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THE ROLE OF HISTIDINE RESIDUES IN THE NON-ENZYMIC COVALENT ATTACHMENT OF GLUCOSE AND ASCORBIC ACID TO PROTEIN

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Copper ions have been suggested to play a role in the non-covalent glycosylation (glycation) of proteins via transition metal-catalysed oxidations. We have further investigated "autoxidative glycosylation" by comparison of the behaviour of dog and bovine serum albumin with respect to the oxidative reactions of glucose and ascorbate. The proteins possess similar numbers of total amino residues available for glucose attachment but dog serum albumin contains fewer histidine groups and also lacks a high affinity copper-binding site. We find that the higher copper-binding capacity of bovine serum albumin is reflected in a lower rate of ascorbate oxidation as well as less protein oxidative damage than is the case for dog serum albumin. We also observe that modification of bovine serum albumin histidine groups by diethylpyrocarbonate enhances ascorbate-mediated protein fluorophore formation.

KEY WORDS: Histidine, copper, protein, non enzymatic, covalent attachment, glucose, ascorbic acid.

INTRODUCTION

The exposure of protein to glucose or ascorbic acid, *in vitro*, is considered a relevant model for the functional degeneration of tissues which occurs in diabetes mellitus and ageing.[1–4] We have previously demonstrated that glucose, in common with other α-hydroxyaldehydes, undergoes a transition metal-catalysed oxidation ("autoxidation"), via its enediol,[5,6] resulting in the formation of protein-reactive ketoaldehydes,[5,6] hydrogen peroxide, and reactive oxygen intermediates which cause protein oxidation.[7] Asorbic acid, a permanent enediol, undergoes a similar, but more rapid, transition metal-catalysed oxidation[8,9] and behaves like glucose with respect to protein modification.[2,10,11] We have now investigated the role of histidine-copper complexation by serum albumins with respect to covalent modifications arising from copper ion-catalysed glucose and ascorbic acid oxidation.

MATERIALS AND METHODS

All materials were of the highest purity available from the Sigma Chemical Company, unless otherwise indicated.

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Oxygen Consumption

The effect of albumins on the initial rate of oxygen consumption by 1 mM ascorbic acid and 1 μM copper sulphate (in the presence or absence of 1 mM diethyl pyrocarbonate) was determined using a "Clarke type" electrode over a 10 minute period.[12,13]

Protein Fragmentation

Bovine and dog serum albumin (Sigma Fraction V) were [^{14}C]-radiomethylated (18,000 dpm/mg).[14] Fragmentation, measured as the production of 5% trichloroacetic acid-soluble (TCA-soluble) radiolabelled peptides was subsequently assessed as previously described.[7,15]

Histidine Modification

The modification of histidine residues in serum albumin was performed with the use of diethyl pyrocarbonate (Aldrich). Two cuvettes with protein and 100 mM potassium phosphate (pH 7.4) were placed in a spectrophotometer (Philips PU 8720 UV/VIS). The spectra (between 237 nm and 320 nm) were recorded. After addition of 1 mM diethyl pyrocarbonate to the sample cuvette, the spectra were recorded at several intervals over a 1 hour period.[16] The number of histidine residues modified were calculated from the difference in absorbance at 240 nm (ε240 N-carbethoxyhistidine = 3200 $cm^{-1}M^{-1}$). Under identical conditions to subsequent investigations, 1 mM diethylpyrocarbonate modified 26 and 17 histidine residues in 0.75 μM bovine serum albumin and dog serum albumin, respectively (equivalent to ca. 95% modification of total histidine content). Similarly, 1 mM diethylpyrocarbonate modified 18 and 12 histidine residues in 15 μM bovine serum albumin and dog serum albumin, respectively (equivalent to Ca. 67% total histidine content).

Determination of the Albumin Bound Copper

Comercial preparations of bovine and dog serum albumin (Sigma Fraction V) were assessed for the presence of contaminating copper by incubating 75 μM albumin with 10 mM diethyl pyrocarbonate over 1 hour in the presence of 100 μM potassium phosphate at 37°C. Released copper was analysed using the Cu^{I} complexing dye 2, 2′-Biquinoline-4, 4′-dicarboxylic acid (Fluka).[17] The assessment of copper was performed in the presence of 10 μM potassium phosphate, (pH 7.4 (previously treated with chelating resin: Sigma), 10 mM dye and 1 mM ascorbic acid. After 30 minutes the absorbance at 560 nm was measured and the copper concentration calculated using copper sulphate standards (0–10 μM). Bovine serum albumin contained 0.18 mol copper/mol protein and dog serum albumin 0.075 mol copper/mol protein.

Generation of Protein Fluorescence

Albumin, previously exposed to ascorbic acid, was precipitated and washed with 5% TCA prior to resuspending in 100 μM potassium phosphate (pH 7.2).[6] The suspension was fluorimetrically assessed by measuring emission between 350 nm and 500 nm when exciting at 350 nm using a Perkin-Elmer LS 5 fluorometer.[6]

Attachment of Radiolabelled Substrate

The extent of incorporation of either D-[U^{14}C]-glucose of L-[carboxy-^{14}C]-ascorbic acid into TCA-insoluble bovine serum albumin was determined as described above for the assessment of protein fluorophores.[6] The absence of protein-reactive impurities in commercially radioactively labelled glucose was verified.[18] Radiolabelled ascorbic acid was used soon after delivery. Reaction mixtures were filter-sterilised (0.22 μm filter) prior to incubation.

Estimation of Residues Available for Glycation

Residues potentially available for the attachment of glucose were estimated by [^{14}C]-formaldehyde titration with a modification of the procedure utilised for radiomethylation.[14] Essentially, 75 μM albumin was incubated, in the absence of added reducing agent, with 100 μM [^{14}C]-formaldehyde (1 μCi/ml) at 37°C, pH 7.4 (100 μM potassium phosphate). After 1 hour, samples were removed and precipitated with 5% TCA. This was then centrifuged (12,000 g). The resulting pellet was resuspended in 50 μM potassium phosphate (pH 7.4) and reprecipitated by adjusting to 5% TCA. This washing procedure was repeated and the extent of [^{14}C]-formaldehyde incorporation into the final TCA-insoluble protein was then determined.

Further details of experimental regimes can be found in legends to figures. All results are from representative experiments.

RESULTS AND DISCUSSION

Histidyl Residues Inhibit Copper-catalysed Oxidation and Albumin Fragmentation

The inhibitory influence of albumin on oxidative reactions has been attributed to copper (II) complexation by histidine residues.[19,20] Dog serum albumin and bovine serum albumin inhibit copper-catalysed ascorbic acid oxidation (Figure 1). However, the inhibitory effect of bovine serum albumin exceeded that of dog serum albumin. The presence of contaminating copper ions bound to the commercial preparation of these albumins cannot account for the greater inhibitory effect of bovine serum albumin on ascorbic acid oxidation, since more copper was already attached to the bovine serum albumin used (0.18 mol/mol) than to the dog serum albumin (0.075 mol/mol). The variation in the level of inhibition by bovine and dog serum albumin may reflect the nature and content of constituent histidine residues within these two albumins, since the histidine content of bovine serum albumin (27 histidine residues per molecule) exceeds that of dog serum albumin (18 histidine residues per molecule)[21] and bovine serum albumin,[21] unlike dog serum albumin,[21,23] possesses a histidine residue with a high affinity for copper. This inhibitory effect is substantially prevented on modification of all histidine residues by diethyl pyrocarbonate (Figure 1). However, the incomplete prevention by histidine modification of albumin suggests that other residues may also be involved in metal complexation by these proteins.[24]

Free radical-mediated fragmentation of serum albumins associated with oxidising ascorbic acid and glucose has been described as "site specific"[7,24] and has been attributed to metal ion complexation at histidine residues,[24,25] permitting oxidation and free radical production in close proximity to the protein. In the absense of histidine modification, ascorbic acid-mediated fragmentation of 15 μM bovine serum

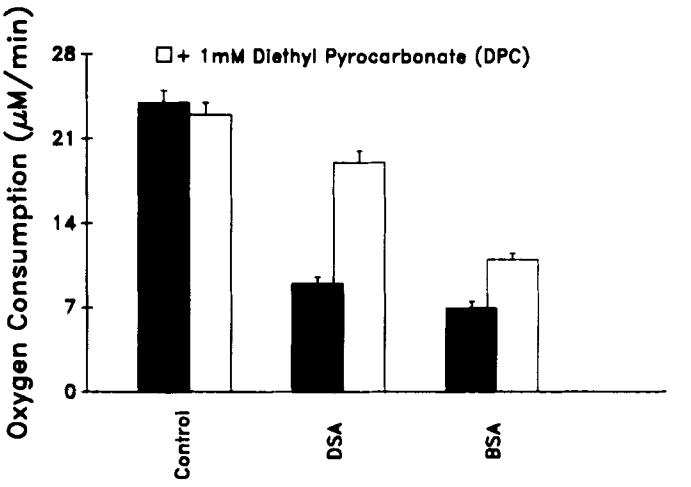

FIGURE 1 The Role of Protein-Histidyl Residues in Copper Catalysed Ascorbic Acid Oxidation. The initial rate of oxygen consumption by 1 mM ascorbic acid in the presence of 1 μM copper was monitored over 10 minutes using a "Clarke type" electrode (Filled Bar). The effect of protein (0.75 μM) modification by 1 mM diethyl pyrocarbonate (diethyl pyrocarbonate) was investigated (Open Bar). All reactions were performed in the presence of 100 mM potassium phosphate (pH 7.4) and at 37°C. A control in which no protein was present is shown. Units are μM oxygen consumed per minute and are a mean $\pm$ standard deviation of triplicate assays. Diethyl pyrocarbonate did not affect ascorbic acid oxidation performed in the absence of protein.

albumin increased between 0 and 25 μM copper (Figure 2A). Interestingly, dog serum albumin, which lacks high affinity histidine residues, is fragmented to a greater degree that bovine serum albumin by both hydrogen peroxide and ascorbic acid in the presence of copper (Table I). Histidine residues of a lower affinity for copper appear to permit more extensive ascorbic acid oxidation and protein fragmentation.

The addition of formate, a hydroxyl radical scavenger, did not affect fragmentation of unmodified albumin at any of the copper concentrations used (shown for 5 μM copper with bovine serum albumin in Figure 2B). The inability of formate to inhibit fragmentation suggests that free radical formation occurs at sites inaccessible to formate, consistent with the site specific nature of free radical formation.[26] In contrast, fragmentation of bovine serum albumin modified with diethyl pyrocarbonate was elevated even at the lowest concentration of added copper (5 μM). The addition of formate led to significant inhibition of this diethyl pyrocarbonate-elevated fragmentation, suggesting that histidine modification results in free radical production at sites accessible to formate. Studies with hydrogen peroxide replacing ascorbic acid resulted in similar observations (not shown).

Histidine Content and Glucose Attachment to Serum Albumins

Bovine and dog serum albumin possess similar numbers of amino residues available for glycation, as assessed by ^{14}C-formaldehyde attachment. Over a 1 hour incubation, 205 $\pm$ 17 μM and 197.7 $\pm$ 5.0 μM radiolabelled formaldehyde became attached to 75 μM bovine and dog serum albumin, respectively. However, exposure to radiolabelled glucose led to differing extents of glucose attachment despite the presence of

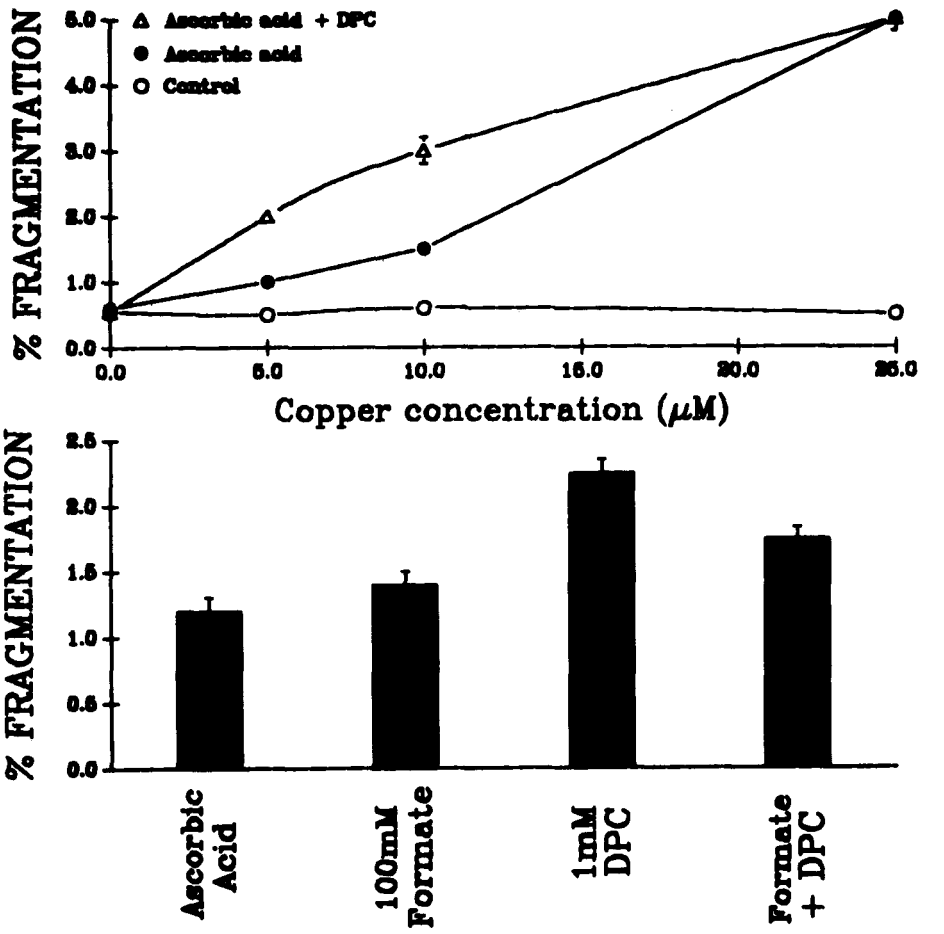

FIGURE 2 Histidine and Copper Dependent Fragmentation by Ascorbic Acid. (A) Fragmentation of radiolabelled bovine serum albumin (15 μM) by 1 mM ascorbic acid in the presence of copper (0–25 μM) was determined after a 3.5 hour period. Reactions were performed at 37°C in the presence of 100 μM potassium phosphate (pH 7.4). The effect of 1 mM diethyl pyrocarbonateis shown. A control in which albumin was incubated in the absence of ascorbic acid is included. Values are expressed as a mean ± standard deviation of triplicate assays. (B) Fragmentation of radiolabelled bovine serum albumin (15 μM) by 1 mM ascorbic acid in the presence of copper (5 μM) was determined after a 3.5 hour period. Reactions were performed at 37°C in the presence of 100 μM potassium phosphate (pH 7.4). The effect of 100 μM formate and 1 mM diethyl pyrocarbonate (with or without formate) is shown. A control in which albumin was incubated in the absence of ascorbic acid is included. Values are expressed as a mean ± standard deviation of triplicate assays.

identical numbers of residues available for glycation (Figure 3). Metal ions are involved in glucose attachment as indicated by the inhibitory effect of the chelator diethylenetriaminepenta-acetic (DTPA).[6]

The exposure of albumin to glucose results in the fragmentation of protein to TCA-soluble peptides, which is also inhibitable by DPTA (Figure 3). However, in contrast to the observation made on monosaccharide attachment, it can be seen that

TABLE I

Susceptibility of Dog and Bovine Serum Albumin to Free Radical Mediated Fragmentation. Dog serum albumin (DSA) or bovine serum (BSA) were exposed at a concentration of 15 μM to 1 mM ascorbic acid or 2.5 mM hydrogen peroxide in the presence of 1 μM copper sulphate over 3.5 hours. Reactions were performed at 37°C in the presence of 100 mM potassium phosphate (pH 7.2). Control reactions containing 1 mM DTPA are shown. Values of % fragmentation to trichloroacetic acid (TCA)-soluble material are expressed as a mean ± standard deviation of triplicate assays.

	Dog Serum Albumin	Bovine Serum Albumin
H_2O_2/Cu^{2+}	4.5 ± 0.1	3.0 ± 0.1
+ 1 mM DTPA	1.0 ± 0.05	0.5 ± 0.05
Ascorbate/Cu^{2+}	3.0 ± 0.1	1.5 ± 0.07
+ 1 mM DTPA	1.0 ± 0.05	0.5 ± 0.05

dog serum albumin is more susceptible to fragmentation by glucose than is the case with bovine serum albumin. It is interesting to note that protein fragmentation decreases the observed level of glucose attachment using TCA-precipitation to separate protein from unbound glucose.[7] Dog serum albumin, with fewer histidine residues and lacking histidine residues with a high affinity for copper, may inhibit the oxidation of glucose to a lesser extent than bovine serum albumin. As a result of this elevated level of glucose oxidation, fragmentation is elevated and the observed level of glucose attachment is decreased. Thus, differences in histidine content and copper affinity may contribute to variation in the response of proteins to glucose.

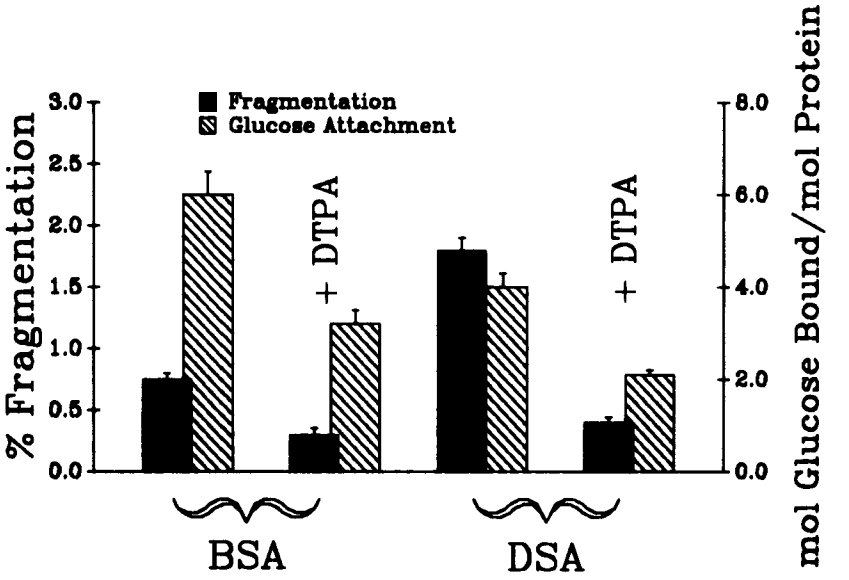

FIGURE 3 Glycation and Fragmentation of Albumins. Dog serum albumin (DSA) or bovine serum albumin (BSA) were exposed at a concentration of 75 μM to 25 mM glucose over 8 days. Reactions were performed at 37°C in the presence of 100 μM potassium phosphate (pH 7.2). Fragmentation (Filled Bar) or glucose attachment (Cross-hatched Bar) was determined in identical experiments in which either radiolabelled protein or glucose were used, respectively. Control reactions containing 1 μM DTPA are shown. Reaction mixtures were filter sterilised (0.22 μM micropore filter) and performed under sterile conditions. Values are expressed as a mean ± standard deviation of triplicate assays.

TABLE II

The Role of Autoxidation in the Attachment of Ascorbic Acid to Protein. Ascorbic acid (100 μM) was incubated with 75 μM bovine serum albumin over 2 hours. The effect of glutathione (reduced an oxidised form), metal chelator (DTPA) or histidine modifying reagent (DCP: diethyl pyrocarbonate) on the incorporation of the label into TCA-insoluble material is shown. All reaction mixtures were carried out in the presence of 100 μM potassium phosphate (pH 7.4) and at 37°C. Reactions were filter sterilised (0.22 μm micropore filter) and performed under sterile conditions. The extent of attachment are expressed as a mean $\pm$ standard deviation of triplicate assays and units are μmol bound/mol protein.

Reaction Mixture	Attachment of Ascorbic Acid
Ascorbic Acid	420 $\pm$ 20
+ 1 mM DTPA	275 $\pm$ 5
+ 1 mM DPC	550 $\pm$ 20
+ 25 mM GSH (reduced glutathione)	250 $\pm$ 40
+ 25 mM GSSG (oxidised glutathione)	390 $\pm$ 10

Histidine Influences Ascorbic Acid-Induced Protein Browning

The attachment of ascorbic acid to bovine serum albumin, performed over relatively short periods of incubation as a result of its rapid rate of autoxidation, enabled us to investigate the role of histidine residues (using diethyl pyrocarbonate modification studies) in the attachment and fluorophore generation by ascorbic acid.[10,27]

Like glycation, ascorbic acid attachment to protein also appears to be dependent

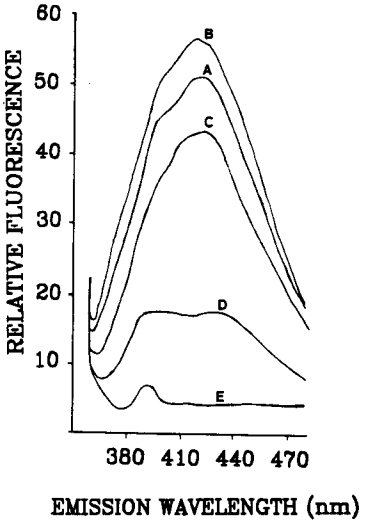

FIGURE 4 Ascorbic Acid-Induced Fluorophore Generation. All reaction mixtures were filter-sterilised (0.22 μm) and experiments were performed under sterile conditions in the presence of 100 μM potassium phosphate (ph 7.4) at 37°C. Bovine serum albumin (75 μM) was incubated with 2 mM ascorbic acid over 24 hours [A]. The effect of 1 mM diethyl pyrocarbonate [B] and 1 mM DTPA [C] on the generation of fluorophores is shown. Representative emission spectra (350–500 nm) upon excitation at 350 nm are shown. Control scans of buffer [E] or bovine serum albumin [D] incubated in the absence of ascorbic acid are included.

upon metal-catalysed oxidation. DTPA, as well as the reduced form of glutathione (possibly by maintaining ascorbic acid in its reduced form),[27] inhibit the attachment of ascorbic acid to bovine serum albumin. Diethyl pyrocarbonate, which increases ascorbic acid oxidation, leads to an increase in attachment to bovine serum albumin (Table II).

Histidine residues on bovine serum albumin also play a role in the generation of ascorbic acid-induced fluorophores (Figure 4). Histidine modification by diethyl pyrocarbonate resulted in an increase in fluorophore generation, presumably by releasing albumin bound copper ions and thus allowing accelerated oxidation of ascorbic acid. The inhibitory effect of glutathione (not shown) and DTPA on fluorophore formation further indicates that oxidation is involved in fluorophore generation by ascorbic acid.[6,27]

Predictably, due to the relatively slow rates of glucose oxidation[6] and the labile nature of diethyl pyrocarbonate,[16] glucose attachment and fluorophore formation were unaffected by diethyl pyrocarbonate modification (data not shown).

CONCLUSION

Glucose and ascorbic acid are both able to undergo a "site-specific" transition metal-catalysed oxidation, producing oxidants which fragment protein, and aldehydes which become attached to protein. Using either proteins with an identical potential for glucose attachment but differing histidine content, or diethyl pyrocarbonate modification of histidine residues, we have demonstrated that histidine residues play an important role in limiting autoxidation and contribute to the covalent modification of protein by glucose and ascorbic acid. It is interesting to note that the albumin residues most likely to be glycated *in vivo* are located in Lys-Lys, Lys-His, Lys-Lys-Lys and Lys-His-Lys sequences.[28] Indeed, variation in histidine content, as well as residues available for glycation, may be another factor responsible for the large variation in the extent of glycation of individual proteins *in vivo* and may dictate specificity of glycation in diseases such as diabetes and ageing.

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