Molecular and Cellular Biochemistry **181:** 63–69, 1998.

Non-enzymatic glycosylation of alkaline phosphatase alters its biological properties

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Received 6 March 1997; accepted 3 October 1997

Abstract

Hyperglycaemia in poorly controlled diabetic patients induces non-enzymatic glycosylation (glycation) of proteins, altering their structure and physiological bioactivity. Alkaline phosphatase (ALP) is a membrane-bound exoenzyme which faces the extracellular compartment. We have investigated the glycation of intestinal alkaline phosphatase *in vitro* and the consequences of such molecular modifications on certain structural and functional characteristics. The effect of glycation on alkaline phosphatase specific activity was determined after incubation of the enzyme with different sugars for various periods of time. The formation of early reversible glycation products was determined by the measurement of fructosamine levels, while the appearance of advanced glycation end products was estimated by spectrofluorometric analysis. A decrease in the specific activity of ALP was associated both with an increase in fructosamine levels and with the appearance of AGE-characteristic fluorescence. Changes in these parameters were found to depend on the incubation time, and on the concentration and glycating capability of the sugar employed. Co-incubation with aminoguanidine slowed down the appearance of protein-linked fluorescence, and additionally curbed the decrease in enzymatic specific activity. A significant correlation between the levels of ALP-fructosamine and ALP-advanced glycation end product was observed. Patterns of protein bands fractionated by SDS-PAGE were essentially identical for the nonglycated controls and the glycated samples. The electrophoretic mobility of the band of alkaline phosphatase on cellulose acetate gels increased as a function of the incubation time and the glycosylating power of the carbohydrate used. The present study provides evidence for the *in vitro* glycation of alkaline phosphatase, and for the consecutive alteration of its activity and structure. (Mol Cell Biochem **181**: 63–69, 1998)

Key words: non-enzymatic glycosylation, advanced glycation end products, alkaline phosphatase, Amadori products

Introduction

One of the major consequences of hyperglycaemia in poorly controlled diabetic patients is an excessive non-enzymatic glycosylation (glycation) of proteins [1, 2]. This process is initiated by a reducing sugar such as glucose, which reacts with free amino groups of proteins resulting in the formation of a Schiff base. A chemical rearrangement then gives rise to the Amadori product, which is a ketoamine. These reactions are reversible, increase with high sugar levels and mainly occur on long-lived proteins. This reaction reaches equilibrium in a period of weeks. With time, Amadori products irreversibly progress to the formation of crosslinked and fluorescent compounds. These compounds, whose chemical structures have not been completely identified, are collectively known as advanced glycation end products (AGEs). The AGEs continue to accumulate over years, and are increased in processes such as diabetes and normal aging [3, 4].

Non-enzymatic glycosylation has been demonstrated to affect a number of proteins, including serum proteins such as apoLDL [5], fibrin [6], albumin [7] and IGFBP-3 [8]; cell

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proteins (hemoglobin, superoxide dismutase) [9, 10]; and extracellular matrix proteins such as collagen [11], myelin [12] and laminin [13]. As a consequence of this glycation, the structure and/or function of these proteins becomes disturbed, altering their physiological bioactivity [14].

Alkaline phosphatase (ALP) is a membrane-bound metalloenzyme consisting of a group of isoenzymes: tissue non-specific, intestinal, placental and germ-cell ALP [15]. The protein is anchored to the cell membrane by a covalent linkage with a glycosyl-phosphatidyl-inositol anchor [16]. In human serum as well as in many other species, ALP predominantly circulates as a hydrophilic homodimer. Although the physiological role of this enzyme is still unknown, three functions have been proposed for the human ALP *in vitro*: phosphohydrolysis of organic phosphomonoesters, phosphotransferase activity and protein phosphatase activity. Skeletal ALP has been implicated both in bone formation and mineralization processes [17]. In intestine, as well as in kidney, ALP has been proposed to regulate the transport of phosphate and calcium [18, 19]. Thus, ALP appears to be implicated in fundamental biochemical processes.

Since ALP is an exoenzyme which faces the extracellular compartment, it is conceivable to suppose that its activity and function could be especially affected by the environmental sugar levels. To assess the rationale of this study, we have investigated the glycation of intestinal alkaline phosphatase *in vitro*. The consequences of such molecular modifications on certain structural and functional characteristics of ALP have also been examined.

Materials and methods

Materials

Alkaline phosphatase from bovine intestine (ALP), α-naphthyl phosphate, fast blue RR, sodium dodecyl sulfate (SDS), acrylamide, coomassie brilliant blue R-250, p-nitrophenyl phosphate (pNPP), aminoguanidine, and other reactives of analytical degree were obtained from Sigma Chemical Co., MO, USA. Test Combination Fructosamine kit was kindly provided by Boehringer Mannheim Argentina.

In vitro *glycation of ALP*

Bovine intestinal ALP (10 mg/ml) was incubated in phosphate buffered saline (PBS) containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and protease inhibitors, pH 7.4 at 37°C with or without 100 mM different sugars for the periods indicated in the legends of the figures and tables [8]. The selected sugars were glucose (G), fructose (F) and ribose (R) since they have different glycosylating capabilities [20]. Thus, they will induce different rates and degrees of glycation on proteins. In selected experiments, aminoguanidine (250 mM) was included in the incubation mixture, to examine the possible participation of AGEs in structural and functional alterations which might appear in ALP as a consequence of its glycation. In other experiments, glucose was added to the incubation mixture at a concentration of 30 mM, to evaluate the dose-dependence of the modification of ALP parameters. At the end of each incubation period, the aliquots of ALP were concentrated and separated from low molecular weight molecules by centrifugation/filtration with Centricon 10 kDa cut-off filter cartridges (Amicon Inc., Beverly, MA, USA).

Determination of glycated products

The formation of early glycation products on ALP was assayed by the fructosamine method (Test combination fructosamine Kit, kindly provided by Boehringer Argentina) [8]. The presence of AGEs on ALP was assessed by their characteristic fluorescence-emission maximum at 440 nm upon excitation at 366 nm. For this assay, the protein concentration of all samples was adjusted to 1 mg per milliliter, and fluorescence measurements were expressed as percentage relative fluorescence compared to that of a 1 μg/ml quinine standard, as we have previously described [21].

Measurement of alkaline phosphatase specific activity

Aliquots of either glycated or non-glycated ALP were assayed incubating approximately 0.001 μg/ml ALP with 5 mM pNPP in 55 mM Glycine/0.55 mM $MgCl_2$ buffer, pH 10.5 at 37°C for 20 min. The production of pNP was determined by optical density at 405 nm [21]. Protein concentration was determined by the Bradford method [22].

Separation of ALP isoforms by electrophoretic methods

To evaluate possible changes in the electrophoretic mobility of ALP during its glycation, aliquots corresponding to 1 μg protein were electrophoresed on gelatinized cellulose acetate (Cellogel, Chemetron Milano, Italy), at a constant current of 1 mA/cm, during 50 min. The enzymatic reaction was then performed by placing the gel on an agar phase containing α-naphthyl phosphate as substrate, at 37°C for 30 min. The product formed on the cellulose acetate gel, α-naphthol, was then allowed to react with Fast Blue RR (also in an agar phase) for 20 min at 25°C. The coloured ALP bands were finally evaluated by densitometric analysis and Rf determination [23].

To test for changes in the molecular weight patterns of ALP during glycation, this protein was also separated by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS using a Laemmli Tris-glycine buffer, pH 8.6 [24]. Aliquots of ALP with different degrees of glycation were boiled for 3 min before loading on a 12.5% acrylamide gel. After electrophoresis, the gels were fixed and stained with 0.1% Coomassie brillant blue R-250. Prestained molecular weight markers were run in a parallel lane.

Statistical methods

At least 3 experiments were performed for each experimental condition. Data are expressed as the mean ± S.E.M. Statistical differences were analysed using Student's *t*-test or analysis of variance when suitable. Linear correlation analysis was performed by Pearson's correlation coefficient.

Results

The effect of glycation on alkaline phosphatase specific activity was determined after incubation of the enzyme with 100 mM of different sugars. As shown in Fig. 1, the decrease in ALP activity depends both on the length of the incubation period and on the relative glycating power of the sugar employed. Small changes in the ALP activity of the control sample were observed during the course of these experiments (45.3 ± 0.7 vs. 39.2 ± 0.9 nmol pNP/min.µg protein; for 1 vs. 35 days).

In order to evaluate the formation of early reversible glycation products, we determined the concentration of fructosamine in all the ALP samples. Figure 2 shows that the increase in fructosamine was dependent both on the time of the incubation and on the glycosylating capacity of each sugar. This increment in every case reached a maximum after 14 days of incubation. No significant differences were found in the degree of glycation in the control samples (31 ± 4 and 26 ± 5 µmol fructosamine/g protein for 1 and 35 days, respectively).

The extent of advanced glycation end product formation on ALP was evaluated by measuring specific fluorescence. After 1 week incubation, fluorescence characteristic of AGEs was detected in ALP samples incubated in the presence of different sugars (Fig. 3). The fluorescence-excitation spectrum of each sample showed a maximum at 440 nm. The excitation intensity at 440 nm increased when ALP was incubated with fructose and ribose. On the other hand, after 7 days incubation the spectra of both the control sample and glucose were found to be closely similar. Table 1 shows the percentage relative fluorescence/mg protein in ALP samples incubated with different sugars at a concentration of 100 mM

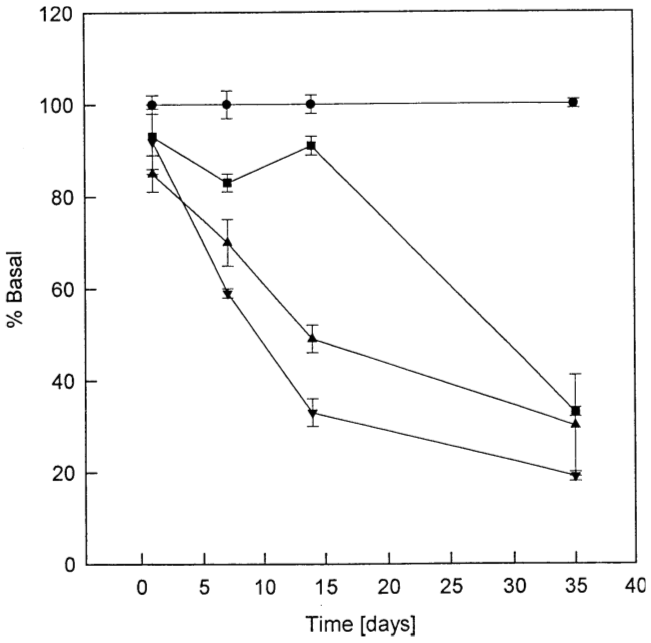

Fig 1. Time-dependent effect of non-enzymatic glycosylation on ALP activity. ALP was incubated in PBS alone (●), or PBS with 100 mM glucose (■), fructose (▲) or ribose (▼) at 37°C for different periods of time. Aliquots were removed at the indicated times and ALP activity was assayed. Data are expressed as a percentage of basal activity, mean ± S.E.M. n = 6.

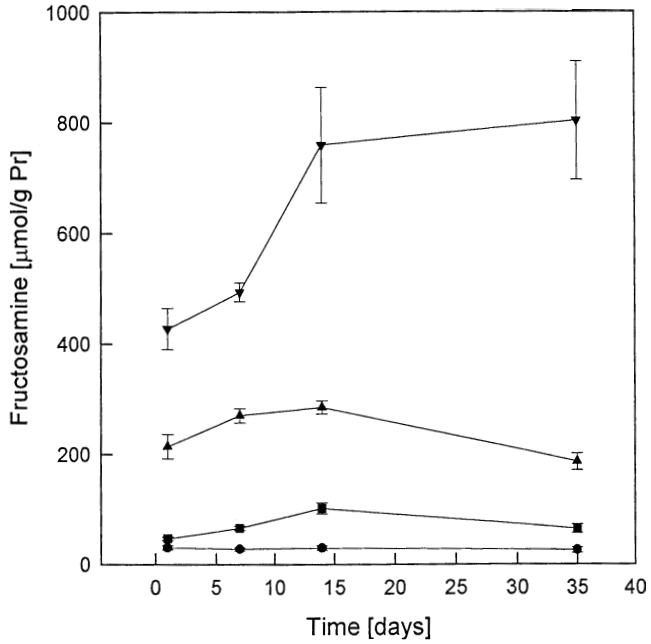

Fig. 2. Time-dependent effect of non-enzymatic glycosylation on fructosamine levels. ALP was incubated in PBS alone (●), or PBS with 100 mM glucose (■), fructose (▲) or ribose (▼) at 37°C for different periods of time. Amadori products formed on ALP were assessed by the fructosamine levels. Data are expressed as mean ± S.E.M. n = 6.

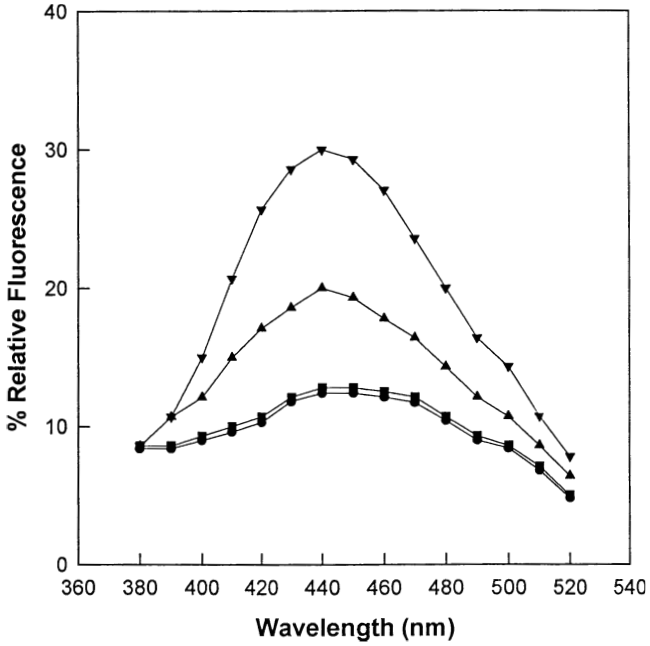

Fig. 3. Fluorescence-emission spectra of control ALP (●) and ALP incubated with 100 mM glucose (■), fructose (▲) or ribose (▼) at 37°C for 7 days. (λexc = 366 nm).

for several incubation periods. Glucose, the weaker glycating agent, only induced AGE accumulation on ALP as from 14 days of incubation. AGE-ALP increased as a function both of the time of incubation and the potency of the glycating sugar, ribose being the strongest of all sugars tested.

Thus, the decrease in the specific activity of ALP samples incubated for different periods was associated both with an increase in fructosamine levels and with the appearance of AGE-characteristic fluorescence. A significant correlation between the levels of fructosamine-ALP and AGE-ALP was observed after 7 ($p < 0.02$), 14 ($p < 0.05$) and 35 ($p < 0.01$) days of incubation.

In an attempt to examine the participation of covalently-bound AGEs in the structural and functional alterations of ALP, 250 mM aminoguanidine was added both to a control ALP sample (no sugar added), and to a sample containing ALP and 100 mM ribose. These samples were incubated for 2 weeks, submitted to centrifugation/filtration as described in Materials and methods, and finally evaluated for enzymatic specific activity and protein-linked fluorescence. Aminoguanidine, a compound which has been shown to inhibit the formation of AGEs but not of Amadori products, was found to reduce by 90% the AGE-associated fluorescence induced by 2 weeks incubation with ribose (Fig. 4). In addition, the presence of aminoguanidine together with ribose increased ALP specific activity to 72% of time-paired control samples (compared to 33% when ALP was incubated 2 weeks with ribose alone). No significant differences were found in these parameters between control samples incubated with or without aminoguanidine (data not shown).

In further experiments, ALP was incubated with increasing glucose concentrations (0, 30 and 100 mM) at 37°C for 5 weeks. In this situation, a dose-dependence was found both for ALP specific activity and for protein-linked fluorescence: as glucose levels rose, so did the inhibition of enzymatic activity and the AGE-specific fluorescence per mg protein (Table 2).

We next evaluated the possible structural changes in ALP which could have occurred as a consequence of its glycation. The patterns of protein bands fractionated by SDS-PAGE were essentially identical for the nonglycated controls and the glycated samples at all incubation times (data not shown). Additionally, samples were electrophoresed on cellulose acetate gels. The bands of ALP activity were visualized by an *in situ* colorimetric reaction as described in Materials

Table 1. Relative fluorescence per mg protein after incubation of ALP at 37°C with different sugars (100 mM) and for different periods of time

	Relative fluorescence at 440 nm (%)*			
Time [days]	Control	Glucose	Fructose	Ribose
7	13.6	13.6	19.3	29.3
14	11.6	18.4	29.5	142.5
35	12.2	22.3	30.8	157.5

*expressed as percent fluorescence relative to a quinine standard sample.

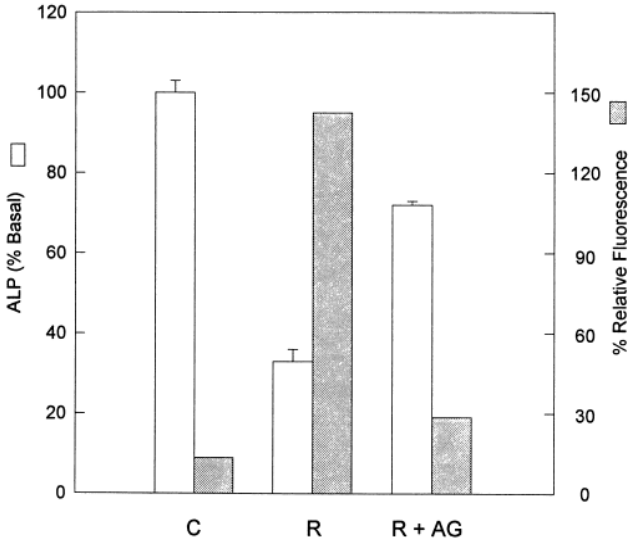

Fig. 4. Effect of the co-incubation of aminoguanidine on ALP specific activity and relative fluorescence at 440 nm. ALP was incubated at 37°C for 2 weeks in PBS alone (C), PBS with 100 mM ribose (R) or PBS with 100 mM ribose and 250 mM aminoguanidine (R+AG). ALP specific activity is expressed as percentage of basal activity, mean ± S.E.M. n = 6, and relative fluorescence is given per mg protein, as described in Materials and methods.

Table 2. Alkaline phosphatase specific activity and relative fluorescence at 440 nm of ALP samples incubated with different concentrations of glucose at 37°C for 5 weeks as described in Materials and methods

Glucose [mM]	ALP [% Basal]	Relative Fluorescence (%)
0	100 ± 1	13
30	54 ± 0.5	19
100	33 ± 1	22

and methods. Figure 5 shows the glycated and non-glycated ALP bands after 14 days incubation with 100 mM of each sugar. A decrease in the intensity of the band, and an increase in its mobility, can be seen in the samples incubated with fructose and ribose. The area under each peak was integrated to estimate ALP specific activity. These values, expressed as percentage of control, were generally found to be similar to those obtained with the ALP assay using pNPP as a substrate (Table 3). This correlation decreased when ALP specific activity was lower than 30% of incubation-time paired control samples. The Rf was determined for the peak of activity in each case. As shown in Table 3, the electrophoretic mobility of the band of ALP activity for each sugar increased as a function of the incubation time. On the other hand, the increase in the Rf for each incubation time was observed to depend on the glycosylating power of the carbohydrate used. The order of potency was G < F < R, in agreement with their effects on the inhibition of ALP activity, on ALP fructosamine levels and on AGE formation in the samples. After 5 weeks incubation with different sugars, a significant linear correlation was found between the changes in the ALP mobility as assessed by the changes in the Rf and either the ALP-fructosamine levels (r^2: 0.992, $p < 0.01$), or AGE-ALP formation (r^2: 0.983, $p < 0.02$).

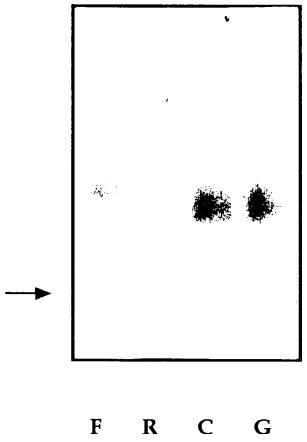

Fig. 5. ALP was incubated in absence of carbohydrate (C) or in the presence of 100 mM glucose (G), fructose (F) or ribose (R) at 37°C for 14 days and submitted to electrophoresis on gelatinized cellulose acetate as described in Material and methods. Arrow indicates the origin.

Table 3. Increment in the electrophoretic mobility of ALP incubated at 37°C with different sugars (100 mM) and for different periods of time

	ΔRf (%)*			
Time [days]	Control	Glucose	Fructose	Ribose
1	0	0	0	0
7	0	0	0	9 (58)
14	0	0	6 (57)	35 (22)
35	0	12 (58)	24 (10)	76 (4)

*expressed as percent changes in the Rf of glycated ALP bands, compared to the corresponding (incubation-time paired) control sample. Values given between brackets when ΔRf > 0 correspond to the intensity of the ALP band measured by densitometric analysis and expressed as percentage of incubation-time paired control samples. No significant differences were found in this parameter between control samples and samples with ΔRf = 0.

Discussion

The non-enzymatic glycosylation of different proteins has been extensively documented with *in vitro* as well as *in vivo* studies, in animal models and human diabetes [1, 2, 14]. This molecular modification, which includes early, intermediate and advanced glycosylation products, alters both the structural and functional properties of proteins. Consequently, it has been suggested that such alterations could be involved in the pathophysiology of diabetic complications. In this study, we have examined the non-enzymatic glycosylation of intestinal alkaline phosphatase. We have chosen this iso-enzyme as a model ALP, and studied its possible *in vitro* glycation together with the consequences of such a modification on certain structural and functional properties.

The measurement of ALP fructosamine levels was used to monitor the accumulation of early (Amadori) glycation products, while the formation of AGEs was estimated by means of spectrofluorometric analysis. The *in vitro* glycation of ALP was found to proceed as a function of the incubation time and of the glycating capability of the sugar employed (Fig. 2, Table 1). A positive correlation was also found between alkaline phosphatase AGE and fructosamine levels. This is consistent with previous studies which have shown AGE formation to be a direct function of the Amadori product concentration at equilibrium [25, 26].

A gradual inactivation of ALP specific activity occurred during incubation of the enzyme with all the monosaccharides tested (Fig. 1). In general, this inhibition of ALP was inversely associated both with fructosamine levels and AGE-specific fluorescence. However, after short incubation periods (e.g. 24 h), visible increases in the levels of Amadori products were found on ALP, without there being any significant changes in its enzymatic activity. On the other hand, co-incubation for longer periods (e.g. 2 weeks) with aminoguanidine, an inhibitor of the formation of AGEs but

not of early glycation products, was found to diminish the appearance of ALP-linked fluorescence, and accordingly also curbed the decrease in ALP specific activity (Fig. 4). These observations suggest that the amino groups initially compromised in the glycation process are not involved in, or are not close to, the ALP active site. As the incubation proceeds, the observed decrease in ALP activity could be due to one or more different causes: direct glycation of sterically less accessible free amino groups in close proximity to the active site; covalent formation of inter- or intramolecular crosslinks due to AGE moieties which, through conformational changes in ALP, could either limit the access of substrates to the enzymes' active site, or could prevent correct interaction between ALP monomers. Nevertheless, as no differences were found in the molecular weight patterns of samples (when submitted to SDS-PAGE), formation of inter-molecular crosslinks must be discarded.

In several studies, sugars other than glucose have been used to accelerate the *in vitro* glycation of proteins, which *in vivo* occurs over a period of months or years [14]. In our experiments, the observed ALP time-dependent inactivation was found to be a direct function of the glycating capability of the monosaccharide employed. Moreover, the order of this relative glycating power was in agreement with previous reports [14] (Ribose>Fructose>Glucose).

In diabetic patients, plasma glucose levels of 100 mM are very rare: they can only appear briefly during the acute hyperglycaemic crises of NIDDM [34]. However, in poorly compensated diabetic individuals it is not infrequent to find plasma glucose concentrations in the 20–30 mM range. In this context, we studied the effect of 30 mM glucose on certain alkaline phosphatase parameters: in this case, after an incubation of 5 weeks, we found an inhibition of ALP specific activity, and the appearance of AGE-specific protein-linked fluorescence (Table 2). Although significant, the modification of these parameters was 69 and 67%, respectively, of those found after a 5 week incubation with 100 mM glucose. These results are consistent with a glucose dose-dependence, both for the inhibition of enzymatic activity, and for the appearance of ALP-linked AGEs.

In various studies, changes have been demonstrated in the structural properties of enzymes and other proteins as a consequence of their glycation [1, 2]. In most of these cases, free ε-amino groups of Lys residues are the main sites for glycation. If this were the case with ALP, the non-enzymatic glycosylation of Lys residues could produce a relative increase in the proteins negative surface charge, which in turn would lead to an increase in its electrophoretic mobility in an alkaline environment. Present evidence lends support to this suggestion: in our experiments, the electrophoretic mobility of the ALP band increased in a time-dependent manner (Fig. 6 and Table 2). The changes in Rf, used to evaluate this effect, were found to correlate directly with ALP fructosamine and AGE levels. However, these alterations in the ALP bands electrophoretic properties could also be at least partly accounted for by AGE-induced conformational changes decreasing the molecules' surface area/volume ratio.

The precise molecular mechanism by which glycation induces these effects remains unclear. Alkaline phosphatases are a family of isoenzymes widely distributed in nature with close amino acid homology of sequence [15]. The human intestinal ALP sequence is well known, as is its active site. The same ALP amino acid residues controlling catalysis (Asp^{91}, Ser^{92}, Arg^{166}), and those which determine binding of the protein to Zn^{2+} and Mg^{2+}, have been found from bacteria to humans [27]. Human intestinal ALP shows 87% positional identity with placental ALP, and 57% amino acid identity with liver/bone/kidney ALP [28]. Intestinal ALP includes 17 lysine residues [28], which are possible candidates for non-enzymatic glycosylation. It is thus likely that the reducing sugar binds to lysine residues close to or involving the active site of the enzyme.

The precise contribution of the *in vivo* glycation of intestinal ALP, if it does occur, to physiological variations in its enzymatic activity remains to be determined. Intestinal ALP activity has been found to depend on genetic, hormonal and dietary factors. In either starch- or sucrose-fed obese spontaneous hypertensive/NIH corpulent rats, which exhibit some of the metabolic and pathological alterations associated with NIDDM, tissue intestinal ALP activity was found to be depressed when compared with starch-fed (but not sucrose-fed) lean control rats [29]. On the other hand, in alloxan insulin-deficient diabetic rats fed *ad libitum*, tissue intestinal ALP activity was increased compared with control rats. However, this was not the case when alloxan diabetic rats were fed a restricted diet, suggesting that increased activity of tissue intestinal ALP is only a secondary effect of insulin deficiency, caused by the increased food intake resulting from insulin deficiency [30]. Moreover, in streptozotocin-induced insulin defficient rats, circulating (serum) intestinal ALP activity was found to be markedly elevated when compared with control rats [31, 32]. Nevertheless, it should be noted that no physiological role has been found for serum ALP: the biologically active enzyme is normally anchored to tissue membranes, and is thus exposed to the extracellular medium. In this context, it is conceivable that ALP, together with other extracellular matrix proteins, could become excessively glycated in diabetic individuals *in vivo* [33]. Thus, it would be possible to hypothesize that membrane-bound ALP, with reduced bioactivity due to excessive glycation, could coexist with the increased serum ALP activity found in insulin-defficient individuals.

The present study provides evidence for the *in vitro* glycation of alkaline phosphatase, and for the consecutive alteration of its activity and structure. Further studies are

required to determine whether ALP becomes glycated in diabetic individuals, and, if so, what would be the functional consequences of these modifications *in vivo*.

Acknowledgements

This work was supported by grants from Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina. We thank Boehringer Mannheim Argentina for the provision of the Test Combination Fructosamine. SBE is member of the Carrera del Investigador, CONICET, Argentina; AMC is member of the Carrera del Investigador, CICPBA, Argentina.

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