Oxidative alterations in the experimental glycation model of diabetes mellitus are due to protein–glucose adduct oxidation

Some fundamental differences in proposed mechanisms of glucose oxidation and oxidant production

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Modification of human serum albumin (HSA) with formaldehyde resulted in a loss of 75% of available lysine residues, but there was no change in histidine content or susceptibility to free-radical-mediated fragmentation. The modified HSA appeared resistant to glycation and glucose-mediated fragmentation. Native HSA inhibited oxidant production by free glucose, as assessed by the hydroxylation of benzoic acid, but modified HSA had little effect. Thus the oxidation of free glucose appeared to be inhibited by glycatable protein, but not by unglycatable protein. Also, a close proximity of glucose to protein (decreased in the case of modified HSA) would seem to be a prerequisite for glucose-mediated protein fragmentation. This latter observation, in particular, led us to examine the role of oxidation of glucose attached to HSA in the production of reactive oxidants and subsequent molecular damage. Glycated HSA, washed free of unbound glucose, became fragmented and generated oxidants capable of hydroxylating benzoic acid and oxidizing cholesteryl linoleate–HSA complexes. Significant levels of benzoate hydroxylation and HSA fragmentation occurred with HSA (10 mg/ml) containing 3.3 mol of glucose bound/mol of HSA. This is equivalent to incubation of 10 mg/ml native HSA with 0.66 mM glucose, conditions which lead to little fragmentation or oxidant formation. The oxidative activity of glycated HSA was dependent on transition-metal concentration. The level of protein-bound glucose appeared to decrease during the oxidant production and protein fragmentation. Thus glucose can oxidize and generate reactive oxidants, whether in solution or attached to protein. We discuss which is the more likely mechanism of glucose oxidation under the near-physiological conditions used to study the effects of protein exposure to glucose in vitro.

INTRODUCTION

Oxidative stress may play a role in tissue damage associated with both diabetes and aging [1,2]. The source of this oxidation is not known, but may be related to an increase in the concentration of transition metals (redox catalysts) and/or substances prone to oxidize and generate H₂O₂ and free radicals, such as glucose [1,2].

Evidence in favour of oxidative stress in diabetes [3] and aging [4] has become extensive. For instance, the transition metals copper [5,6] and iron [7,8] increase in plasma concentration with age and in diabetes, suggesting a decreased ability to sequester these trace elements [1]. Albeit tightly regulated, an increase in either of these metals may imply an increase in non-sequestered forms able to undergo redox reactions [1]. Thus a role for transition metals, known redox catalysts, in disease processes associated with diabetes and aging has been suggested [1,2]. In addition, a number of the antioxidant defences in diabetic individuals seem to be compromised. These include decreased plasma levels of glutathione [9], ascorbic acid [10] and uric acid [11] as well as decreases in cellular vitamin E [12].

The in vitro exposure of macromolecules to concentrations of glucose representative of hyperglycaemia is often used to investigate the way in which glucose might cause functional degeneration of tissues occurring in aging and diabetes mellitus [13]. During such studies, proteins undergo structural alterations and develop novel fluorophores [2,3,14] similar to those found in tissue alterations in diabetes [15]. However, the covalent attachment of glucose to amino groups alone is no longer sufficient to account for structural changes observed during such in vitro studies [1,2]. Two alternative mechanisms by which glucose may induce structural changes in proteins have been proposed.

Although they bear certain similarities, they also possess fundamental differences.

Firstly, protein-bound products of the Amadori reaction may subsequently degrade, in a transition metal-catalysed process, to yield H₂O₂, reactive oxidants and further protein-reactive aldehydes. The production of oxidants by the oxidation of glucose–protein adducts has been termed glycoxidation and is outlined in Scheme 1 [16,17].

The second mechanism involves free glucose, unattached to protein, which, when in solution, is also prone to transition-metal-catalysed oxidation [1,2]. This also results in the generation of H₂O₂ [18], reactive oxidants equivalent in reactivity to the hydroxyl radical [19] and protein-reactive dicarbonyl compounds [20]. This process of oxidation of free glucose has been termed glucose autoxidation and is outlined in Scheme 2.

Thus, the theory of glucose autoxidation proposes that oxidants and protein-reactive aldehydes are formed as a result of transition-metal-catalysed glucose oxidation which occurs independently of attachment to protein. However, the theory of glycoxidation proposes that the oxidative processes occur after glucose has become attached to protein. Whether one mechanism of oxidation occurs in preference to the other is uncertain.

Both processes require the presence of transition metals, the formation of enediol intermediates and enediol–transition-metal complexes. Both result in the production of dicarbonyl compounds and superoxide radicals. The latter leads to the formation of hydrogen peroxide and other reactive free-radical derivatives and eventually results in macromolecular alterations [12,16,19,20].

Here, using techniques similar to those used in previous studies on glucose autoxidation, we consider the role of free-radical production by glucose in solution and glucose adducts in
MATERIALS AND METHODS

All reagents were of the highest purity available from Sigma Chemical Co. unless otherwise indicated. In all cases, reaction mixtures were filter-sterilized (0.22 µm-pore-size filter) prior to incubation and reactions were performed at 37 °C in the presence of 100 mM potassium phosphate, pH 7.4.

Measurement of protein structural alterations

Human serum albumin (HSA; Sigma Fraction V) was 14C-radiomethylated (18000 dpm/mg). Fragmentation was measured as the production of 5% trichloroacetic acid-soluble radio-labelled peptides and monitored by SDS/PAGE as previously described [19,22]. The extent of incorporation of D-[U-14C]glucose into trichloroacetic acid-insoluble HSA was determined as previously described [20]. The absence of protein-reactive impurities in commercial radioactively-labelled glucose was also verified as previously described [20].

Hydroxyl-radical detection

The hydroxylolation of benzoate by glucose was measured by fluorescence (308 nm excitation; 410 nm emission) as previously described [19,22]. The level of fluorescent hydroxylation products is expressed as salicylic acid equivalents [19,22].

Albumin modification

Approx. 75% of the lysine residues in HSA were modified by exposure of 100 mg/ml radiolabelled HSA in the presence of 100 mM potassium phosphate, pH 7.4, to 10 mM formaldehyde and 10 mM sodium cyanoborohydride over a period of 24 h at 37 °C. After dialysis, the protein was analysed for both lysine and histidine content. Histidine content was determined using diethyl pyrocarbonate as previously described [23]. HSA which had undergone lysine modification exhibited less than 5% histidine loss.

Determination of available lysine content

The reaction of 2,4,6-trinitrobenzenesulphonic acid (TNBS) with primary amino groups within proteins was performed as previously described [24]. Protein was adjusted to 1 mg/ml in 4% (w/v) Na2CO3, pH 8.5. An equal volume of protein was added to 0.1% TNBS in water, which was then incubated at 40 °C for 2 h. The mixture was heated at 120 °C after adding concentrated HCl (3:1, v/v) for 1 h. After cooling, in order to remove unchanged reagent and-picric acid, an equal volume of water was added and the mixture extracted twice with an equal volume of diethyl ether. Residual diethyl ether was removed from the aqueous phase by heating in a water bath for 5 min. The absorbance of the aqueous fraction was read at 346 nm against a blank carried through the same procedure. The amount of trinitrophenyl derivatives of lysine was calculated from a standard curve obtained with unmodified protein. The absolute level of lysine formation is dependent upon the availability of transition metals. The way in which reducing conditions induced by sodium cyanoborohydride affect enedial formation is included. The role of enedial complexation with transition metals is also shown (b). The reaction scheme is an adaptation of that proposed by Sekari et al. [16].
present was also calculated using a value of $1.46 \times 10^4 \text{M}^{-1} \cdot \text{cm}^{-1}$ for the molar absorption coefficient of trinitrophenyl-lysine [24].

**Determination of albumin-bound copper**

Commercial preparations of HSA (Sigma Fraction V) were assessed for the presence of contaminating copper by incubating 75 \( \mu \text{M} \) HSA with 10 mM diethyl pyrocarbonate over 1 h in the presence of 100 mM potassium phosphate at 37 °C. Released copper was detected using the Cu(I)-complexing dye 2,2'-biquinoline-4,4'-dicarboxylic acid (Fluka). The assessment of copper was performed in the presence of 10 mM potassium phosphate (pH 7.4)/10 mM dye/1 mM ascorbic acid. After 30 min the absorbance at 560 nm was measured and the copper concentration was calculated by using Cu(II) (CuSO\(_4\)) standards (0–10 \( \mu \text{M} \)) put through the same procedure [23]. HSA contained 0.18 mol of copper/mol of protein.

**Artificial lipoprotein preparation and oxidation**

Artificial lipoproteins, consisting of HSA and cholesterol linoleate, were prepared as previously described [25]. The molar ratio of lipid to HSA (native and glucose-modified) was 60:1. HSA was used at 10 mg/ml in PBS. Cholesterol linolate was first dissolved in acetone (BDH; AnalaR) and then added to 10 mg/ml HSA with vortex mixing and then sonicated for 1 min. Acetone was subsequently evaporated by gassing with a stream of \( \text{N}_2 \).

**Lipid hydroperoxide measurement**

Lipid hydroperoxides present in methanolic extracts of cholesterol linolate–HSA complexes in which any \( \text{H}_2\text{O}_2 \) had previously been removed by incubation with catalase [26,27] were determined by the oxidation of Fe(III) to Fe(II) under acidic conditions and in the presence of Xylenol Orange, as previously described [26,27].

**RESULTS AND DISCUSSION**

**Exposure of formaldehyde-modified albumin to glucose**

The oxidation of glucose in solution (glucose autoxidation) is known to be inhibited by the presence of albumin. Albumin has been shown to reduce the production of glucose-derived protein-reactive dicarbonyl compounds [20], \( \text{H}_2\text{O}_2 \) [18] and hydroxyl radicals [19]. These effects were assumed to result from the copper-binding capacity of albumin, since oxidation of glucose is transition metal-dependent.

In the present study, formaldehyde-modified HSA, on which 75 % of the lysine content was modified, had an unaltered affinity for copper and an unchanged susceptibility to Cu(II)-catalysed \( \text{H}_2\text{O}_2 \)-mediated fragmentation. The latter is shown in Figure 1 (lanes 1 and 2) in which fragmentation by peroxide and Cu(II) led to identical extents of protein scission. This was further confirmed by measuring trichloroacetic acid-soluble peptides (results not shown). Formaldehyde-modified HSA also appeared to be resistant to both glucose attachment, which was virtually abolished (results not shown), and glucose-mediated fragmentation. The latter was shown by the formation of trichloroacetic acid-soluble peptide generation (Figure 2) and by SDS/PAGE (Figure 1, lanes 3–10). In control reactions, during which formaldehyde-modified HSA (Figure 1, lane 3) and native HSA (Figure 1, lane 6) were incubated with glucose, Cu(II) and EDTA, far less peptide fragments are evident. However, there is an apparent increase in this low-level protein fragmentation in the case of formaldehyde-modified HSA, suggesting that ex-

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**Scheme 2 Glucose autoxidation**

An outline of oxidation of glucose in solution ('glucose autoxidation') is shown (a). The process is rate-limited by enediol formation, and oxidant formation is dependent on the availability of transition metals. Also shown is the involvement of metal–enediol complexes [21] (b) and the effect of a reducing environment brought about by agents such as sodium cyanoborohydride on the formation of enediol [1,19,20].
Throughout, incubations (37 °C) were performed in 100 mM potassium phosphate, pH 7.4; HSA was used at 1 mg/ml, glucose at 25 mM, H2O2 at 2.5 mM, EDTA at 1 mM and Cu(II) at 100 μM. Lane 1, HSA exposed to H2O2 and Cu(II) for 30 min; lane 2, formaldehyde-modified HSA exposed to H2O2 and Cu(II) for 30 min; lane 3, formaldehyde-modified HSA exposed to glucose, Cu(II) and 1 mM EDTA for 4 weeks; lane 4, formaldehyde-modified HSA exposed to glucose and Cu(II) for 4 weeks; lane 5, HSA exposed to glucose and Cu(II) for 4 weeks; lane 6, HSA exposed to glucose, Cu(II) and 1 mM EDTA for 4 weeks. MM, molecular-mass (M) markers (14–95 kDa).

**Figure 1** Fragmentation of native and modified albumin by glucose

Native (continuous line) and formaldehyde-modified HSA (broken line) was incubated at 2 mg/ml at pH 7.4 for 2 weeks. The effect of 25 mM glucose and 1 mM benzoic acid is shown. Fragmentation in the presence of various copper concentrations (0, 1, 10 and 50 μM) is shown on a logarithmic x-axis. Sterility was maintained by filter-sterilizing solutions and using sterile vessels. Fragmentation was monitored as described in the text. Values are means ± S.D. for three experiments.

**Figure 2** Modified albumin is less susceptible to fragmentation by oxidizing glucose

Figure 3 Modifed albumin does not significantly inhibit oxidant production by glucose

The effect of various copper concentrations and the presence of native and modified HSA (2 mg/ml) on the generation of oxidants by 25 mM glucose is shown. Oxidants able to hydroxylate benzoic acid to fluorescent products was measured after 2 weeks. Cu(II) concentrations are shown on a logarithmic scale on the x-axis. Values are means ± S.D. for three experiments. CHO-HSA, formaldehyde-modified HSA.

tensive lysine modification of formaldehyde leads to a small increase in autolytic hydrolysis of HSA.

Previous studies have shown that fragmentation of albumin resulting from exposure to glucose was time-dependent and detectable by SDS/PAGE [19]. Exposure to glucose for a period of 4 weeks leads to the production of fragments of a defined size. Figure 1 shows the peptide fragments generated on exposure to H2O2 or glucose in the presence of Cu(II). In all instances peptide fragments appear to be identical. This is in keeping with the proposed chemistry of both glucose autooxidation and glycoxidation in which H2O2 is the source of oxidants which cleave proteins [16,18,19,28]. The uniform size of fragments produced on exposure to peroxide is thought to be the result of site-specific peroxide decomposition. This is dependent on the histidine residues in albumin, which are the sites of copper complexation [28]. Thus both native and lysine-modified HSA generated identical peptide fragments when exposed to glucose or H2O2 in the presence of Cu(II), as shown in Figure 1.

When modified by formaldehyde, albumin was resistant to glucose-mediated fragmentation, despite an unaltered histidine content and susceptibility to peroxide. This leads us to suggest that an alternative manner in which albumin might inhibit glucose autoxidation, the oxidation of free glucose in solution, is by the sequestration of glucose (open-chain hydroxaldehyde) in the form of Schiff bases. If this is indeed the case, then perhaps the oxidation of glucose (attached to protein) described by glycoxidation may actually compete with the oxidation of glucose in solution, described by glucose autoxidation.

Figure 2 shows that benzoic acid, a reagent often used to monitor the production of oxidants similar in reactivity to the hydroxyl radical [19], inhibited fragmentation of both native and formaldehyde-modified HSA. The extent of inhibition seemed greatest for the fragmentation of formaldehyde-modified HSA. On the other hand, oxidant production by glucose in the presence of formaldehyde modified HSA was virtually unaffected when compared with the inhibitory effect of native HSA, and approached 80% of that produced by glucose in the total absence of protein (Figure 3).
The effect of glucose-mediated fragmentation, as described by Bruckner et al. [19], implies that attachment of glucose is a prerequisite for protein oxidation. This apparent necessity for the proximity of glucose for protein fragmentation leads us to suggest that oxidation of glucose after attachment (glycoxidation) to protein may be the process of importance to glycation studies pertinent to near-physiological conditions.

**Glycoxidation and protein fragmentation**

To study the effect of glucose oxidation once attached to protein (glycoxidation), investigations were performed using HSA which had previously been exposed to 25 mM glucose for 2 weeks. These conditions resulted in 3.3 mol of glucose bound/mol of HSA. The protein was then separated from unbound glucose by dialysis and sequential washing in 5% trichloroacetic acid. The washed precipitated protein was then used after resuspension in 100 mM potassium phosphate (pH 7.4). Alternative methods of preparing glycated protein, including boronate affinity chromatography and extensive dialysis, were also conducted and provided similar findings (results not shown).

In the presence of 10 μM Cu(II), glycated HSA (10 mg/ml) became fragmented to trichloroacetic acid-soluble peptides (Figure 4). The level of glucose attached to HSA, used in these studies, equates to a reaction mixture consisting of 10 mg/ml HSA to 0.66 mM glucose and 10 μM Cu(II). Previous studies have shown that such an exposure leads to little or no detectable fragmentation over similar times and conditions [2,19]. Indeed, at high protein concentrations (10 mg/ml and above) even 25 mM glucose only results in a little fragmentation (Figure 5). Thus it would appear that protein-bound glucose is able to undergo oxidative reactions, resulting in protein oxidation, at rates in excess of unattached glucose.

The exposure of glycated HSA to Cu(II) was accompanied by the loss of protein-bound glucose (Figure 6), suggesting that protein fragmentation of glycated HSA occurred at the expense of HSA-bound glucose. Both fragmentation and the loss of attached glucose was inhibited by the presence of EDTA, suggesting that both processes are transition-metal-dependent. A decrease in the level of glucose remaining attached to albumin probably resulted from a loss of glycated non-precipitable peptide resulting from fragmentation. This suggests the possibility that fragmentation occurs close to the site of glucose attachment. This effect of fragmentation upon the observed level of glucose attachment has previously been described [19].

However, exposure of glycated HSA to buffer alone also resulted in loss of protein-bound glucose, which was inhibitable by EDTA (Figure 6), despite an absence of detectable levels of fragmentation. Thus, decreases in the level of attached glucose may not be solely due to fragmentation and loss of glycated non-precipitable peptide. The reversible nature of Schiff-base formation (Scheme 1) may have contributed to the observed decrease in the level of glucose remaining attached to HSA. However, it is difficult to envisage a manner in which EDTA could affect the reversible nature of Schiff-base formation. Perhaps the EDTA affects a metal-catalysed process (possibly oxidation) in which HSA-bound glucose becomes an unbound product. The loss of HSA-bound glucose is likely to be due to a combination of protein fragmentation [19], the reversible nature of Schiff-base formation and perhaps the oxidation of bound glucose to products unattached to protein.

**Oxidant production by glycated albumin**

To show whether glycated HSA can generate reactive oxidants, glycated HSA (10 mg/ml) was exposed to glucose with or without
EDTA, or in the absence of sodium cyanoborohydride. After washing with trifluoroacetic acid, HSA was then incubated with benzoic acid in the presence of 10 µM Cu(II) for a period of 10 days.

Benzoate hydroxylation increased over time with glycated HSA which had previously been exposed to glucose under non-reducing conditions (Figure 7). The presence of EDTA during HSA glycation resulted in a lower level (~10% lower) of benzoic hydroxylation. HSA previously glycated in the presence of EDTA was glycated to a lesser degree (4.5 mol of glucose bound/mol of HSA) than in the absence of EDTA (6.5 mol of glucose bound/mol of HSA). This may have been the cause of this lower level of oxidant formation. The lower level of glucose attachment to HSA in the presence of EDTA is in agreement with previous studies suggesting that the oxidation of glucose may partially contribute to the attachment of glucose to protein [20] during in vitro glycation studies.

Control studies in which HSA was previously glycated in the presence of sodium cyanoborohydride (9 mol of glucose bound/mol of HSA) or exposed to buffer alone or sodium cyanoborohydride alone, led to little or no benzoate hydroxylation. Sodium cyanoborohydride is a reducing agent able to reduce Schiff bases selectively and thus increases the level of glucose attachment. It also prevents endiol formation, which is a prerequisite for oxidant formation by both mechanisms of glucose oxidation (see Schemes 1 and 2) [20]. In all instances, oxidant formation was inhibited by the presence of 1 mM EDTA (Figure 7 and, again, suggests that oxidant formation by protein-bound glucose is transition-metal-dependent.

Further investigations were performed to show that oxidant production by glycated protein can lead to the oxidation of unsaturated fatty acids. HSA previously glycated by pre- incubation with combinations of glucose, EDTA and sodium cyanoborohydride was used. Pretreated HSA was complexed with cholesteryl linoleate to form artificial lipoproteins which were then incubated with 10 µM of Cu(II) overnight. Table 1 shows that glycated HSA led to an elevated level of lipid peroxide formation. On the other hand, HSA glycated in the presence of sodium cyanoborohydride or pre-exposed to buffer alone resulted in lower levels of lipid peroxide. The addition of EDTA to the overnight exposures of cholesteryl linoleate–HSA complexes inhibited peroxide formation.

Thus the exposure of cholesteryl linoleate to glycated HSA

![Figure 6 Exposure of glycated albumin to copper results in a loss of protein-bound glucose](image)

Native HSA (25 mg/ml) was exposed to radiolabelled 25 mM glucose for a period of 2 weeks. This protein was then dialysed and precipitated with 5% trichloroacetic acid. The precipitate was then resuspended in 100 mM potassium phosphate and precipitated once more prior to final resuspension in 100 mM potassium phosphate, pH 7.4. Glucose remaining attached to the trichloroacetic acid-washed HSA was equivalent to 3.3 ml glucose bound/mol of HSA. The level of glucose remaining attached to HSA was then monitored over a period of 2 weeks. The effect of 10 µM copper with or without 1 mM EDTA, as well as a control incubation of buffer alone, is included. Values are means ± S.D. for three experiments.

![Figure 7 Oxidant production by glycated albumin](image)

The level of glucose attachment was determined after 2 weeks pre-treatment of 25 mg/ml HSA with (1) buffer alone (HSA), (2) radiolabelled 100 mM glucose (6.5 mol of glucose bound/mol of HSA) (HSA-glucose) and (3) glucose with 1 mM EDTA (5.5 mol of glucose bound/mol of HSA) (HSA-glucose/EDTA). Pretreatment of controls by incubation over 24 h with buffer alone, 10 mM sodium cyanoborohydride and 10 mM sodium cyanoborohydride with glucose (9 mol of glucose bound/mol of HSA) were also conducted (pretreatment controls). Pre-treated HSA was resuspended at 10 mg/ml and then incubated with 1 mM benzoic acid and 10 µM Cu(II). The generation of fluorescent benzoic acid hydroxylation products was then monitored over a period of 10 days (a). The effect of including 1 mM EDTA, during incubation with benzoic acid is shown (b). Values are means ± S.D. for three experiments.

**Table 1** Cholesterol linoleate oxidation is enhanced by glycated albumin

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>− EDTA</th>
<th>+ 1 mM EDTA</th>
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<tbody>
<tr>
<td>HSA</td>
<td>6.0 ± 0.1</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>HSA-glucose</td>
<td>7.4 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>HSA-glucose + EDTA</td>
<td>7.5 ± 0.2</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>HSA-glucose + NaCNBH₃</td>
<td>4.8 ± 0.3</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>HSA + NaCNBH₃</td>
<td>4.4 ± 0.3</td>
<td>4.2 ± 0.4</td>
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resulted in an enhanced level of copper-catalysed lipid peroxidation. Autoxidative glycation, the oxidation of glucose which is unattached to protein, has been shown to induce lipid peroxidation in low-density lipoprotein (LDL) [1,27]. Whether glucose contributes to LDL oxidation after its attachment to the protein moiety of LDL remains unclear but, from these studies, seems likely. The implications for glucose-mediated lipid oxidation have already been discussed in terms of the elevated incidence of atherosclerosis in diabetes and the role of LDL oxidation in this disease [1,16,27].

Concluding remarks

Evidence in favour of both mechanisms of glucose oxidation have been reported [1,16,17,19]. In either mechanism, observations support the catalytic role of transition metals in oxidant production [1,16,17,19]. To date, studies using transition-metal chelators indicate that glucose autoxidation may contribute to the attachment of glucose to protein [1,19,20]. However, glucose or glycated protein can both generate oxidants which can lead to protein oxidation and lipid peroxidation [16,17,27]. Here, we also show that proteins resistant to glucose attachment are also resistant to fragmentation, despite the presence of glucose-derived oxidants.

Thus, observations made in these studies lead us to propose that, although glucose autoxidation does indeed occur, inhibited by glycatable protein but not by unglycatable protein [18–20], it is unlikely to be the major mechanism of oxidant formation pertinent to the exposure of protein to glucose under near-physiological conditions. Previous studies have shown that glycation and oxidation occur simultaneously and are inextricably linked [2]. We propose that the oxidation of protein–glucose adducts, as opposed to glucose itself, is the reason for the inextricable link between glucose attachment to protein and oxidation.

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