understand the progression of the Maillard reaction in the body. A number of works have confirmed the generation of dicarbonyls via glucose autooxidation.[22,23] It appears that dicarbonyls can be generated on degradation of the Amadori product, a key intermediate in the Maillard reaction.[24,25] Researchers have suggested that methylglyoxal and glyoxal can be generated from the degradation of the Schiff base adduct that is initially formed on reaction of glucose with an amine.[26,27] Methylglyoxal can also be formed by enzymatic routes from dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.[28] There appears to be no data regarding in vivo implications of biacetyl, a closely related α-dicarbonyl compound (Fig. 1), in terms of the Maillard reaction. However, biacetyl has, for a number of years, been employed as a standard arginine modifier,[29,30] and this modification is thought to be a readily reversible process.[31] It has also been noted that lysine residues can be modified by biacetyl.[30]

Within the extensive literature on the reaction of proteins with α-dicarbonyl compounds, little attention has been paid to the relationship of the structural features of these molecules and their rate of crosslink formation. We have previously noted that methylglyoxal is a highly reactive crosslinker and that this reactivity is not matched by the closely related compound, glyoxal.[32] It is intriguing that such a small structural change can have such dramatic consequences for crosslinking. Thus, the first section of this paper aims to address the structural requirements of small α-dicarbonyl compounds that result in effective crosslinking by examining the reaction of glyoxal, methylglyoxal and biacetyl with defined protein substrates. In tandem with this work, Meade et al. have investigated the effect of a variety of dicarbonyl compounds on crosslinking, with a view to gaining a further understanding of the structural requirements for effective crosslinks and elucidating the underlying pathways involved in their synthesis. These results are reported in a subsequent paper.[33]

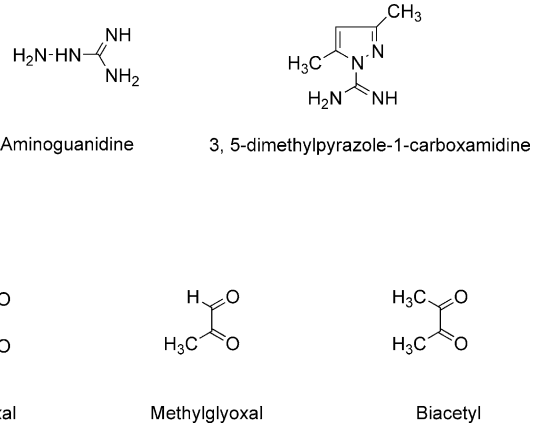


Figure 1. Structure of the three α-dicarbonyl compounds employed in this study and the two dicarbonyl trappers that have been used to examine the crosslinking-function relationship.

Although efforts have been undertaken to address the extent to which particular amino acids participate in the crosslinking process, no systematic study has yet been undertaken of the precise amino acid requirements for reaction. Most research points to reaction involving lysine residues,[18] and arginine residues,[20] but whether both amino acids are required, or whether the amino acid requirements change for different crosslinking moieties, has not been investigated in detail. To begin to address this question, we have examined the reaction of each of the three α-dicarbonyl compounds with somatostatin, an arginine-free peptide that contains two lysine residues, and renin substrate tetradecapeptide, which lacks lysine, along with ribonuclease, an enzyme containing both arginine and lysine residues.

In addition to defining structural requirements for protein crosslinking with α-dicarbonyl compounds, this paper seeks to address the relationship of crosslinking and function of a model enzyme. Any therapeutic strategy designed to inhibit ageing by preventing or reversing protein crosslinks makes the tacit assumption that loss of function is due to protein crosslinking, and, further, that preventing crosslinking will maintain function. With rare exceptions,[34] this assumption has yet to be rigorously tested. Nevertheless, it forms the basis for the development of a class of Maillard inhibitors which act to break protein crosslinks, therefore effecting a proposed return of function to the target protein,[7,35,36] although these results have been the source of some debate.[37,38] Along with the crosslink breakers, compounds such as aminoguanidine[39] (Fig. 1) are reported to trap reactive dicarbonyl species,[40] sequestering them before they can react with the protein. More recently it has been speculated that effectiveness of proposed dicarbonyl trapping and crosslink breaking compounds as AGE inhibitors may be due to other characteristics such as metal ion chelation properties, which may provide an additive effect.[41] In most AGE inhibition literature, the assumption remains that prevention of crosslinking will maintain protein function. This assumption is tested herein.

In order to examine the crosslinking-function relationship, the three α-dicarbonyl compounds were reacted with a model enzyme. Following literature precedent, ribonuclease (RNase A) was chosen as a well characterised enzyme, and the loss of activity was monitored and related to the degree of crosslinking. This provided a system in which to test the efficacy of two proposed dicarbonyl trappers—aminoguanidine and 3,5-dimethylpyrazole-1-carboxamidine (DMPC)[42]—during glycation of ribonuclease.

Results

To effect the extent of crosslinking that has been implicated in cataract formation and other biological diseases[43,44] on

an appropriate time scale, the concentration of the α-dicarbonyls selected was 130 mM. Whilst the concentration is not likely to be met in vivo, it allowed us to undertake a vigorous comparison of our chosen substrates and provide 'proof of concept' chemistry that may be accessible in vivo.

Reaction of defined peptide substrates with glyoxal, methylglyoxal and biacetyl

In order to assess the ability of glyoxal, methylglyoxal and biacetyl to crosslink oligopeptides of defined amino acid sequence, we employed our previously published methods[45] with minor modification, to enable the analysis of very low molecular weight material by electrophoresis. Incubations were carried out over 7 days, with daily sampling. Somatostatin and renin substrate tetradecapeptide were chosen as commercially available peptides that lack arginine and lysine respectively. In order to establish the role of the N-terminus, an *N*-protected form of renin substrate tetradecapeptide was also employed.

Reaction with somatostatin. The incubations with glyoxal and biacetyl did not result in detectable crosslinking after one week (data not shown), nor was any colour change observed during the course of the reaction. Methylglyoxal, on the other hand, showed a clear ability to crosslink somatostatin as determined by 1-D SDS-PAGE analysis (Fig. 2). During the course of the incubation, the solution showed substantial browning. It is evident that by day 3, a large amount of crosslinking occurred as evidenced by the smearing in the lane at this incubation time. By day 7, the aggregates formed via the Maillard reaction were too large to enter the gel and remained in the wells. Crosslinking was not observed in the controls. Thus, under these conditions, methylglyoxal is able to crosslink proteins in the absence of arginine, but glyoxal and biacetyl are not.

Reaction with renin substrate tetradecapeptide. In the case of the renin substrate tetradecapeptide samples, the only incubations to exhibit evidence of crosslinking on a 1-D SDS-PAGE gel were those that had been incubated with glyoxal. A representative result is depicted in Figure 3. Interestingly, neither the methylglyoxal-treated renin, nor that treated with biacetyl exhibited any degree of crosslinking. All three incubations showed some degree of browning, with glyoxal and biacetyl producing darker colours than the methylglyoxal incubation. Comparison to the protein-free controls suggested that this browning could not be accounted for by the α-dicarbonyl itself browning over time.

We hypothesised that the crosslinking observed in the glyoxal incubation may not be entirely due to the arginine residue, but involved the N-terminus. This theory was tested by repeating the incubations with *N*-protected renin substrate tetradecapeptide (Fig. 4). No crosslinking was observed for any of the other α-dicarbonyls tested (data not shown).

Thus, in contrast to the results obtained for somatostatin, glyoxal, under these conditions, is able to crosslink proteins in the absence of lysine, via the N-terminus, whereas methylglyoxal and biacetyl are not. The presence of arginine alone was not sufficient to allow

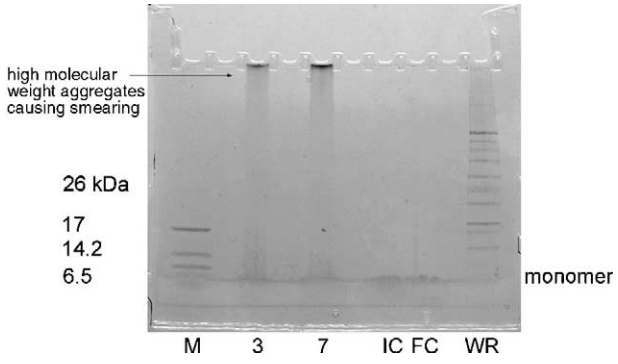


Figure 3. 4–20% SDS-PAGE gel of renin substrate tetradecapeptide following glycation by glyoxal. From left to right; M = ultra low molecular weight marker, 3 = day 3 incubation of glyoxal with renin substrate tetradecapeptide, 7 = day 7 incubation of glyoxal with renin substrate tetradecapeptide, IC = renin substrate tetradecapeptide incubated control, FC = renin substrate tetradecapeptide frozen control, WR = wide range marker.

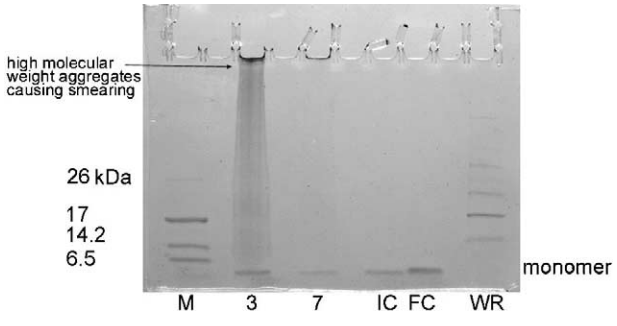


Figure 2. 4–20% SDS-PAGE gel of somatostatin following glycation by methylglyoxal. From left to right; M = ultra low molecular weight marker, 3 = day 3 incubation of methylglyoxal with somatostatin, 7 = day 7 incubation of methylglyoxal with somatostatin, IC = somatostatin incubated control, FC = somatostatin frozen control, WR = wide range marker.

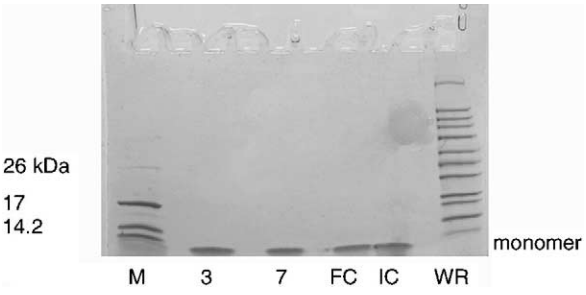


Figure 4. 4–20% SDS-PAGE gel of *N*-protected renin substrate tetradecapeptide following glycation by glyoxal. From left to right; M = ultra low molecular weight marker, 3 = day 3 incubation of glyoxal with *N*-protected renin substrate tetradecapeptide, 7 = day 7 incubation of glyoxal with *N*-protected renin substrate tetradecapeptide, FC = *N*-protected renin substrate tetradecapeptide frozen control, IC = *N*-protected renin substrate tetradecapeptide incubated control, WR = wide range marker.

crosslinking for any of the compounds tested. This is, to our knowledge, the first time that such specificity in structural requirements for protein crosslinking by α-dicarbonyls has been demonstrated.

Reaction of ribonuclease with glyoxal, methylglyoxal and biacetyl

Comparative crosslinking ability. RNase A contains both lysine and arginine residues in its sequence, and was reacted with the same series of α-dicarbonyls to determine the relative crosslinking rates in the presence of both amino acids. Figure 5 shows a typical result depicting the extent to which RNase A was crosslinked by methylglyoxal, glyoxal and biacetyl. The results confirm those from previous studies in this laboratory in which methylglyoxal was found to be a great deal more reactive than glyoxal or biacetyl.[32] The latter two compounds were found to have similar reactivities with respect to each other.

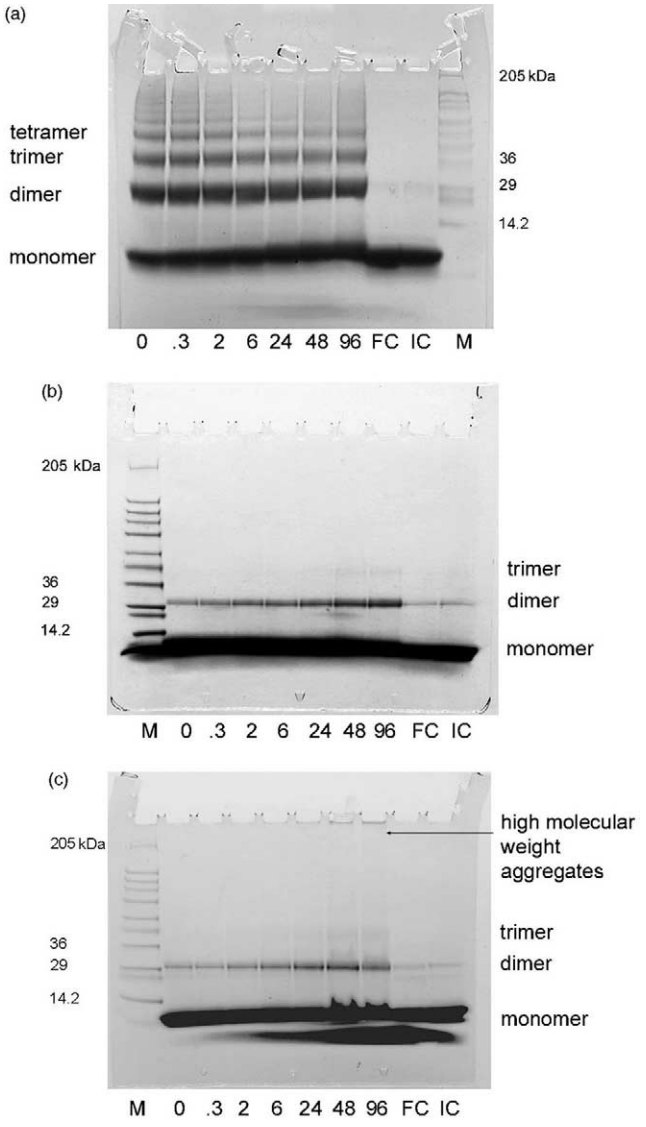


Figure 5. 8–16% SDS-PAGE gels of (a) methylglyoxal, (b) glyoxal, (c) biacetyl incubated with RNase A. Abbreviations are as follows: M = wide range marker, 0–96 = hours of incubation time of dicarbonyl with RNase A at 37 °C, IC = RNase A incubated control, FC = RNase A frozen control.

It is evident from Figure 5a that the crosslinking process mediated by methylglyoxal is almost immediate, as the crosslinking in the time 0 incubation is almost as marked as the 96 h sample. The fact that methylglyoxal is able to crosslink RNase A more effectively than either somatostatin or renin substrate tetradecapeptide, suggests that, perhaps, the majority of crosslinks formed contain both lysine and arginine, consistent with literature proposals that these crosslinks include MODIC.[3] Figure 5b and c, show the similar reactivity of biacetyl and glyoxal.

The effect of Maillard inhibitors on crosslinking. In order to gauge the capacity of two reported Maillard reaction inhibitors, aminoguanidine and DMPC, to prevent crosslinking, the inhibitors were included in dicarbonyl-RNase A incubations, and the effect on crosslinking monitored. During replicate experiments, batch-wise variation of RNase A activity was observed, manifesting as a variation in enzymatic activity within the control incubations. Thus the replicate data was not pooled. However, the overall trends were observed for each experiment. Figure 6 depicts typical results from an incubation of aminoguanidine and methylglyoxal with RNase A and, when compared to Figure 5a, corroborate the previously reported results, that the inclusion of aminoguanidine in protein-sugar incubations causes a marked inhibition of protein crosslinking.[46] On visual inspection of the incubations, those containing methylglyoxal and aminoguanidine only were found to exhibit the same degree of browning as those which also had the RNase A present, suggesting the presence of a chromophore produced on the reaction of methylglyoxal with aminoguanidine. Glyoxal- and biacetyl-mediated RNase A crosslinking was also inhibited in the presence of aminoguanidine (data not shown).

The effect of DMPC on the crosslinking process was also measured. Figure 7 exemplifies the ability of the inhibitor to repress crosslinking caused by methylglyoxal, with the inhibition of oligomeric protein formation that

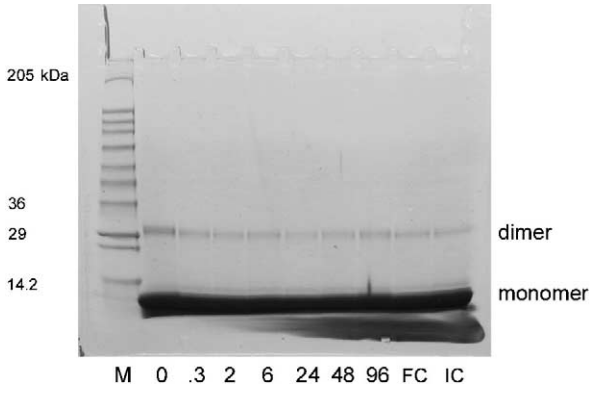


Figure 6. 8–16% SDS-PAGE gel of methylglyoxal incubated with RNase A in the presence of aminoguanidine. Abbreviations are as for Figure 5.

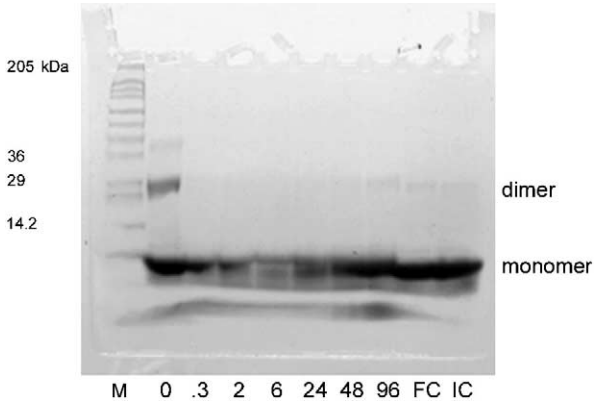


Figure 7. 8–16% SDS-PAGE gel of methylglyoxal incubated with RNase A in the presence of DMPC. Abbreviations are as for Figure 5.

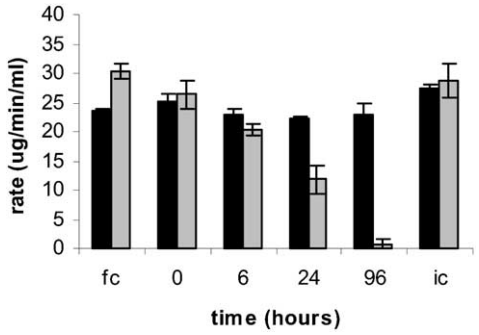


Figure 8. RNase A activity over 96 h following incubation with methylglyoxal in the presence, (■) or absence of aminoguanidine (■). Bars indicate the average of data generated from a triplicate reading of a sample. Error bars represent the standard error of the mean for the triplicate reading. *x* axis: FC (0) = RNase A frozen control, 0–96 = time in hours, IC (96) = RNase A incubated control. Rate expressed as μg/mL of RNA cleaved per minute.

was observed in the absence of inhibitor (Fig. 5a). As with aminoguanidine, DMPC effectively inhibited crosslinking of RNase A mediated by glyoxal and biacetyl, confirming the report of Niigata et al. that this compound is an effective inhibitor of crosslinking.[42]

The relationship of protein crosslinking and enzyme function. Activity of RNase A following incubation with α-dicarbonyls. With the crosslinking capacities of the three α-dicarbonyl compounds established, and the efficacy of two inhibitors of this process demonstrated, our attention turned to the relationship of these crosslinking events to enzyme function. The activity of RNase A was monitored using the methylene blue assay reported by Greiner-Stoeffele,[47] which enabled accurate and reproducible measurement of the enzyme's activity after being subjected to various treatments. The method employed in this study is useful in that the kinetics of a complex system can be easily assayed.[48] As the samples are measured at a wavelength of 688 nm, the contribution of other biomolecules to rate is virtually excluded. The activity of RNase A after glycation has previously been studied,[14] however this was undertaken with glucose in the presence of phosphate buffer, the latter of which has been found to have an effect on the kinetics of glycation.[49] Figure 8 depicts the activity of RNase A following incubation with methylglyoxal. The reduction of activity over time following glycation is obvious. By 96 h, the RNase A is almost completely inactive, as compared to the control RNase A incubation which, in the absence of methylglyoxal, maintains its activity over this time period.

Similar trends were noted when RNase A was incubated with biacetyl and glyoxal over the same time period, although the inactivation was not as complete (Table 1). The observation that RNase A is inactivated by glyoxal is in agreement with those results recently published by Voyizan et al., who quote around a 20% loss in activity after 288 h of incubation.[34]

The effect of Maillard inhibitors on the activity of RNase A. The enzyme activity of the RNase A incubations with each of the α-dicarbonyl compounds in the presence of aminoguanidine and DMPC was also monitored using the methylene blue assay. The data, shown in Fig. 8, clearly demonstrates that that the activity of RNase A in the presence of aminoguanidine is retained following incubation with methylglyoxal. Retention of RNase A activity by biacetyl- and glyoxal-treated RNase containing inhibitor was also observed (Table 1). This suggests that aminoguanidine may indeed be effective at retaining protein function in vivo, and that anti-ageing therapies based on this compound may prove effective, side effects nonwithstanding.[50]

Table 1. Comparison of RNase A crosslinking with catalytic activity

Dicarbonyl	Aminoguanidine		DMPC		No inhibitor	
	Cross-linking[a]	Rate of reaction (μg RNA/mL/min)[b]	Cross-linking	RNA cleavage (μg/mL/min)	Cross-linking	RNA cleavage (μg/mL/min)
Methylglyoxal	–	22.9±0.4	–	9.4±3.1	+++	3.8±1.6
Glyoxal	–	29.3±1.7	–	25.4±0.9	+	17.9±0.7
Biacetyl	–	30.2±1.0	–	23.6±0.5	+	22.1±0.9
No dicarbonyl[c]	–	29.5±0.6	–	27.1±1.3	–	28.8±1.1

[a]Positive crosslinking results were those which showed increased crosslinking compared to controls.
[b]Rate calculated from methylene blue RNase A activity assay. Errors are ±SEM of triplicate readings. Data is that from the 96-h incubations for each dicarbonyl.
[c]Controls values are an average of results yielded for each differing α-dicarbonyl series, Error is the standard error of the mean for these readings. Rate expressed as μg/mL of RNA cleaved per min.

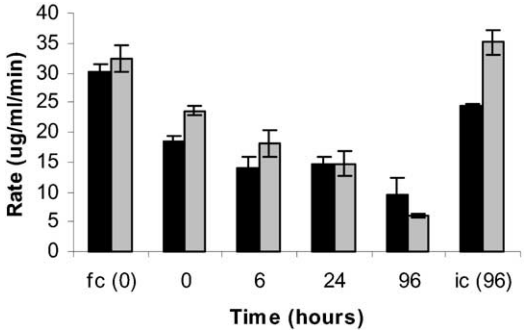


Figure 9. RNase A activity over 96 h following treatment with methylglyoxal presence (■) or absence of DMPC (■). Bars indicate the average of data generated from a triplicate reading of a sample. Error bars represent the standard error of the mean for the triplicate reading. *x* axis: FC (0) = RNase A frozen control, 0–96 = time in hours, IC (96) = RNase A incubated control. Rate expressed as μg/mL of RNA cleaved per minute.

Interestingly, however, the analysis of those incubations that had been carried out between methylglyoxal and RNase A, in the presence of DMPC did not show retention of enzymatic activity, despite the obvious success of this compound in inhibiting protein crosslinking. Figure 9 shows the data from the incubation of RNase A with methylglyoxal in the presence of DMPC. The samples treated with DMPC alone were consistently lower than those without addition of inhibitor, suggesting slight inhibition of the RNase A by DMPC. However, the drop in activity cannot be solely accounted for by the addition of crosslinking inhibitor.

In contrast, analysis of RNase A samples that had been incubated with biacetyl and glyoxal in the presence of DMPC were found to retain activity in the presence of inhibitor, with DMPC appearing to be more effective in the glyoxal incubations. These results are summarised in Table 1.

Thus, the ability of a compound to inhibit crosslinking may not necessarily correlate with the ability of such a compound to protect the protein function. The literature associated with crosslinking inhibition as an anti-ageing strategy should thus be interpreted with caution.

Discussion

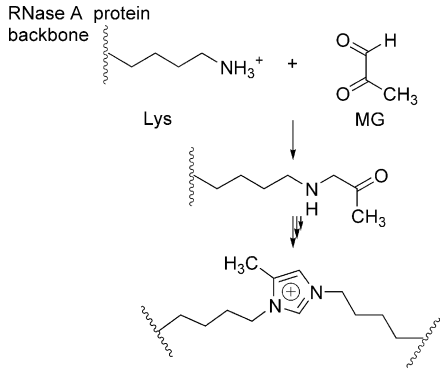
The molecular details of protein crosslinking via the Maillard reaction have remained elusive, despite extensive research, due to the inherent difficulties associated with the analysis of complex mixtures of proteinaceous products. In order to circumvent these problems, we have employed a new approach in which a range of starting materials is carefully selected to yield new insights into the requirements for efficient protein crosslinking. Specifically, we have examined the selectivity of three α-dicarbonyl compounds (methylglyoxal, glyoxal and biacetyl) toward lysine residues, arginine residues and the N-terminus of proteins. This approach has successfully yielded new information as to the precise structural requirements for protein crosslinking by α-dicarbonyls.

All three α-dicarbonyls were capable of crosslinking RNase A, an enzyme containing both lysine and arginine residues. Methylglyoxal proved to be the most reactive of the α-dicarbonyls studied, with the crosslinking proceeding to a much greater extent than for either glyoxal or biacetyl. The reasons for methylglyoxal, with one aldehyde moiety and one ketone moiety, being so much more reactive than either glyoxal, with two aldehyde moieties, or biacetyl, with two ketone moieties, is unclear. Further work to account for this phenomenon is reported by Meade et al. in the following paper.[33]

The most striking feature of our results is perhaps not the relative rates, but the specificity of the reactions observed: small structural differences in the α-dicarbonyl moiety lead to a large change in amino acid requirements. Methylglyoxal was the only α-dicarbonyl of the series that was able to crosslink a lysine-containing peptide in the absence of an arginine residue. This is consistent with the previously reported imidazolium crosslinks derived from the reaction of lysine with methylglyoxal that have been characterised and detected in vitro and in vivo,[2,17,18,51,52] and may be contributors to the crosslinking observed in this study.

In the absence of lysine, crosslinking by methylglyoxal was not observed. This is consistent with the literature consensus that methylglyoxal reacts preferentially with arginine over lysine,[43,53–56] if these are arginine modifications that do not lead to crosslinking, such as argpyrimidine, carboxymethylarginine and imidazolone compounds.[57–60] To the authors' knowledge, there have not been any reports of arginine-arginine crosslinks mediated by methylglyoxal, although one has been reported involving glyoxal, but this requires the aid of a carbonyl compound, not present in our reactions.[61] That glyoxal is able to crosslink the lysine-free peptide, via the N-terminus, whereas methylglyoxal cannot, was not predicted. Presumably this reflects a subtle balance between competing reaction pathways, and perhaps the rapid reaction of methylglyoxal with arginine to form argpyrimidine renders it unavailable for the slower crosslinking reaction with the N-terminus. Glass and Pelzig have reported reversible modification of arginine residues by glyoxal under particular experimental conditions and the modification of guanidine, a functional moiety of arginine, by glyoxal in aqueous conditions has been noted by Bengelsdorf.[62,63] In the same manner as methylglyoxal-arginine modifications, it appears from the literature that the majority of the glyoxal-mediated arginine modifications are also non-crosslinking in nature.[9,64] However, if the reaction with glyoxal is more easily reversed than that with methylglyoxal, then this may account for the difference in behaviour between the two systems. Whether the glyoxal crosslink in this system is between the two N-termini of renin substrate tetradecapeptide, or between one N-terminus and an arginine residue, is, to date, unclear.

Our structure–activity study leads us to two unequivocal conclusions. Firstly, that arginine is not essential for crosslinking to occur with α-dicarbonyls, as evidenced



Scheme 1. Possible reaction pathways for lysine modification by methylglyoxal reaction with lysine in vitro.

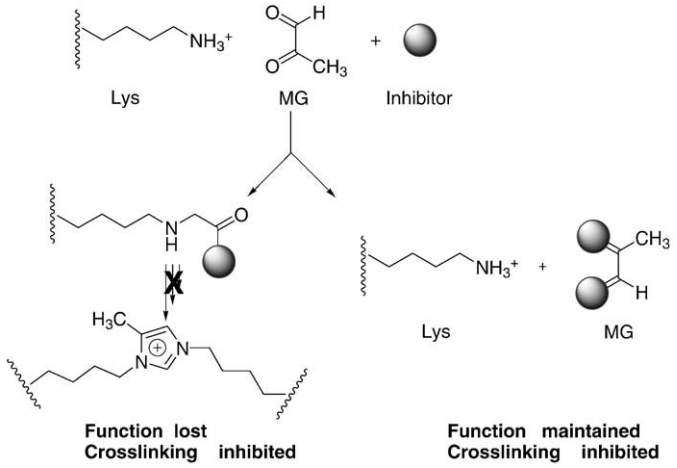
from the work with methylglyoxal and somatostatin. Secondly, that a free amino group, either as the N-terminus or as a lysine residue is required for protein crosslinking under these conditions, in this series. We can further speculate that the presence of both lysine and arginine residues, for example in RNase A, leads to the most efficient crosslinking, but that an excess of arginine residues leads to the predominance of non-crosslinking modifications, such as argpyrimidine. Perhaps most importantly, this study has shown that results from the crosslinking of one molecule cannot be extrapolated to predict the behaviour of another, however closely related. This is an extremely important observation when designing novel therapeutic strategies based on simple model systems in the laboratory.

All three α-dicarbonyl molecules were found to impair the function of ribonuclease on the same timescale as protein crosslinking, consistent with previous reports of treatment with either methylglyoxal or glyoxal.[65] In order to establish whether the loss in function was a direct result of the crosslinking process, or a co-incident event, two previously reported crosslinking inhibitors were selected, aminoguanidine and DMPC. Aminoguanidine is reported to be an effective inhibitor of the progression of the Maillard reaction and has found to be effective in a number of in vitro and in vivo experiments.[6,39,46] The mode of action was initially proposed to be reaction with glucose, rendering the latter unable to react with amines,[39] but has more recently been attributed to its ability to react with α-dicarbonyls, such as those used in this study.[40,66] 3,5-Dimethylpyrazole-1-carboxamidine has also been proposed as a Maillard reaction inhibitor, but is less well-characterised.[42]

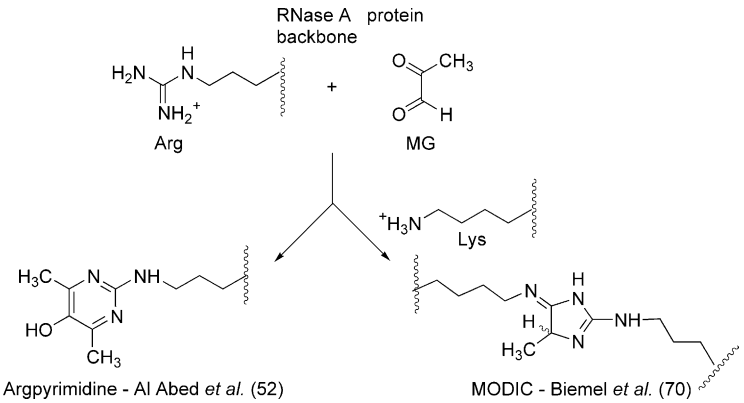
As expected, both aminoguanidine and DMPC inhibited the protein crosslinking reaction by all three α-dicarbonyl compounds with RNase A. In the presence of aminoguanidine, enzyme activity was retained, suggesting that this compound does indeed afford some protection against loss of protein function. However, in the presence of DMPC, despite the inhibition of protein crosslinking on incubation with RNase A and methylglyoxal, activity of the protein was lost. Thus an ability to inhibit protein crosslinking by the Maillard reaction does not necessarily guarantee that the damaging effects of protein glycation on protein function have been circumvented.

Schemes 1 and 2 summarise these findings and suggests a possible explanation for the difference in behaviour between the two inhibitors. Our results support the view that aminoguanidine is effective at inhibiting crosslinking and preventing inactivation of the protein, since it reacts with α-dicarbonyls at a much faster rate than they are able to react with proteins.[40] This appears not always to be the case with DMPC, which does not react with methylglyoxal quickly enough to prevent it from reacting with the protein. Although it does render the intermediate incapable of protein crosslinking, the damage, in terms of loss of protein function, is already done, as outlined in Scheme 2. A more detailed mechanistic analysis of the formation of MOLD is outlined by Meade et al. in the following paper.[33]

It is tempting to speculate on the mechanism of inactivation of RNase A by methylglyoxal. Inactivation may be arising from reaction with lysine via steps outlined in Schemes 1 and 2 or by a similar mechanism. Another



Scheme 2. Possible reaction pathways of lysine on reaction with methylglyoxal in the presence of a Maillard reaction inhibitor in vitro.



Scheme 3. Possible reaction pathways for arginine modification by methylglyoxal in vitro.

possibility is that methylglyoxal may react with arginine residues (Scheme 3), such that these modifications may ultimately result in the modification of enzymatic activity.

Interestingly, Takahasi suggested that as RNase A could be inactivated by phenylglyoxal, a selective arginine modifier under certain conditions, the loss in function in the enzyme was due to modification of arg 39.[67] One must consider the possibly of arginine-lysine crosslinks (Scheme 3)[52,70] which could also potentially modify catalytically important lysine and arginine residues.

In summary, these results demonstrate that preventing protein crosslinking does not necessarily preserve enzyme activity. These results cast doubt on the likely efficacy of some purported anti-ageing compounds in vivo, and emphasise the danger of generalising the results from simple model studies. Based on these results, the efficacy of Maillard inhibitors as anti-ageing therapeutics is likely to depend on many complex factors, the outcome of which will depend on not only the individual carbonyl moiety in question, but also the specific protein affected.

Experimental

Materials

Bovine pancreatic ribonuclease A Type XII-A (RNase A), somatostatin (AGCKNFFWKTFTSC), porcine renin substrate tetradecapeptide (DRVYIHPFHLLVYS), porcine *N*-acetyl renin substrate tetradecapeptide, methylglyoxal (40% aqueous solution), biacetyl (99% aqueous solution), glyoxal (trimer: dihydrate), MOPS buffer, aminoguanidine–HCl, ultra low molecular weight marker and wide range marker were all purchased from Sigma (St Louis, MO, USA). Methylene blue and NaOH were from BDH chemicals (Poole, UK). 3,5-Dimethylpyrazole-1-carboxamidine nitrate was purchased from Aldrich (Milwaukee, WI, USA). Yeast RNA was purchased from Boehringer (Germany). 4–20 and 8–16% Tris-glycine SDS-PAGE gels were obtained from Gradipore (NSW, Australia). Spectrophotometric assays were performed with an HP 8452A Diode Array spectrophotometer and data analysed using UV-Visible Chemstation software (Agilent Technologies).

Incubation of model proteins with α-dicarbonyl compounds

For each of the replicate incubations of RNase A, somatostatin or renin substrate tetradecapeptide with each α-dicarbonyl the following procedure was undertaken. Stock solutions were prepared by solubilising the protein in dH_2O to give a final concentration of 50 mg/mL with the pH adjusted to 7 using 0.1 M NaOH. In the case of *N*-protected renin substrate, solubilisation necessitated the addition of a minimal amount of DMSO. Aliquots of each solution were diluted 1:1 with a 260 mM α-dicarbonyl solution to give final concentrations of 25 mg/mL protein and 130 mM α-dicarbonyl. The resultant solution was aliquoted into Eppendorf tubes and incubated at 37 °C for the prescribed period of time then stored at −20 °C prior to analysis. Controls containing protein only were prepared by diluting an aliquot of the stock solution 1:1 in dH_2O. One sample was frozen at time 0 in liquid N_2, whilst the other was incubated with the α-dicarbonyl-containing samples and removed at the conclusion of the incubation. An α-dicarbonyl only control was prepared by diluting the stock solution of α-dicarbonyl 1:1 in dH_2O and incubated for the same time period as those containing somatostatin. All RNase incubations were repeated at least in triplicate with triplicate analysis of each incubation. Low molecular weight peptide incubations were repeated in duplicate with duplicate analysis of each incubation.

Where required, aminoguanidine and DMPC were added to the incubations to give a final concentration of 130 mM. Aminoguanidine–HCl was first solubilised in a 1:1 solution of DMSO/dH_2O. 3,5-Dimethylpyrazole-1-carboxamidine has traditionally been employed as a guanidinylating agent of proteins.[68,69] However, its capacity to convert lysine residues to homoarginine residues is restricted to a prescribed pH that is not attained in the system that is under study in this paper. Appropriate control incubations confirmed that DMSO

did not influence the crosslinking process. Controls containing protein and inhibitor in the absence of α-dicarbonyl, and α-dicarbonyl and inhibitor in the absence of protein were also included.

SDS-PAGE analysis of glycated samples

SDS-PAGE electrophoresis was performed using the method outlined by Fayle et al.[45] with the following modifications: ultra-low molecular weight marker was diluted 1:4 in 2 × treatment buffer before loading; 10 μL of treated oligopeptide was loaded onto a 4–20% gradient Tris–glycine SDS-PAGE gel; the RNase A samples were analysed on an 8–16% gradient Tris–glycine SDS-PAGE gel. Both were electrophoresed at a constant voltage of 150 V for 1.5–2 h. Again all RNase samples were analysed by electrophoresis in triplicate with dulpicate analysis of low molecular weight peptides.

Methylene blue assay to determine RNase A activity[47]

The RNase A enzyme activity was undertaken as outlined as described by Greiner-Stoeffele.[47] The working concentration of RNA employed in the assay was 600 μg/mL (in methylene blue buffer). To 1 mL of this RNA solution, 10 μL of RNase A sample (diluted in MOPS buffer, concentration of 10 μg/mL) that had been subjected to glycation, or otherwise, was added. The subsequent rate of RNA cleavage was measured and calculated. All controls were diluted and assayed in exactly the same manner as for the incubations of interest. A background rate reading was also taken which consisted of the solvent, generally water, in place of the glycated proteins. The appropriate adjustments to the assay were made when the 1:1 DMSO/dH_2O-containing incubations were examined. DMSO did not appear to inhibit RNase A activity. Each incubation: methylglyoxal and RNase, methylglyoxal RNase A and DMPC and methyglyoxal RNase A and aminoguanidine were prepared and assayed at least in triplicate. During each assay, triplicate readings were recorded for each sample.

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