Protein Modification

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A Systematic Study of Selective Protein Glycation

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Abstract: *Glycation is a non-enzymatic post-translational modification (PTM) that remains poorly understood, largely because it is unknown how it occurs selectively. Using mass spectrometry, it was possible to evaluate total glycation levels, identify distinct glycated products, assign unique glycation sites, and correlate these data with chemical and structural features for a panel of proteins glycated in vitro. It was determined that the extent of glycation does not correlate with pK_a or surface exposure at reactive sites. Rather, the data reveal that primary sequence dictates the overall likelihood that a site will become glycated, while surrounding structure further sculpts the glycation outcome. Clustered acidic residues were found to prevent glycation, whereas a combination of tyrosine and polar residues appear to promote glycation. This work contributes important new knowledge about the molecular features that govern selective glycation.*

Glycation is a non-enzymatic post-translational modification (PTM) in which sugars or sugar-derived metabolites are covalently attached to protein amino or guanidino groups through the Maillard reaction.[1,2] This process yields a chemically heterogeneous set of modifications known as advanced glycation end-products (AGEs; Figure 1).[3,4] Glycation is a hallmark of molecular aging associated with neurodegenerative, cardiovascular, and metabolic diseases, and age-related diseases of the skin and eye.[5–10] AGE-dependent changes in function have been reported for several proteins, including collagen,[11] α-lens crystallin,[12] Hsp27,[13] and p300.[14] Although such studies demonstrate that glycation impacts protein activity and may influence disease development, we do not currently understand what controls the susceptibility of certain proteins to become glycated. This remains an open question preventing a molecular understanding of the biological role of glycation. Prior work has established that glycation occurs selectively for many proteins, including hemoglobin,[15–17] human serum albumin,[18,19] ribonuclease,[20] and α-lens crystallin.[12] However, each study has focused on one or two proteins only, and/or was performed using different conditions that preclude direct comparison. As a result, these remain isolated instances that have not coalesced into a general appreciation for how selective glycation arises.

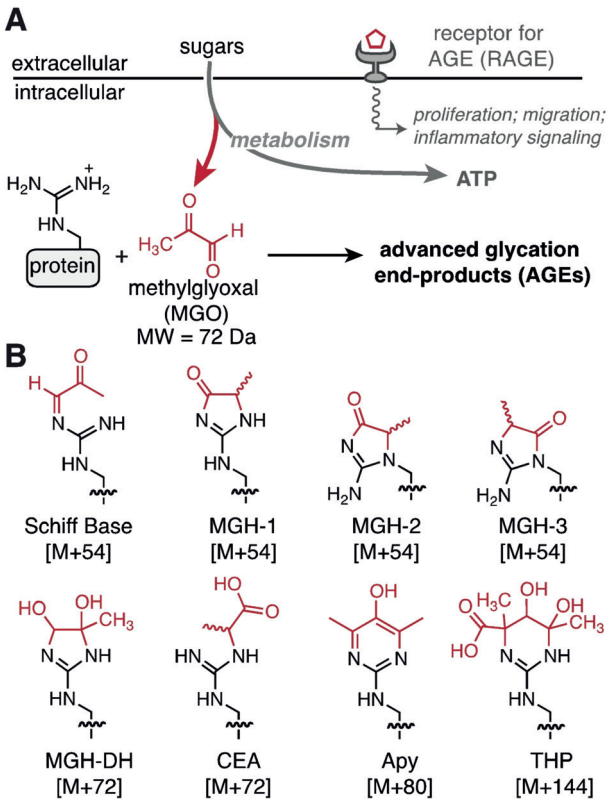

Figure 1. A) Glycation by methylglyoxal (MGO) yields intracellular and extracellular advanced glycation end-products (AGEs). B) Potential AGEs form between MGO and arginine, including hydroimidazolones (MGH-1–3), dihydroxyimidazolidine (MGH-DH), carboxyethylarginine (CEA), argpyrimidine (Apy), and tetrahydropyrimidine (THP). The Schiff base is also shown.

Herein we report a methodical study of selective glycation for an array of proteins in vitro using mass spectrometry (MS). Though cell-based proteomic studies are well-suited for identifying AGE-modified proteins,[21–25] they struggle with the heterogeneity intrinsic to cellular glycation. For instance, the cell contains many biologically relevant aldehydes,[26] each of which can influence the preferred sites of glycation and can form numerous distinct and/or isomeric AGEs.[16–18] Thus, cataloging cellular glycation events may not reveal the underlying chemical features that govern preferential glycation. In contrast, our approach offers a practical, advantageous alternative by limiting the number of glycating agents at play, avoiding artifacts that arise from differential protein expression levels, and simplifying the identification of any, not only expected, AGEs. Additionally, glycation levels can be compared directly, as all proteins are modified using identical

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conditions. As a result, it was possible to assess the total extent of glycation, identify discrete glycation adducts, catalogue locations where glycation occurs, and cross-reference with chemical and structural features at each site. This enabled us to discern, and experimentally validate, key trends that control selective glycation. Therefore, this work has significantly advanced our collective understanding for how glycation is able to occur selectively, even in the absence of an enzyme.

To begin, we assessed the reaction between a panel of purified proteins (Supporting Information, Table S1) and methylglyoxal (MGO) in vitro. MGO is a 1,2-dicarbonyl with enhanced electrophilicity and is among the most potent and prevalent glycating agents in vivo.[27–29] Upon treatment with MGO, numerous adducts and variable extents of glycation were observed by LC-MS (Supporting Information, Table S1, Figures S1–S4). We found that the amount of modification did not correlate with the total number of nucleophilic residues, the number of Lys or Arg, or pI (Supporting Information, Figure S5). The lack of any correlations at the intact protein level suggested that individual chemical and structural features at each site control the glycation outcome.

Sites of selective glycation were identified by tryptic digestion after MGO treatment. Glycation at Arg or Lys prevents trypsin cleavage at that site, allowing us to identify modified peptides as missed cleavages (Supporting Information, Figure S6). Owing to potential changes in ionization following glycation, and the large number of distinct and/or isomeric products, we calculated approximate conversion by comparing the amount of unmodified peptide in MGO treated and untreated samples (Supporting Information, Equation S1; Table S2).[30] This data was used to generate frequency logos depicting the five residues flanking each side of the reactive site (Figure 2A). To confirm that MGO concentration does not influence the site-selectivity of the reaction, the same analysis was performed using a range of MGO concentrations (50 μM–1 mM) for a select group of proteins. We found that glycation occurred at the same sites for all concentrations tested, though the amount of glycation increased with the MGO concentration (Supporting Information, Figure S7). Next, we used structural information (available for all but four proteins) to identify residues within 5 Å of each glycated site (Figure 2B). Although this analysis did not reveal a consensus sequence (Figure 2A), the frequency of residues surrounding our glycated sites differed from those neighboring all Arg in our input set and from those adjacent to a set of glycated Arg manually curated from the literature (Supporting Information, Figure S8). This suggested that our data set holds important clues about selective glycation.

Compared to prior studies, our data set is unique in that it enabled further analysis based on each AGE adduct observed, the known structural features, and the extent of glycation at each site. A full account of this analysis, grouped by AGE identity or structure, can be found in the Supporting Information, Figures S9–S11 along with additional discussion.

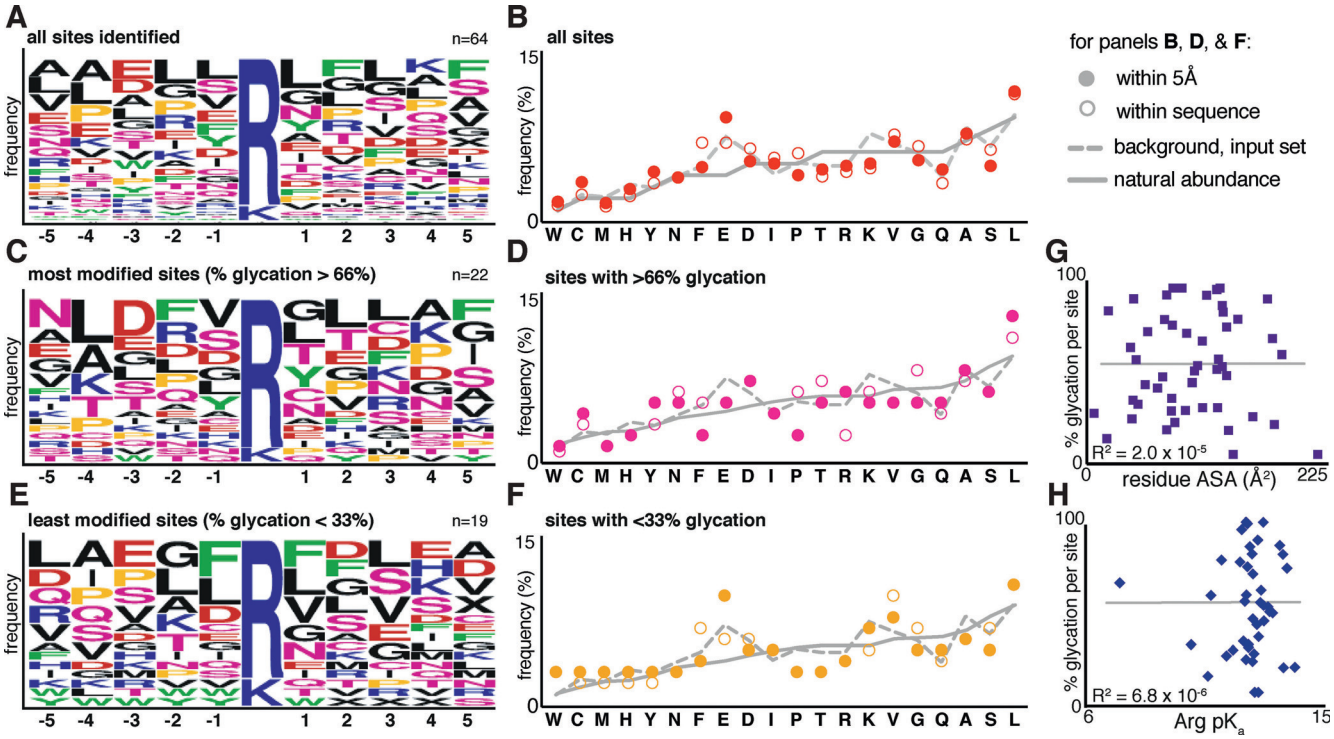
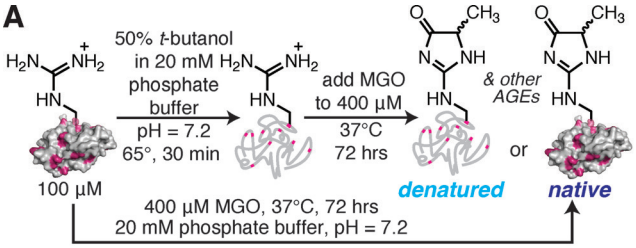


Figure 2. A) Frequency logo of flanking residues surrounding all glycated sites identified in this study. B) Occurrence of residues found within the nearby sequence (open circle) or 5 Å radius (filled circle) for all glycated sites (red), as compared to the natural abundance (solid gray) and background abundance of our input set (dashed gray). The same analysis was performed for the most extensively modified C),D) (>66% glycation, pink), and least modified sites E),F) (<33% glycation, yellow). Correlation between the extent of glycation at each site and G) the accessible surface area (ASA) for each residue or H) the individual Arg pK_a.

The greatest differences were observed when the identified sequences were sorted by the extent of glycation (> 66 % and < 33 % modified, Figure 2 C–F). The most apparent difference was the increased occurrence of Tyr in the set of "most-modified" sequences; Tyr has been suggested previously to promote glycation.[18] This can be contrasted with the striking presence of Phe in the "least-modified" group. Additionally, there was a notable decrease in the frequency of acidic residues found surrounding glycated sites in the "most-modified" sequences. Several studies have highlighted the enrichment of acidic residues surrounding glycated sites,[15,21,31,32] which is also reflected in our complete data set and the "literature" set of glycated sequences (Figure 2 A,B; Supporting Information, Figure S8). However, the importance of negative charge has been controversial, as some reports suggest that acidic residues are detrimental,[15] but others propose that they promote glycation.[21,31,32] In our case, the decrease in the frequency of acidic residues in the "most-modified" sequences was concomitant with an increase in the occurrence of Asn, Cys, Pro, Ser, and Thr. Although none of these differences met the criteria for statistical significance, the observed trends led us to build several hypotheses about features that could influence glycation. In particular, this analysis suggests that the presence of tyrosine and polar groups, and perhaps proline, could enable glycation at a particular site.

Next, we incorporated structural considerations into our analysis. We determined the residue accessible surface area (ASA) using the VADAR algorithm, which calculates the residue surface area that a water molecule can "touch".[33] The pK_a for each glycated Arg was also estimated using PROPKA, a widely used empirical program for predicting pK_a.[34] We found no correlation between the extent of glycation at each site and ASA (Figure 2 G) or pK_a (Figure 2 H). Although this conflicts with prior hypotheses that Arg pK_a perturbations govern glycation,[18,32,35–37] it was not surprising to us based on the diversity of our glycated sequences. Moreover, if glycation were purely driven by nucleophile pK_a, preferential glycation would be observed at the N-terminus or Lys, rather than Arg. From a mechanistic standpoint, this likely reflects that imine formation, the first step in the Maillard reaction, occurs readily and reversibly on amino and guanidino groups throughout the protein.[1,2,38–41] Instead, selective glycation is more likely driven by polar or ionizable groups that promote later, rate-determining rearrangements, which may vary depending on the mechanism through which each adduct forms.[42,43]

To disentangle the effect of sequence from that of structure on the glycation outcome, we examined the glycation of proteins that were unfolded prior to MGO treatment. We focused on ubiquitin, ribonuclease A, and myoglobin, as these proteins exhibited high, medium, and low levels of glycation, respectively (Supporting Information, Figure S1). We confirmed that commonly used chaotropic denaturants, such as guanidinium chloride and urea, completely prevented glycation (Supporting Information, Figure S12). As an alternative, we established that 50 % *tert*-butanol does not significantly alter total levels or distributions of AGE adducts, and can be used to denature proteins (Supporting Information, Figure S12, Figure S13 A,B). For proteins that were unfolded using this method prior to MGO treatment, we observed modestly increased levels of glycation by intact MS (Supporting Information, Figure S13 C–E). While this was most apparent for myoglobin, western blot analysis of glycated ribonuclease and ubiquitin confirmed that more glycation occurred for unfolded proteins (Supporting Information, Figure S13 F). Following proteolytic digest of the denatured proteins, all of the same sites were modified, but each contained fewer distinct AGE products (Figure 3). In other words, though the overall number of glycated sites was lower for folded proteins, the total number of adducts was higher. This demonstrates that when structure is lost, there is less diversity in the type of AGEs that form. Moreover, we did not observe glycation at every possible Arg or Lys, suggesting



B

protein	entry	sequence	adducts observed (Da) denatured	native
myoglobin	1	QEVLI**R**LFTGH	54, 72	54
2 total Arg	2	ALELF**R**NDIAA	54, 72	54, 72,144
19 total Lys	A	LNVWG**K**VEADI	72	--
ribonuclease A	3	AAKFE**R**QHMDS	54, 72	54, 72,126,144
5 total Arg	4	NLTKD**R**CKPVN	54, 72	54, 72, 80, 126
11 total Lys	5	NLTKD**R**CKPVN	54, 72	54, 72, 144
	6	SITDC**R**ETGSS	54	54, 72,126,144
	7	ETGSS**K**YPNCA	72	54, 72, 126
	B	QMMKS**R**NLTKD	54	--
ubiquitin	8	PPDQQ**R**LIFAG	54,72	54, 72,126,144
4 total Arg	9	QLEDG**R**TLSDY	54,72	54, 72,126
7 total Lys	10	LHLVL**R**LRXXX	54,72	54, 72,126
	11	LVLRL**R**XXXXX	54,72	54, 72, 126

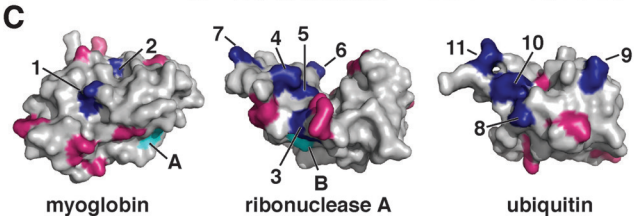


Figure 3. A) Reaction depicting the glycation of native or denatured proteins. Pink residues denote all Arg and Lys. B) Identified glycation sites, along with the products observed (Δ Da), for native and unfolded proteins ($n = 4$). Entry numbers reference the Supporting Information, Table S2. C) Protein structures for myoglobin (1ymb), ribonuclease A(1fs3), and ubiquitin (1ubq) highlighting sites of selective glycation. Indigo: sites glycated under native conditions. Cyan: additional sites glycated when denatured. Pink: remaining Arg and Lys.

that selective glycation occurs even for unstructured targets. Taken together, these results reveal that primary sequence dictates overall reactivity, while nearby structure sculpts the distinct outcome at each site.

Building on these findings, we chose to evaluate further the effect of primary sequence on glycation using short, unstructured peptides. We synthesized three peptides: **1** (Ac-AEELE**R**EFLAV), **2** (Ac-LADFE**R**TGLLS), and **3** (Ac-FTPSA**R**SQTSY), which were designed to mirror trends on either extreme of our data set (Figure 2C–F) including abundant negative charge and Phe (**1**), the presence of Tyr, Pro, and polar residues (**3**), or an intermediate combination (**2**). After 24 h treatment with MGO, all formed the most commonly observed adducts in our protein data set ([M + 54], [M + 72]), and [M + 144]). However, **2** was the only to form an [M + 90] adduct, whereas **3** was the only to yield [M + 126]. The most glycation was observed for **3**, followed by **2** and **1** (Figure 4). These data confirm that differences in primary sequence result in differences in both overall glycation and the distribution of AGEs. This result is significant because past work has considered sequence and structure together, making it extremely difficult to examine them as independent variables that, on their own, provide specific contributions to the glycation outcome. Moreover, these results reveal that multiple acidic residues within the same sequence are detrimental to reactivity, even though they were abundant in our modified sequences. They also suggest that Tyr and polar residues can promote glycation.

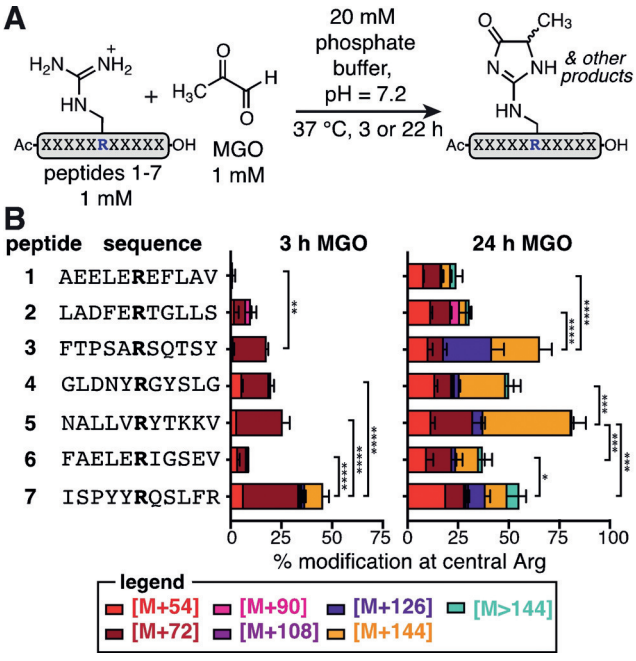


Figure 4. A) Depiction of the glycation of synthetic peptides with MGO. B) Distribution of glycation products observed for **1**–**7** after treatment with MGO ($n \geq 3$). Careful MS/MS analysis was performed in addition to our standard quantification protocol (see the Supporting Information). This enabled the quantification of glycation that had taken place at only the central Arg for peptides **5** and **7**, which both contain multiple potential glycation sites. However, all trends remained the same when considering "total glycation", which considers modification at all sites (Supporting Information, Figure S14). Tukey HSD performed separately for **1**–**3** and **4**–**7**; $p < 0.005$ (**), $p < 0.0005$ (***), $p < 0.0001$ (****).

Our next goal was to use these findings to inform an experiment that could test of our understanding of the rules governing selective glycation. To do so, we selected peptides **4** (Ac-GLDNY**R**GYSLG) and **5** (Ac-NALLV**R**YTKKV) to prepare from our set of highly modified sequences (Figure 2C–F; Supporting Information, Table S2). As both contained at least one Tyr and several polar residues, they were expected to be substantially modified. From the least modified set, we selected peptide **6** (Ac-FAELE**R**IGSEV), which we anticipated to be minimally glycated owing to its three acidic residues. We also chose peptide **7** (Ac-ISPYY**R**QSLFR), which did not match the trends expected for sequences with low reactivity. Instead, we hypothesized that the multiple Tyr and polar groups would promote glycation. Indeed, after 24 h of treatment with MGO, **5** was the most glycated, while **4** also exhibited moderate reactivity (Figure 4). As expected, **6** was the least modified and **7** displayed moderate reactivity similar to that of peptide **4** after 24 h of MGO treatment. Strikingly, at shorter incubation times (3 h), peptide **7** yielded the highest levels of glycation for any peptide tested. These studies provide important validation about features that control selective glycation: **1**–**3** permitted us to test experimentally the trends we uncovered; these results enabled us to correctly predict relative levels of glycation for select isolated peptides (**4**–**7**).

To further evaluate the specific sequence contributions that govern selective glycation, a series of point mutants to peptide **4** were prepared (Figure 5; Supporting Information, Figure S15). We chose to explore these effects for peptide **4** because it contains two Tyr and multiple polar residues that were determined to be beneficial for glycation (Figures 2 and 4). However, like many of the glycation sites we identified (Supporting Information, Table S2), it also possesses a single Asp, providing an opportunity to reconcile the role of an isolated negative charge in influencing the glycation outcome. First, we evaluated the effect of removing the two Tyr and replacing them with Phe, Ser, or Asp (peptides **4a**, **4b**, and **4c**, respectively; Figure 5A). For all of these variants, the extent of glycation was decreased relative to that observed for peptide **4**. A larger decrease in the amount of glycation was observed for peptide **4a** (Tyr to Phe) than for **4b** (Tyr to Ser), suggesting that the hydroxyl group of Tyr has a greater effect than its aromatic core in influencing glycation. Moreover, the reduction in glycation was most dramatic for peptide **4c**, which introduced two additional acidic residues. Together, these observations provide further confirmation that multiple negative charges within a sequence are detrimental for glycation, and support the importance of Tyr and polar residues as beneficial for glycation.

Our subsequent studies aimed to assess the role of substitution in several positions throughout the peptide **4** scaffold (peptides **4d**–**n,** Figure 5B–D). Although we established that removal of Tyr was detrimental to glycation, we found that introduction of an additional Tyr led to either no change or slightly lower levels of glycation (**4d** and **4i**). The same was true for the introduction of polar residues such as Gln (**4f**) or Ser (**4g** and **4m**). Indeed, none of the point

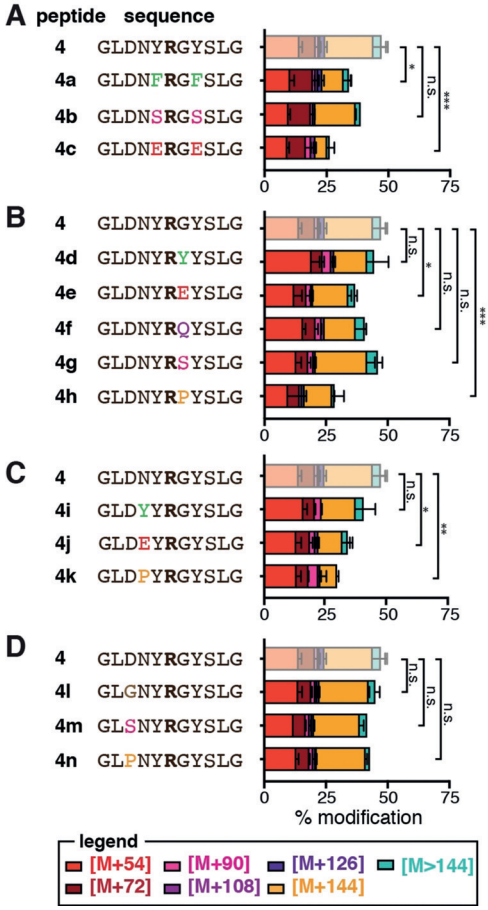


Figure 5. Distribution of glycation products observed for a series of single- and double-point mutations to peptide **4** (peptides **4a**–**n**) after 24 h treatment with MGO ($n \geq 3$). For the reaction scheme, please refer to Figure 4A. To aid in interpretation, these data are grouped by the position, relative to Arg, where substitutions were made: A) Position −1/+2 (**4a**–**c**); B) Position +1 (**4d**–**h**); C) Position −2 (**4i**–**k**); and D) Position −3 (**4l**–**n**). Tukey HSD comparing to peptide **4**; $p < 0.05$ (*), $p < 0.005$ (**), $p < 0.0005$ (***).

mutants we prepared led to increased levels of glycation compared to peptide **4**. However, we found that the introduction of Pro led to a significant decrease in the amount of glycation observed (**4h** and **4k**). Additionally, the introduction of Glu (**4e** and **4j**) consistently led to significantly less glycation relative to peptide **4**. However, when the single negative charge in peptide **4** was substituted with Gly, Ser, or Pro (**4l**–**n**), levels of glycation remained fairly constant. This suggests that, while clustered acidic residues consistently decrease levels of glycation, a sole negative charge may be important for glycation. Taken together, these experiments provide further validation that Tyr and polar residues are helpful for glycation, whereas the introduction of multiple acidic residues is disadvantageous. As a result, this is the first report, to our knowledge, to experimentally validate guidelines for selective glycation that have the potential to be generalized across different substrates.

Past studies of selective glycation with MGO have rationalized findings individually without further experimental validation (see the Supporting Information, Figure S8 for a complete reference list). This has led to many ideas about features that might promote glycation but few, if any, that can be generalized. Therefore, our goal was to perform a systematic study that would contribute robust information about how selective glycation arises. We conclude that primary sequence primarily governs the propensity of a site to be glycated, whereas the surrounding structure refines the specific glycation outcome. Our experiments validated that clustered negative charge is detrimental to glycation, and suggests that a combination of tyrosine and polar residues are beneficial. Our future work will focus on fully reconciling the effect of individual negative charges, which frequently appear in glycated sequences that we, and others,[15,21,31,32] have identified. We will also evaluate further how the positional preferences for surrounding residues affect the distribution and/or identities of AGEs that form. Further studies to define the influence of structure are also underway. Such knowledge will enable the development of new tools that can be used to advance our understanding of glycation as a functional PTM.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: advanced glycation end-product · chemoselectivity · glycation · methylglyoxal · protein modification

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