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SHORT COMMUNICATION

Effect of pH, phosphate and copper on the interaction of glucose with albumin

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Protein glycation is believed to play an important role in the development of long-term disorders associated with diabetes. Previous studies have shown that copper could activate this process; however, these experiments were performed under non-physiological conditions. In this study, *in vitro* experiments were carried out at near-physiological conditions to examine the catalytic activity of copper on the interaction of albumin with glucose. Changes in pH and phosphate buffering capacity were shown to affect albumin glycation. Under stable pH conditions, copper activates albumin glycation only at low protein concentrations (< 30 g l^{-1}). Copper had no effect on albumin glycation at higher protein concentrations probably because the metal is chelated by the protein.

***Keywords*: copper, albumin, glycation**

Introduction

In vitro experiments have previously demonstrated that transition metals play a key role in the development of the Amadori products in glycoxidation [1]. These deleterious reactions affect proteins exposed to high glucose concentrations, and could account for the changes that occur in diabetes [2]. The way in which copper may activate these advanced Maillard reactions is still debated. It could catalyze glucose autoxidation [3], or activate the conversion of the Amadori product into deoxyglucosone compounds [4]. But experiments that simulate the long-term pathogenesis of tissues exposed to hyperglycaemia, use exaggerated copper and/or glucose to protein ratios [5–7], and these concentration ratios could favour non-physiological chemical pathways. The glucose : albumin ratio (g/g) is often set at 4.5–45 whereas it is 0.015–0.05 in the plasma of diabetics. The copper : albumin ($\mu M\, g^{-1}$) ratio is set at 1–100, although the ratio in human plasma is 0.04. This is important because proteins are potent chelators of copper and a high protein : metal ratio greatly inhibits the activity of the metal in radical-mediated reactions [8, 9]. The lack of effect of copper on the advanced Maillard reaction when albumin ($60\, g\, l^{-1}$) is incubated with 1.5 μM copper (the plasma concentration) could be because the albumin chelates the metal [10].

Moreover, many experiments have been carried out with phosphate concentrations as low as 50 mM, which may not have been sufficient to overcome the acidification caused by the glycation. This could be significant, as a low pH inhibits the advanced Maillard reaction [11]. We have therefore examined the effect of pH, phosphate buffering capacity and copper concentration on the production of advanced glycated end products (AGEs) in albumin-glucose mixtures.

Materials and methods

Bovine serum albumin was purchased from Euromedex (France). Glucose was obtained from Prolabo (France) and diethylenetriaminepentaacetic acid (DTPA) from Sigma. All other chemicals were purchased from Janssen.

Bovine albumin ($0.4–60\, g\, l^{-1}$) was incubated in sterile tubes, at 37 °C, with two concentrations of glucose (140 and 500 mM). The mixtures contained 0.1% sodium azide or were sterilized by filtration Millex-GS 0.22 μm (Millipore). The concentrations of phosphate buffer were 10, 25, 50, 100 and 200 mM at pH 7.4, and the pHs varied between 6.2 to 7.4 with a 100 mM phosphate buffer. The samples were incubated with 0–25 μM $CuSO_4$; any copper in the buffer was chelated by adding 1 mM DTPA. All experiments were done in triplicate. The data or their means are shown in the figures.

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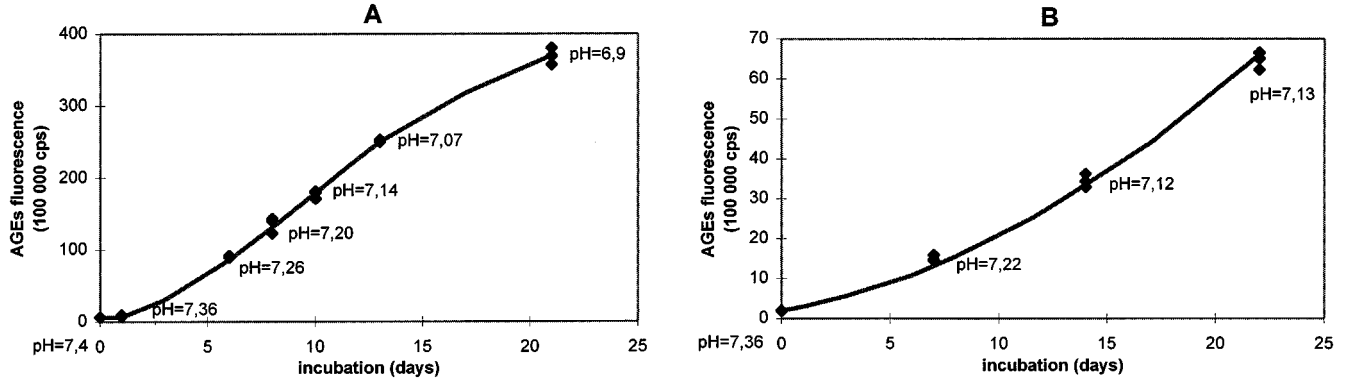

Figure 1. Changes in AGEs fluorescence (336–415 nm) and pH of albumin-glucose mixtures with time. Albumin (10 g l^{-1}) was incubated in (A) 50 mM phosphate buffer, pH 7.4 with 500 mM glucose; (B) 100 mM phosphate buffer with 140 mM glucose at pH 7.36. All experiments were done in triplicate. The relative standard deviation was always lower than 5%.

The fluorescence of AGEs was measured using a Fluoromax-Spex spectrofluorometer (ISA, Jobin-Yvon) in protein solutions diluted ($\leqslant 3$ g l^{-1}) to avoid any quenching by light scattering. Emission spectra were recorded (excitation: 336 nm; emission 415–425 nm) and results are expressed as 10^5 counts per second (cps) after correction for dilution. The fluorescence of the buffer alone was around 0.02×10^5 cps. The influence of the pH (6.2–7.4) on the fluorescence quantum yield of AGEs was checked.

Results

Acidification of the reaction mixture

The fluorescence due to AGEs formed by 10 g l^{-1} albumin plus 500 mM glucose in 50 mM phosphate buffer increased non-linearly (sigmoidal) with incubation time (Figure 1A). The pH decreased from 7.40 at $t = 0$ to 6.90 at $t = 21$ days, whereas the pH of the buffer alone or the protein alone or the sugar alone did not change.

Incubation in 100 mM phosphate buffer and 140 mM glucose reduced the drop in the pH to 0.23 U over 22 days (Figure 1B). Under these conditions, the increase in AGEs fluorescence appeared exponential.

Effect of pH on AGEs production

Samples containing 10 g l^{-1} albumin and 25 or 500 mM glucose were incubated at pH 6.20, 6.50, 7.00 and 7.40 in 100 mM phosphate buffer for 20 days. The pH of the samples with the high glucose concentration decreased slightly (0.3 U). The fluorescence increased linearly (correlation coefficient: $r = 0.995$) with pH, at both glucose concentrations (data not shown). It was confirmed in other experiments that the fluorescence intensity that was characteristic of AGEs, was not itself pH-dependent (between 6.2 and 7.4).

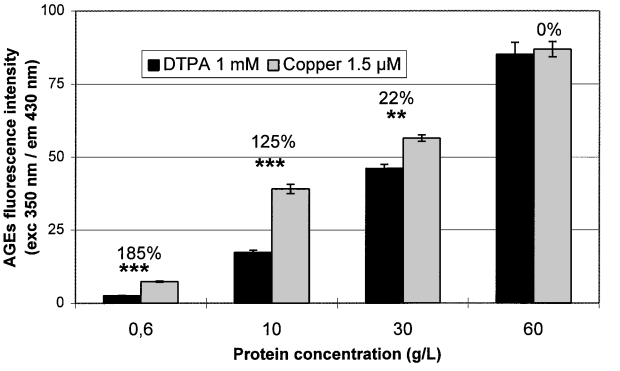

Figure 2. Effect of addition of copper 1.5 µM on the AGEs fluorescence produced by albumin-glucose mixtures. AGEs fluorescence (340 nm–420 nm) was measured after incubating various concentrations of albumin (10 g l^{-1}) with 500 mM glucose at pH 7.4 (in 100 mM sodium phosphate containing 0.67 µM copper) plus 1 mM DTPA (dark bars) or 1.5 µM copper (*ie* final copper concentration 2.17 µM) (clear bars) for 7 days. The fluorescence data were obtained in triplicate and are presented as 10^5 cps. The standard deviation and the statistics (Variance t-test: $p < 0.005$ = ***; $p < 0.01$ = **) are represented at the top of the bars. The percentage activation by copper was calculated for each albumin concentration on the basis of the fluorescence obtained with 1 mM DTPA.

Effect of phosphate buffers

There was an inverse linear relationship ($r = 0.993$) between the phosphate buffer concentration and the drop in pH of samples containing 10 g l^{-1} albumin and 500 mM glucose over 25 days. The drop in pH was small (0.27 units) in 200 mM phosphate, but much larger (0.77 units) with 10 mM phosphate.

Effect of DTPA

Figure 2 shows the influence of albumin concentration on the production of AGEs in solutions with DTPA (1 mM)

or copper (1.5 μM). The pH never decreased more than 0.1 U. Copper increased AGEs fluorescence for albumin concentrations below 60 $g l^{-1}$, and the increase was inversely proportional to the albumin concentration. Higher copper concentrations (3–25 μM) had no additional effect on albumin fluorescence, whatever the albumin concentration.

Discussion

pH greatly influences the advanced Maillard reaction rate [11, 12]. We confirm that the AGEs fluorescence of albumin-glucose solutions increases linearly with pH over a physiological pH range (pH 6.2–7.4) at 37 °C. Albumin incubated for 20 days produced 10 times more fluorescence at pH 7.4 than at pH 6.2. The greater rate of advanced glycation could result from a faster glucose autoxidation rate with increasing pH (3) and/or from greater isomerization into the more reactive sugars, fructose or mannose [13–15].

Glycation of albumin caused acidification of the incubation medium, mainly because of a decrease in free amino groups on the protein [12] and the release of sugar-derived acid molecules [16]. The acidification of the medium was proportional to the extent of reacting amino groups. Buffering prevented the drop in pH, but the rapid glycation that occurred when albumin was incubated with high glucose concentrations (500 mM), caused a significant decrease in pH, even at relatively high phosphate concentrations (data not shown). This slowed down glycation and, under these conditions, AGEs fluorescence increased in a sigmoid manner (see Figure 1A) instead of developing exponentially as a function of time [10]. The buffering capacity of the medium therefore has a considerable effect on the glycation rate.

The phosphate anion concentration may also have a direct effect on the glycation rate. Watkins *et al.* [17] showed that phosphate anions activated protein glycation probably by a neighboring catalyst effect. Phosphate anions could also activate glycation by shifting the sugar from the cyclic form to the open chain [18].

The activation of the advanced Maillard reaction by phosphates may also be due to traces of metal ions complexed by these anions. Ahmed *et al.* [19] reported an increase in the fluorescence of albumin-glucose mixtures with increasing phosphate concentrations, which did not develop when DTPA was added [20]. They conclude that metal ions chelated by phosphates are responsible for this activation, and hence that metals are needed for the conversion of the Amadori product into AGEs. However, the incubation conditions used to demonstrate this effect used phosphate buffer concentrations of 50–300 mM, and it seems unlikely that the pH remained unchanged with phosphate concentrations below 200 mM. A drop in pH during the long incubation period could give rise to the different rates of glycation as observed by Ahmed *et al.* [19]. While AGEs production is normally exponential when the pH is stable, Ahmed *et al.* reported linear and even sigmoidal increases in AGEs fluorescence, suggesting inhibition of glycation due to the decreasing pH.

We also observed that the AGEs fluorescence of low albumin concentrations is increased in 100 mM phosphate buffer (this contains 0.67 ± 0.03 μM copper, as measured by atomic absorption spectroscopy) in the observed 1 mM DTPA as compared to when DTPA is present (data not shown). This indicates that the metal activity of the buffer could also contribute to activation of the advanced Maillard reaction. However, the copper effect is inversely proportional to albumin concentration, and is no longer seen with 60 $g l^{-1}$ albumin, confirming previous data [10]. This could be because the protein, like DTPA, chelates copper [8, 9].

All experiments in which copper has been shown to activate advanced glycation have been carried out with protein concentrations of less than 10 $g l^{-1}$, and often around 1 $g l^{-1}$ [5–7, 21]. In these cases, copper is poorly chelated and can catalyze glycoxidation. As the protein concentration in most human tissues is above 60 $g l^{-1}$, the real action of copper may be doubtful. However, contrary to the assumption of Ahmed *et al.* [19], we show that AGEs are still significantly produced in the presence of the metal chelator DTPA and high concentrations of albumin, demonstrating that advanced glycation can occur in the absence of copper. These results suggest that the glucose concentration and the pH should have a greater influence on protein glycation *in vivo* than does copper.

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