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Understanding non-enzymatic aminophospholipid glycation and its inhibition. Polar head features affect the kinetics of Schiff base formation

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ABSTRACT

Non-enzymatic aminophospholipid glycation is an especially important process because it alters the stability of lipid bilayers and interferes with cell function and integrity as a result. However, the kinetic mechanism behind this process has scarcely been studied. As in protein glycation, the process has been suggested to involve the formation of a Schiff base as the initial, rate-determining step. In this work, we conducted a comparative kinetic study of Schiff base formation under physiological conditions in three low-molecular weight analogues of polar heads in the naturally occurring aminophospholipids *O*-phosphorylethanolamine (PEA), *O*-phospho-DL-serine (PSer) and 2-aminoethylphenethylphosphate (APP) with various glycating carbonyl compounds (glucose, arabinose and acetol) and the lipid glycation inhibitor pyridoxal 5′-phosphate (PLP). Based on the results, the presence of a phosphate group and a carboxyl group in α position respect to the amino group decrease the formation constant for the Schiff base relative to amino acids. On the other hand, esterifying the phosphate group with a non-polar substituent in APP increases the stability of its Schiff base. The observed kinetic formation constants of aminophosphates with carbonyl groups were smaller than those for PLP. Our results constitute an important contribution to understanding the competitive inhibition effect of PLP on aminophospholipid glycation.

1. Introduction

The reactions between a carbohydrate and a amino group in a protein, lipid or nucleic acid are known as 'non-enzymatic glycation of biomolecules' and their products have been deemed responsible for a number of medical pathologies associated to aging,[1] Alzheimer's disease,[2] artherosclerosis[3] and diabetes.[4]

Non-enzymatic glycation starts with the formation of a Schiff base which then rearranges into a more stable ketoamine: an Amadori product. The Amadori product subsequently evolves via a complex series of reactions leading to the formation of a heterogeneous family of compounds known as 'advanced glycation end-products' (AGEs). By oxidation, the sugars, the Schiff base, and or the Amadori compound can form AGE precursors and radical species ($O_2^{\cdot-}$ and ·OH) which accelerate the glycation process and have pro-oxidant effects on other molecules.[5] In fact, some studies showed that glycated aminophospholipids increase oxidation reactions and degradation either of the phospholipid or other biomolecules.[6,7] It has been reported that the glycation of proteins causes fragmentation of the polypeptide chain and formation of toxic aggregates.[8] It also induces nucleic acids mutations during DNA replication,[9] and structural changes in the lipid bilayer.[10]

Non-enzymatic glycation in aminophospholipids has been the subject of study since the 1990s. The naturally occurring aminophospholipids acting as targets in glycation reactions are phosphatidylethanolamine (PE) and phosphatidylserine (PS), both of which are present in mammal cell membranes. Bucala et al.[11] were the first to show that PE reacts with glucose to form phospholipid-linked AGEs. Subsequently, Ravandi et al.[12] examined PE glycation in red cells and plasma from diabetic individuals and found 10–16% of all PE in their samples to be in glycated form. Other authors have characterized various AGEs formed in PE–glucose reaction models,[13–16] and Fountain et al.[17] quantified glucose-linked PE and PS in samples from diabetic patients. In 2001, Breitling et al.[18] identified and quantified Schiff-PE and Amadori-PE adducts in erythrocytes from healthy and diabetic individuals. The last years, Miyazawa et al. have analysed Amadori-glycated phosphatidylethanolamine in human erythrocytes and blood plasma.[19,20] Recently, our group by using theoretical calculations has studied Schiff base formation between various carbonyl and amino compounds,[21–24] and on the surface of an aminophospholipid layer.[25]

One of the therapeutic strategies used to prevent the diseases derived from lipid glycation is based on the use of molecules that can inhibit the glycation process. In 1993, Bucala et al.[11] found

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aminoguanidine to inhibit protein and lipid glycation. Recently, Higuchi et al.[26] detected in vivo formation of PE–PLP adducts in human red cells and found the use of a PLP supplement in the diet for diabetic rats to substantially reduce their physiological levels of Amadori-PE adducts; based on these results, they suggested that pyridoxal 5′-phosphate (PLP, a vitamin B_6 derivative) might be an effective inhibitor of lipid glycation. Subsequently, other authors provided in vitro confirmation that urea protects from lipid-derived AGEs,[27] albeit less efficiently than PLP.[28]

In any case, lipid glycation has been the subject of little kinetic research.[12,15,18,29] In this work, we undertook a study of the glycation kinetics of aminophospholipids analogues with a view to improving current understanding of the mechanism behind lipid glycation; to this end, we focused on lipid polar heads in order to facilitate their study in aqueous media. Figure 1 shows the structure of the three model substances used [*O*-phosphorylethanolamine (PEA), *O*-phospho-DL-serine (PSer) and 2-aminoethylphenethylphosphate (APP)], which were reacted with three strong glycating carbonyl compounds (glucose, arabinose and acetol) in addition to PLP.

A comparison of our kinetic results allowed us to elucidate the influence of the chemical nature of aminophospholipid polar heads on their reactivity, and to assess the reactivity of PLP with aminophospholipid polar heads in relation to carbonyl glycants. These results, together with others obtained in previous work,[30] provide useful insight into the inhibitory effect of vitamin B_6 derivatives on biomolecular glycation.

2. Results

Aminophospholipid glycation was studied in three model compounds of low molecular weight: PEA, PSer and APP. PEA and PSer are analogues of polar heads in PE and PS, respectively. Also, APP has the same polar head as PEA, but possesses a hydrophobic tail by effect of its terminal phosphate group being esterified with a phenethyl group that confers the compound the typical amphiphilicity of naturally occurring aminophospholipids. The three model compounds are water-soluble and possess an amino group acting as glycation target.

Accurately fitting the experimental kinetic results required the potentiometric determination of the ionization constants of the three model compounds at 37 °C. In this way, we found pK_a for the carboxyl group in PSer to be 2.04 ± 0.05; the second ionization constant for the phosphate group in PEA and PSer 5.81 ± 0.02 and 6.02 ± 0.05, respectively; and the ionization constant for the amino group (pK_{a4} in Scheme 1) 10.24 ± 0.02 for PEA and 10.10 ± 0.04 for PSer. These values are similar to previously reported values.[31,32]

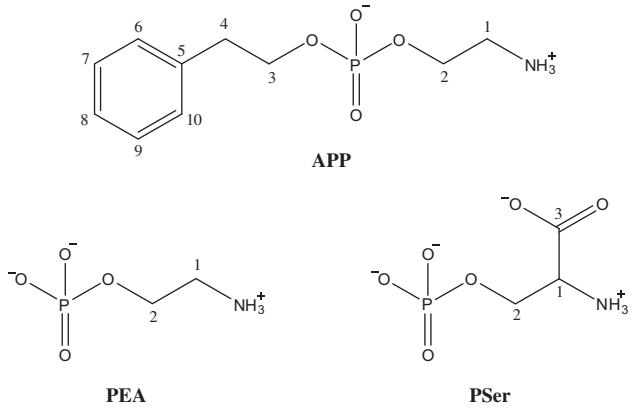


Figure 1. Structural formulae of APP (2-aminoethylphenethylphosphate), PEA (*O*-phosphorylethanolamine) and PSer (*O*-phospho-DL-serine).

We also determined pK_a for the amino group in APP, which was 9.00 ± 0.05.

We examined the reactivity of the three model compounds against three carbonyl compounds of biomedical interest, namely: glucose, which promotes the glycation of biomolecules (particularly in hyperglycemic individuals[33]); arabinose, which is a sugar formed by glucose degradation in the body and a suggested precursor of some AGEs;[34] and acetol, which is an inhibitor of glyceraldehyde 3-phosphatedehydrogenase[35] and present at elevated concentrations in diabetic ketosis.[36] We also studied the reactions of the model compounds with PLP, which is another potent inhibitor of lipid glycation by virtue of its protecting aminophospholipid polar heads from carbonyl glycants via the formation of a Schiff base.[26]

2.1. Reactions between glycating carbonyl compounds and aminophosphates

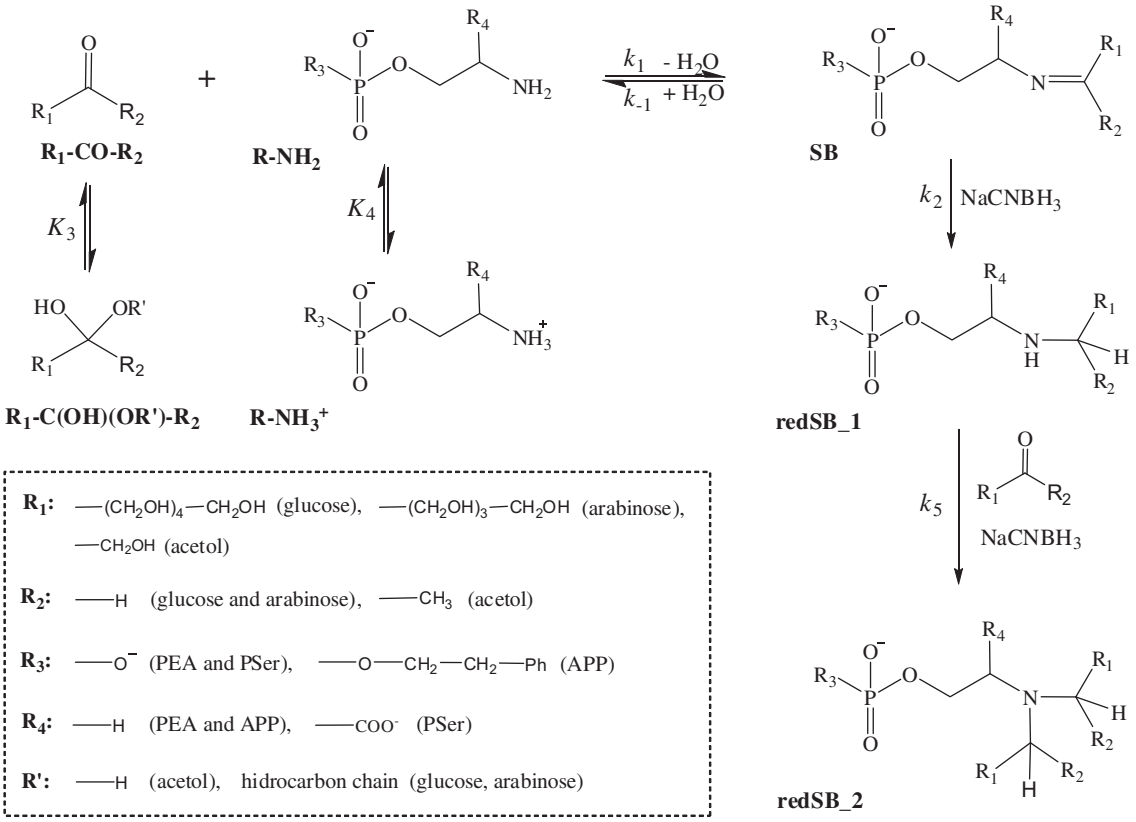
Scheme 1 shows the proposed kinetic mechanism for the reactions of the model compounds with the carbonyl reagents. The nucleophilic attack of the amino group on the carbonyl group produced a Schiff base (SB) in hydrolytic equilibrium with the reactants. These reactions were studied in the presence of $NaCNBH_3$, which is a selective reducing agent for imino groups at neutral pH,[37,38] in order to facilitate scavenging of the resulting Schiff base and the determination of its kinetic formation constant.[30]

Figure 2A shows the temporal variation of the chromatograms for the reaction between glucose and APP. The initial chromatogram contained a major signal for APP (t_R 14.3 min) that decreased with time as a new signal at t_R 12.3 min corresponding to the reduced Schiff base (redSB_1) appeared. After a long enough incubation time, a new signal at t_R 10.8 min corresponding to the reaction product between redSB_1 and a second glucose molecule, redSB_2, was observed. All reaction products were isolated and characterized by 1D and 2D-NMR spectroscopy, and mass spectrometry (see Section 5). Condensation of a second molecule of carbonyl compound with a secondary amine (carbinolamine) was previously demonstrated by other authors.[39]

Figure 2B shows the chromatograms for the reaction between arabinose and APP. As can be seen, the signal for APP (t_R 14.1 min) decreased with time as that for the reduced Schiff base (t_R 12.6 min) appeared. RedSB_2 formation was not observed during the studied time frame. The results for the reaction between APP and acetol were similar to those for the reaction with arabinose (data not shown).

Figure 3 shows the temporal variation of the ^{1}H NMR spectra for the reaction of PEA with arabinose in the region from 3.0 to 3.45 ppm. As can be seen, the triplet at 3.21 ppm, corresponding to H_2–C(1) in PEA, decreased simultaneously with the appearance of two signals downfield. By analogy with the ^{1}H NMR results for the reduced Schiff bases isolated in the reaction of APP with glucose (see Section 5), which exhibited a downfield shift in the signal for H_2–C(1), these signals were assigned to the reduced Schiff bases redSB_1 and redSB_2 from PEA and arabinose. The reaction kinetics was monitored via temporal changes in the H_2–C(1) signal for the reactant. The reaction between PEA and acetol was studied by monitoring changes in the H_2–C(1) signal for PEA by ^{1}H NMR.

The reaction of PEA with glucose, and those of PSer with glucose and arabinose, was monitored by ^{1}H, ^{13}C-HMQC since the 1D-^{1}H spectra exhibited overlapped signals for the reactants. Figure 4 shows the 2D-^{1}H,^{13}C-HMQC spectra for the PSer–glucose reaction mixture in the presence of $NaCNBH_3$; the initial spectrum is shown in blue and that recorded after 16 days of reaction in red. HC(1)–C(1) and H_2C(2)–C(2) cross-peaks in PSer decreased with time, simultaneously with the appearance of new signals at 68 ppm for the species contributing to the formation of redSB_1. The signals for glucose exhibited no change.



Scheme 1.

The temporal variation of the HPLC, 1D and 2D-NMR signals was used to determine aminophosphate concentration in each reaction. Fitting such a variation to Eq. 8 allowed the corresponding observed rate constants, k_{obs}, to be calculated. The rate constants obtained from NMR values are affected by the isotopic effect of the solvent (D_2O). In order to compare the results in H_2O and D_2O, we determined such an effect by using the Schiff base formation between APP and arabinose as a model reaction. The k_{obs} value thus obtained was 0.35 M^{-1} h^{-1} in D_2O and 0.47 M^{-1} h^{-1} in H_2O. The resulting isotopic effect, k^H/k^D = 1.3, is consistent with previously reported values for ketimines.[40] Table 1 shows the k_{obs} values for PEA and PSer after correction for the isotopic effect. The microscopic kinetic formation constants for the Schiff bases (k_l) shown in Table 1 were calculated by introducing the corresponding k_{obs} values in Eq. 7.

2.2. Reactions between PLP and aminophosphates

Scheme 2 shows the kinetic mechanism for the Schiff base formation between PLP and the aminophosphates studied. The formation process was monitored via the absorbance at 420 nm, which has been associated to the formation of PLP Schiff bases.[41] Fitting the experimental values to Eq. 10 allowed the kinetic formation (k_f) and hydrolysis (k_h) constant, and the equilibrium constant (K_{eq}), at pH 7.4 for the Schiff base of each aminophosphate to be calculated (see Table 1).

3. Discussion

3.1. Influence of the chemical nature of the aminophosphate on its chemical reactivity

The quantitative results obtained are shown in Table 1. As can be seen, the microscopic kinetic formation constants for the Schiff bases (k_1) of the aldehyde groups in glucose and arabinose were 3 orders of magnitude greater than that for the ketone group in acetol, which is suggestive of a difference in electrophilicity between the reactive carbonyl groups. A similar trend was previously observed in their reactions with pyridoxamine (PM).[42] The increased reactivity of glucose in these systems is a result of their electrophilicity being raised by effect of intramolecular hydrogen bonding net.[42]

APP was the least reactive of the three model aminophosphates. In fact, its k_l value was 2–5 times smaller than that for PEA and PSer. Also, the three aminophosphates were less reactive by up to one order of magnitude than the dipeptide Ac-Phe-Lys against the same glycants.[30] Therefore, the presence of phosphate groups in the polar heads of aminophospholipids reduces the reactivity of their amino groups. Also, similarly to amino acids in relation to primary amines,[43,44] the presence of a carboxyl group in PSer resulted in slightly reduced formation constants for its Schiff bases in relation to PEA.

Based on the k_{obs} values obtained (Table 1), acetol was the strongest glycant. The efficiency of the glycants was dependent on the proportion of the reactive carbonyl form with respect to the cyclic or hydrated form. Accordingly, glucose was the least efficient sugar owing to the very high proportion of its non-reactive cyclic form (99.99%).[45] k_{obs} was roughly five times greater for APP than for PEA and PSer by effect of its increased proportion of free amino groups at physiological pH (2.45% vs 0.14% in PEA and 0.20% in PSer). The fact that k_{obs} was slightly smaller for PSer than for PEA is consistent with the results of in vitro tests conducted by Fountain et al.[17] which revealed the amount of glucose-glycated product to be greater with PE than with PS. However, these authors found the amount of glycated PS in cell red membranes, where PE and PS are present at a 2:1 ratio, to be three times greater than that of glycated PE. The microscopic constants (k_l) obtained in this work suggest that the polar heads in PS are less reactive than those in PE;

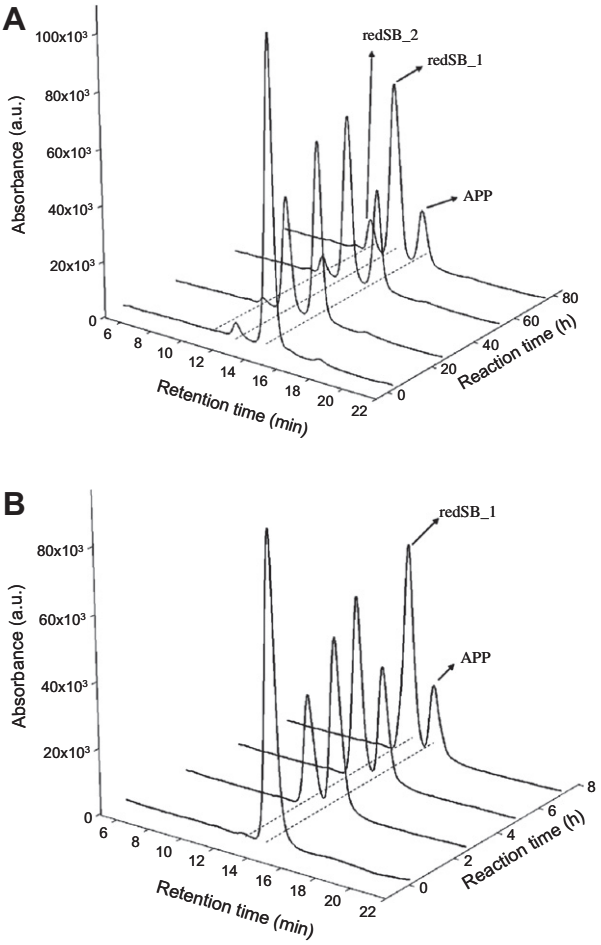


Figure 2. Time-dependent HPLC chromatograms for the reactions of 10 mM APP with 400 mM glucose (A) and 400 mM arabinose (B) in the presence of 100 mM $NaCNBH_3$ in phosphate buffer at pH 7.4 at 37 °C as obtained with UV–vis detection at 254 nm.

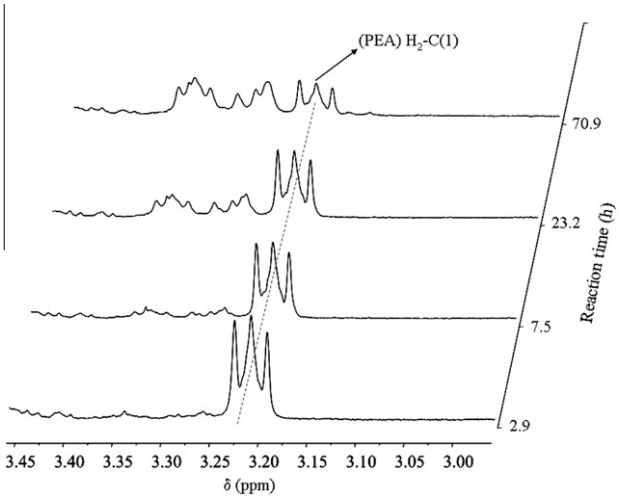


Figure 3. ^1H NMR spectra for a reaction mixture of 10 mM PEA and 200 mM arabinose in the presence of 100 mM $NaCNBH_3$ at pD 7.4 at 37 °C.

therefore, the differences observed in in vivo tests can be ascribed to a change in pK_a for the amino group of these compounds in the lipid membrane. Studies on vesicles from various aminophospholipid mixtures have shown that membrane composition influences

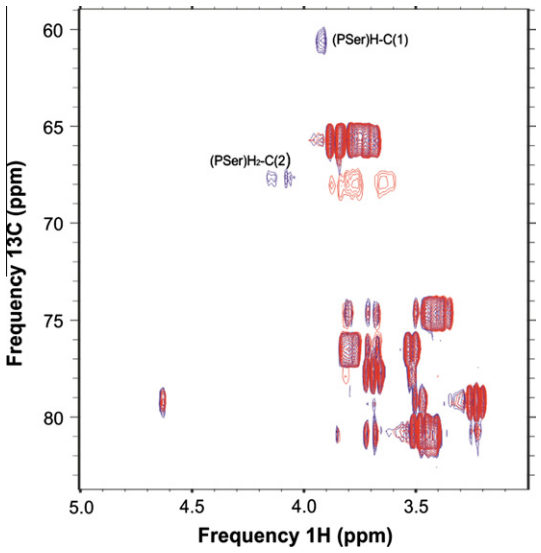


Figure 4. 2D-^1H, ^{13}C-HMQC spectra for a reaction mixture of 20 mM PSer and 0.42 M glucose in the presence of 0.1 M $NaCNBH_3$ at pD 7.4 at 37 °C. The blue line represents the spectrum at $t = 0$ and the red line that after 16 h of reaction.

their pK_a and suggested that the amino group in PE has a slightly higher pK_a in PS-containing vesicles.[46,47]

3.2. PLP competes with carbonyl groups for aminophospholipid polar head groups

PLP is one of the strongest inhibitor of aminophospholipid glycation known to date.[28] Careful study of the reactions between aminophosphates and PLP has allowed its inhibitory effect under physiological pH and temperature conditions to be assessed. Table 1 shows the observed formation (k_f) and hydrolysis constants (k_h) for its Schiff base and the equilibrium constant (K_{eq}) for the process. As can be seen, k_f for PLP was 4–5 orders of magnitude greater than the k_{obs} values for the other carbonyl compounds under physiological conditions. The high stability of the Schiff base of PLP is a result of intramolecular hydrogen bonding between the phenolate ion and the protonate imino nitrogen,[48] which occurs in none of the Schiff bases formed with the other carbonyl groups.

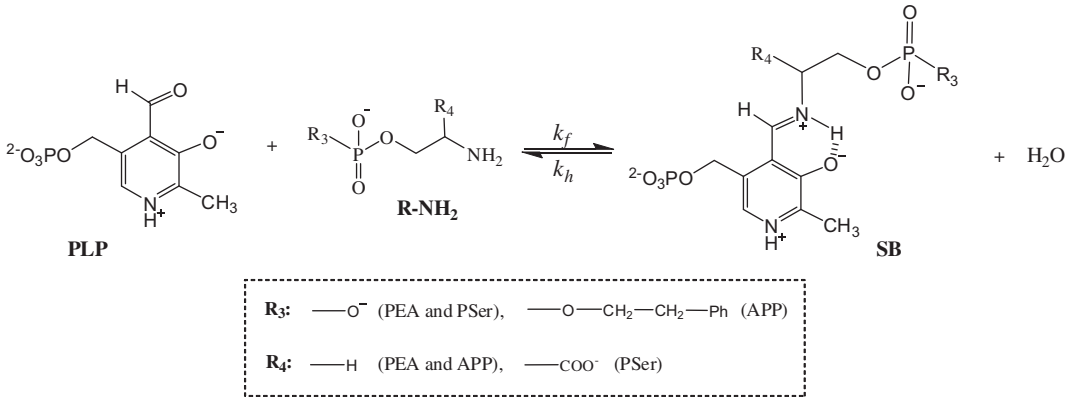
Similarly to the reactions with the previous glycants, the nearness of the phosphate group to the amino group in aminophosphates also influences their reactions with PLP. A comparison of the results for the reactions of PLP and PSer (Table 1) with reported data for serine[49] ($k_f = 3.0 \times 10^3\ M^{-1}\ h^{-1}$ and $k_h = 19\ h^{-1}$ at 25 °C, which, based on the activation energies determined in similar studies,[50,51] should be roughly twice greater at 37 °C), reveals that the presence of the phosphate group reduces k_f and increases k_h. In fact, the equilibrium constants, K_{eq}, for PEA and PSer are smaller than those for glycine ($100\ M^{-1}$)[50] and serine ($160\ M^{-1}$)[49] at 25 °C. These results are consistent with those of a recent study where excess negative charge in the amino acid chain was found to decrease the equilibrium constant for the reaction with PLP at pH 7.35,[52] as well as with the fact that K_{eq} was smaller for PSer than for PEA by effect of the former additionally bearing a carboxylate group. This effect was previously observed in the reaction of PLP with various amino acids and primary amines,[43,44] where the presence of the carboxyl group hindered formation of an imino bond but facilitated hydrolysis of the resulting Schiff base.

The increased k_f value of APP relative to PSer and PEA can be ascribed to its smaller pK_a and its containing a non-polar group. The k_f value for APP was similar to that for tryptophan—an amino acid also possessing an aromatic ring—under identical pH and

Table 1
Kinetic and equilibrium constants for the reactions of aminophosphates with glycating carbonyl compounds and PLP at pH 7.4 at 37 °C

Compound		APP	PEA	PSer	[c]Ac-Phe-Lys	[c]PM
Acetol ([a]$K_3 = 4.9 \times 10^1$)	k_{obs} ($M^{-1}\,h^{-1}$)	9.3×10^{-1}	1.8×10^{-1}[d]	–	8.6×10^{-1}	2.9
	k_1 ($M^{-1}\,h^{-1}$)	3.9×10^1	1.3×10^2	–	4.3×10^2	8.5×10^1
Arabinosa ([b]$K_3 = 3.0 \times 10^{-4}$)	k_{obs} ($M^{-1}\,h^{-1}$)	4.7×10^{-1}	8.7×10^{-2}[d]	7.8×10^{-2}[d]	5.4×10^{-1}	3.4
	k_1 ($M^{-1}\,h^{-1}$)	6.4×10^4	2.0×10^5	1.3×10^5	8.7×10^5	2.9×10^5
Glucosa ([b]$K_3 = 2.0 \times 10^{-5}$)	k_{obs} ($M^{-1}\,h^{-1}$)	5.3×10^{-2}	1.7×10^{-2}[d]	9.2×10^{-3}[d]	4.6×10^{-2}	2.9×10^{-1}
	k_1 ($M^{-1}\,h^{-1}$)	1.1×10^5	5.9×10^5	2.3×10^5	1.0×10^6	3.7×10^5
PLP	k_f ($M^{-1}\,h^{-1}$)	1.4×10^4	4.5×10^3	3.4×10^3	–	–
	k_h (h^{-1})	5.9×10^1	8.2×10^1	1.1×10^2	–	–
	K_{eq} (M^{-1})	2.3×10^2	5.5×10^1	3.0×10^1	–	–

[a] From Ref. 59.
[b] From Ref. 45.
[c] From Ref. 30.
[d] These values are corrected for the solvent isotope effect, calculated as $k^H/k^D = 1.3$.



Scheme 2.

temperature conditions ($k_f = 1.2 \times 10^4\ M^{-1}\ h^{-1}$).[50] The last result is consistent with those of previous studies by our group, which revealed the formation rate constant for the Schiff base of PLP with the non-polar compound *n*-dodecylamine at neutral pH to exceed that for polar amino acids.[43,53] On the other hand, the presence of non-polar groups hinders the hydrolysis of the imino bond (k_h). As a result of both effects, the equilibrium constant for APP was greater than those for PEA and PSer. Similar K_{eq} values were previously reported for *n*-hexylamine at 30 °C (245 M^{-1})[51] and ε-aminocaproic acid at 25 °C (220 M^{-1}).[53]

Pyridoxamine (PM), a B_6 vitamer with a methylamino group instead of the carbonyl group in PLP, is one other effective inhibitor of glycation and lipoxidation processes in biomolecules. In previous work, we determined the kinetic constants for the reactions of PM with various carbonyl compounds and provided quantitative evidence for the origin of competitive inhibition of protein glycation by PM.[30,54,55] As can be seen from Table 1, k_1 was 2–5 times greater for PM than for APP, but only two times greater than for PSer and ±1.5 than for PEA. Also, k_{obs} for PM (Table 1) exceeded the values for the aminophosphates, which can be ascribed to its increased proportion of free amino form at pH 7.4 (3.5%).[42] Therefore, PM can competitively inhibit aminophospholipid glycation under physiological conditions.

4. Conclusions

Based on the kinetic formation constants (k_{obs}) determined in this work, the presence of a phosphate group in the model compounds studied hinders the formation of a Schiff base in relation to amino acids and peptides, and the additional presence of a carboxyl group in α with the amino group further hinders the reaction. Also, esterifying the phosphate group with a substituent bearing a hydrophobic chain (e.g., APP, which is amphiphilic) favours formation of the Schiff base.

The k_{obs} values for the reactions of PLP with aminophosphates at physiological pH and temperature were 4 orders of magnitude greater than those for the reactions of aminophosphates with the other carbonyl compounds. The difference can be ascribed to intramolecular hydrogen bonding in the Schiff base of PLP. k_{obs} for the reactions of PM with the glycants exceeded the values for those involving the aminophosphates. Based on the results, aminophospholipid glycation can be inhibited by the action of PM and PLP, which act synergistically via two different mechanisms. Thus, PLP reacts reversibly with aminophospholipids to form a Schiff base which does not evolve to an Amadori compound—and hence prevents the formation of AGEs. On the other hand, PM reacts more rapidly with glycating carbonyl compounds than do aminophospholipids, thereby inhibiting structural alterations in the latter. These results constitute a substantial contribution to a better understanding of the inhibitory effect of B_6 vitamer on aminophospholipid glycation.

5. Experimental

5.1. Materials

D-Glucose, D-arabinose, D_2O (99.9% D), pyridoxal 5′-phosphate hydrate (PLP), *O*-phosphorylethanolamine (PEA) and *O*-phospho-DL-Serine (PSer) were purchased from Sigma–Aldrich; acetol was obtained from Fluka; sodium cyanoborohydride ($NaCNBH_3$),

potassium dihydrogen phosphate and sodium hydroxide 0.1 M standard solution were obtained from Acros Organics. All reagents were used as received.

5.2. High performance liquid chromatography (HPLC)

HPLC analyses were conducted on a *Shimadzu-LC 10AT* chromatograph equipped with a *Rheodyne 7725i* universal injector and a *Shimadzu SPD-10AV* UV/Vis detector. The column was a *Tracer Excel 120 ODSB* model (25 × 0.46 cm, 5 μm). The target compounds in the reaction mixtures were separated by using MeOH/water-20 mM potassium phosphate (pH 6.5) in various gradient modes (flow rate 1 ml/min) and detection at 254 nm.

5.3. UV–vis spectra

Absorption spectra were recorded on a *Shimadzu UV-2401 PC* double-beam spectrophotometer. The buffer solution background spectrum was used as spectral reference. Quartz cells of 1 cm path length were used to obtain electronic spectra. The cell's temperature were adjusted at 37 ± 0.1 °C using a *Shimadzu* thermostat *TCC-240A* model.

5.4. NMR spectroscopy

NMR spectra were recorded on a Bruker AMX-300 spectrometer, using sample tubes of 5 mm in diameter and 3-(trimethylsilyl)-1-propanesulphonic acid (DSS) as an internal reference. All the chemical shifts for ^{1}H (δ_H) and ^{13}C (δ_C) are given in parts per million and coupling constants (J) in Hertz. The solutions used for products characterization were prepared in D_2O, and were adjusted at pD 7.4 (pD = $-\log[D^+]$) with 0.5 M phosphate buffer. Tests were of the one-dimensional ^{1}H NMR, ^{13}C NMR and polarization transfer (DEPT-135) and two-dimensional type (^{1}H,^{13}C-HMQC).

5.5. Synthesis of 2-aminoethylphenethylphosphate (APP)

APP was prepared by following the procedure of Argirov[56] with slight modifications. The product was isolated by decantation with benzene to facilitate extraction of all water-soluble compounds. The aqueous fraction was freeze-dried and redissolved in the minimum volume of water. This was followed by HPLC purification on a Kromasil 100 semi-preparative column (25 × 1.00 cm, 5 μm) that was eluted with a MeOH/Milli-Q water binary gradient at a flow-rate of 2.5 ml/min for detection at 254 nm. Finally, the collected fraction was freeze-dried to obtain a product of mp 220–223 °C in 5% yield. ^{1}H NMR: δ 7.37 (m, 5H, H-C(6)–H-C(10)), 4.11 (q, 2H, $^3J_{H3\text{-}H4}$ = 6.4 Hz, $^3J_{H3\text{-}P}$ = 6.4 Hz, H-C(3)), 3.79 (q, 2H, $^3J_{H2\text{-}H1}$ = 5.5 Hz, $^3J_{H2\text{-}P}$ = 5.5 Hz, H-C(2)), 3.07 (t, 2H, $^3J_{H1\text{-}H2}$ = 5.1 Hz, H-C(1)), 2.96 (t, 2H, $^3J_{H4\text{-}H3}$ = 6.5 Hz, H-C(4)); ^{13}C NMR, DEPT-135, HSQC: δ 141.43 (s, C(5)), 131.91 (s, C(6) and C(10)), 131.38 (s, C(7) and C(9)), 129.36 (s, C(8)), 69.51 (d, $^2J_{3,P}$ = 5.8 Hz, C(3)), 64.33 (d, $^2J_{2,P}$ = 5.2 Hz, C(2)), 42.54 (d, $^3J_{1,P}$ = 8.0 Hz, C(1)), 38.83 (d, $^3J_{4,P}$ = 7.2 Hz, C(4)); ESI$^+$: *m/z* 246.11 $[M+H]^+$ (100%), 268.09 $[M+Na]^+$ (50%), 491.21 $[2M+H]^+$ (32%), 513.22 $[2M+Na]^+$ (44%). Theoretical elemental analysis for $C_{10}H_{16}NO_4P$ (MW 245.21): C, 48.98; N, 5.71; H, 6.58; O, 26.10. Experimental Elemental Anal.: C, 48.73; N, 5.51; H, 6.43; O, 25.86.

5.6. Reaction mixtures for the kinetic study of Schiff base formation between aminophosphates and glycating carbonyl compounds

The kinetic study of Schiff base formation with aminophosphates involved three different carbonyl compounds (viz. D-glucose, D-arabinose and acetol) in the presence of $NaCNBH_3$ for selective reduction of imino groups at neutral pH and scavenging of Schiff bases.[37,38]

The reactions between APP and carbonyl compounds were studied by HPLC. Samples were mixtures of the reactants in 0.5 M phosphate buffer at pH 7.4 that were thermostated at 37 °C. Reactant concentrations were 10 mM APP, 100 mM $NaCNBH_3$ and 400 mM carbonyl compound—by exception, acetol was used at 200 mM.

Reactions of PEA and PSer with carbonyl compounds were studied from ^{1}H NMR, and ^{1}H, ^{13}C-HMQC spectra, all recorded at 37 °C. Reaction mixtures were prepared in 0.5 M phosphate buffer, using D_2O at pD 7.4 as solvent throughout. The reactant concentrations used in the reactions studied by 1D-^{1}H NMR were 10 mM PEA and 0.80 M acetol (or 0.20 M arabinose); and those used for 2D monitoring 20 mM PEA (or PSer) and 0.40 M D-glucose (or 0.40 M D-arabinose). A $NaCNBH_3$ concentration of 0.10 M was added in all cases.

Formation of the reduced form of the Schiff base (redSB_1) was demonstrated by using NMR in 0.5 M phosphate buffer at pD 7.4 to characterize the major product of the reaction between APP and D-glucose following isolation by HPLC after 4 days of reaction and freeze-drying. ^{1}H NMR: δ 7.37 (m, 5H, H-C(6)–H-C(10)), 4.11 (H-C(3)), 4.11 (H-C(2′)), 3.82 (H-C(2)), 3.80 (H-C(3′)), 3.80 (H_a-C(6′)), 3.67 (H-C(5′)), 3.64 (H-C(4′)), 3.63 (H_b-C(6′)), 3.20 (H_a-C(1′)), 3.16 (H-C(1)), 3.13 (H_b-C(1′)), 2.96 (H-C(4)); ^{13}C NMR, DEPT-135, HSQC: δ 141.43 (s, C(5)), 131.90 (s, C(6) and C(10)), 131.37 (s, C(7) and C(9)), 129.35 (s, C(8)), 73.56 (s, C(4′)), 73.38 (s, C(5′)), 73.18 (s, C(3′)), 70.72 (s, C(2′)), 69.55 (d, $^2J_{3,P}$ = 5.8 Hz, C(3)), 65.36 (s, C(6′)), 63.08 (d, $^2J_{2,P}$ = 5.2 Hz, C(2)), 52.07 (s, C(1′)), 50.22 (d, $^3J_{1,P}$ = 7.6 Hz, C(1)), 38.80 (d, $^3J_{4,P}$ = 7.4 Hz, C(4)). ESI$^+$: *m/z* 486.28 $[M-H+2K]^+$ (100%), 470.28 $[M-H+K+Na]^+$ (24%). Assignation of the spectral signals confirmed that the isolated product was the Schiff base of APP with glucose (Fig. 5A).

The reaction of APP with glucose gave a second product that was isolated by HPLC after 17 days of reaction and freeze-drying. The relative intensities of the NMR signals for that compound were consistent with the addition of a second glucose molecule to the initial reduced form of the Schiff base to form redSB_2 (Fig. 5B). ^{13}C NMR, DEPT-135, HSQC: δ 141.54 (s, C(5)), 131.94 (s, C(6) and C(10)), 131.39 (s, C(7) and C(9)), 129.36 (s, C(8)), 73.60 (s, C(4′)), 73.55 (s, C(5′)), 73.27 (s, C(3′)), 69.89 (s, C(2′)), 69.58 (d, $^2J_{3,P}$ = 5.8 Hz, C(3)), 65.40 (s, C(6′)), 62.58 (d, $^2J_{2,P}$ = 5.4 Hz, C(2)), 59.07 (s, C(1′)), 57.03 (d, $^3J_{1,P}$ = 7.7 Hz, C(1)), 38.81 (d, $^3J_{4,P}$ = 7.4 Hz, C(4)). ESI$^+$: *m/z* 596.25 $[M+Na]^+$ (100%), 574.26 $[M+H]^+$ (84%).

5.7. Reaction mixtures for the kinetic study of Schiff base formation between PLP and aminophosphates

PLP solutions were prepared daily in 0.5 M phosphate buffer at pH 7.4 and stored in the dark. Their exact concentrations, which spanned the range (1.2–1.4) × 10^{-4} M, were determined by dilution with 0.1 M NaOH and measurement of their absorbance at 388 nm (ε = 6600 mol^{-1} cm^{-1}).[57]

Solutions containing PEA, PSer or APP at a 0.1 M concentration were made in 0.5 M phosphate buffer adjusted to pH 7.4 with NaOH. Reaction mixtures were prepared by diluting an appropriate volume of concentrated aminophospholipid model solution with pre-thermostated buffer and adding PLP last. The aminophosphate concentrations in the measuring cell were 50–260 times greater than the PLP concentrations. Reactions were monitored by UV–vis spectroscopy, by measuring the absorbance at 420 nm. As confirmed in each run, the differences between the initial and final pH of the reaction mixture never exceeded ±0.04 pH units. pH measurements were made with a Crison GLP21+ pH-meter, using

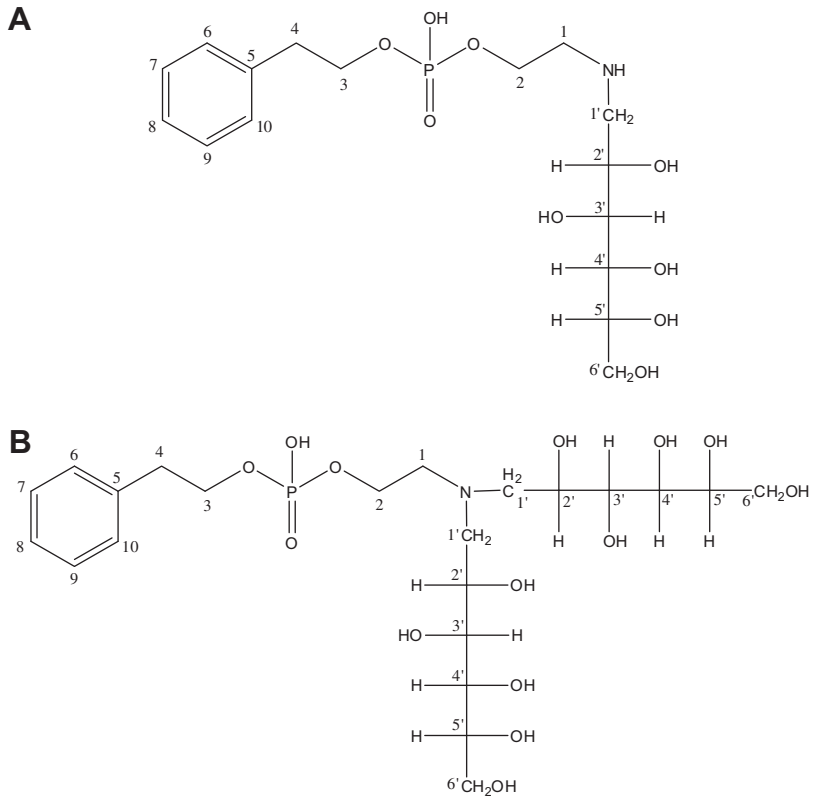


Figure 5. Structure of the compounds redSB_1 and redSB_2, obtained as products of the reaction between APP and glucose in the presence of $NaCNBH_3$ at pH 7.4 at 37 °C.

a model 52 09 glass electrode 6 mm in diameter that was previously calibrated with aqueous buffers at 37 °C.

5.8. Determination of ionization equilibrium constants

The ionization constants for PEA, PSer and APP were determined by potentiometric titration with a Metrohm Titrino 718 pH-Stat of 10 ml of an 0.01 M aqueous solution of each compound adjusted to a total ionic strength of 0.1 M at 37 °C. The titrant was 0.1 M NaOH. Titrations were performed in duplicate and ionization equilibrium constants calculated by using the software SigmaPlot v. 10.0.[58]

5.9. Determination of Schiff base formation rate constants

Scheme 1 depicts the kinetic mechanism of the formation of the Schiff bases of the aminophosphates with the carbonyl compounds and their subsequent reduction by $NaCNBH_3$ added to the solutions. The rate of aminophosphate disappearance fitted the following equation:

$$\frac{-d[\mathrm{R{-}NH_2}]}{dt} = k_1[\mathrm{R{-}NH_2}][\mathrm{R_1{-}CO{-}R_2}] - k_{-1}[\mathrm{SB}] \quad (1)$$

which can be simplified to the following expression by applying the steady-state approximation to the Schiff based under the assumption $k_2 >>> k_{-1}$:

$$\frac{-d[\mathrm{R{-}NH_2}]}{dt} = k_1[\mathrm{R{-}NH_2}][\mathrm{R_1{-}CO{-}R_2}] \quad (2)$$

However, each reagent was involved in another equilibrium. Thus, carbonyl compounds in solution can be in a reactive form (R_1–CO–R_2) or non-reactive form [R_1–C(OH)(OR′)–R_2]. Also, the latter can be a hydrated species (R′ = H) as in acetol or a cyclic species (R′ = hydrocarbon chain) as in glucose and arabinose. Therefore, the equilibrium constant (K_3 in Table 1) is given by

$$K_3 = \frac{[\mathrm{R_1{-}CO{-}R_2}]}{[\mathrm{R_1{-}C(OH)(OR')}{-}\mathrm{R_2}]} \quad (3)$$

The protonation equilibrium constant for the aminophosphates can be expressed as

$$K_4 = \frac{[\mathrm{R{-}NH_2}][\mathrm{H_3O^+}]}{[\mathrm{R{-}NH_3^+}]} \quad (4)$$

Application of a mass balance and substitution of Eq. 2 yields

$$\frac{-d[\mathrm{R{-}NH_2}]}{dt} = \frac{k_1K_3K_4[\mathrm{R_1{-}CO{-}R_2}]_T[\mathrm{R{-}NH_2}]_T}{[\mathrm{H_3O^+}] + K_4 + [\mathrm{H_3O^+}]K_3 + K_3K_4} \quad (5)$$

which can be rearranged to

$$\frac{-d[\mathrm{R{-}NH_2}]}{dt} = k_{\mathrm{obs}}[\mathrm{R_1{-}CO{-}R_2}]_T[\mathrm{R{-}NH_2}]_T \quad (6)$$

where k_{obs} is defined as:

$$k_{\mathrm{obs}} = \frac{k_1K_3K_4}{[\mathrm{H_3O^+}] + K_4 + [\mathrm{H_3O^+}]K_3 + K_3K_4} \quad (7)$$

Under pseudo first-order conditions (i.e., $[R_1\text{–CO–}R_2 \gg [R\text{–}NH_2]$), Eq. 6 can be integrated to obtain

$$\ln\frac{[\mathrm{R{-}NH_2}]_t}{[\mathrm{R{-}NH_2}]_0} = k_{\mathrm{obs}}[\mathrm{R_1{-}CO{-}R_2}]_T t \quad (8)$$

Fitting the experimental data for the reactions with the carbonyl compounds to Eq. 8, and using the initial concentration of each, allowed the observed kinetic constant (k_{obs}) to be calculated.

Scheme 2 depicts the mechanism for the reaction between PLP and the aminophosphates. The mathematical treatment used to determine the kinetic constants is described in detail elsewhere.[49] Briefly, integration of the kinetic equations for this mechanism and

application of the Beer–Lambert law at a wavelength of zero absorption for each aminophosphate yielded

$$\ln\frac{A_\infty - A_0}{A_\infty - A} = -\ln\frac{ab - xx_e}{x_e^2} + k_{ap}t \quad (9)$$

where A_0, A and A_∞ are the absorbances at time 0, t and equilibrium; parameters a and b are the initial concentrations of PLP and amino compound, respectively; and x and x_e are the Schiff base concentrations at time t and equilibrium, respectively. Taking into account that in our experimental conditions $ab \gg xx_e$, k_{ap} was calculated from the slope of a plot of $\ln(A_\infty - A_t)$ versus time. Changing the aminophosphate concentration over a wide range while keeping the PLP concentration constant provided k_{ap} values that were fitted to

$$k_{ap}^2 = [k_h + k_f(a+b)]^2 - 4abk_f^2 \quad (10)$$

were k_f and k_h are the kinetic formation and hydrolysis constant, respectively, for the Schiff base. Finally, the equilibrium constant, K_{eq}, was calculated as the ratio k_f/k_h.

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