JOURNAL OF PHYSICAL ORGANIC CHEMISTRY
J. Phys. Org. Chem. 2004; **17:** 537–540
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/poc.772

Effect of buffer carbonate and arsenate on the kinetics of glycation of human hemoglobin†

Herminia Gil,* Blanca Vásquez, Mary Peña and Jorge Uzcategui

Departamento de Química, Universidad de los Andes, Mérida 5251, Venezuela

Received 30 July 2003; revised 1 October 2003; accepted 1 October 2003

ABSTRACT: The glycation of hemoglobin is catalyzed by buffer carbonate and arsenate. The reaction of hemoglobin with glucose exhibits identical rates in protium and deuterium oxides, both for the buffer-independent rate and for the first-order rate in carbonate and arsenate buffer. When D-glucose-2-*h* is compared with D-glucose-2-*d*, the kinetic isotope effect for the buffer-independent rates is ~ 2, whereas the buffer-dependent rate constants show no isotope effects. The absence of both substrate and solvent isotope effects for the buffer-dependent term are indicative that a functional group on the hemoglobin is the proton-abstracting base in the Amadori rearrangement. The catalytic constant (k_B) of arsenate is double that of carbonate. A different base group on the hemoglobin may be involved in the abstraction of proton 2 in the Amadori rearrangement. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: glycation; hemoglobin; catalysis; carbonate; arsenate; isotope effects

INTRODUCTION

Studies with a number of proteins indicate that glycation is not a random chemical modification of amino groups in proteins. There is preferential glycation of β-chain terminal valine residues in hemoglobin[1] and a selective modification of a limited set of lysine residues in hemoglobin,[1] albumin[2] and lipoprotein.[3] As is shown in Scheme 1, the initial reversible condensation step of glucose and hemoglobin forms an aldimine or Schiff base, which is able to undergo a nearly irreversible intermolecular Amadori rearrangement.[4]

Several factors have been reported that can influence the rate of glycation of hemoglobin: pH,[4–6] glucose concentration[4] and catalysis by phosphate buffer,[7–9] 2,3-diphosphoglycerate[5,10–12] and recently by 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate.[13] Clearly, inorganic and organic phosphates play an important role in determining the kinetics and specificity of glycation of hemoglobin. However, the catalyses of inorganic and organic phosphates in the glycation of hemoglobin are different. The role of organic phosphates is the abstraction of the proton in the Amadori rearrangement,[13] but the role of inorganic phosphate is not a classical, proteolytic general acid–base catalysis, showing that a functional group on the hemoglobin is the abstracting base in the Amadori rearrangement.[8]

In order to have a much clearer understanding of the chemical mechanism for the formation of glycated hemoglobin, we performed a kinetic analysis of buffer carbonate and arsenate in order to establish whether buffer carbonate and arsenate catalyze the glycation of hemoglobin and their role is the same as that of buffer phosphate.

EXPERIMENTAL

Human hemoglobin, glucose-2-*h*, glucose-2-*d* and glucohemoglobin kit were obtained from Sigma Chemical, deuterium oxide from Aldrich, sodium dihydrogen arsenate (NaH_2AsO_4), disodium hydrogen arsenate ($Na_2HAsO_4{\cdot}7H_2O$) and sodium chloride from Merck, sodium bicarbonate from BDH and sodium carbonate from J. T. Baker Acrodisc filters (0.2 μm) were purchased from Gelman Sciences.

Carbonate buffer solutions of pH 8.78 were prepared by mixing appropriate volumes of 25 mM sodium bicarbonate, 25 mM sodium carbonate and 0.15 M NaCl. Arsenate buffer solutions of pH 7.3 were prepared by mixing appropriate volumes of 10 mM sodium dihydrogen arsenate and 10 mM of disodium hydrogen arsenate. Buffers in D_2O were prepared similarly. Hemoglobin solutions were prepared by dissolving a weighed sample of hemoglobin in known volumes of 0.15 M NaCl. The reaction mixtures were prepared by mixing known volumes of hemoglobin with different concentrations of buffer carbonate and arsenate, 0.15 M NaCl, pH 7.3, and

**Correspondence to:* H. Gil, Departamento de Química, Universidad de los Andes, Mérida 5251, Venezuela.
E-mail: herminia_gil@latinmail
Contract/grant sponsor: Consejo de Desarrollo Científico y Humanistico; *Contract/grant numbers:* CDCHT-C-779-96-08A; CVI-ADG-C-24-95.
†Selected paper part of a special issue entitled 'Biological Applications of Physical Organic Chemistry dedicated to Prof. William P. Jencks'.

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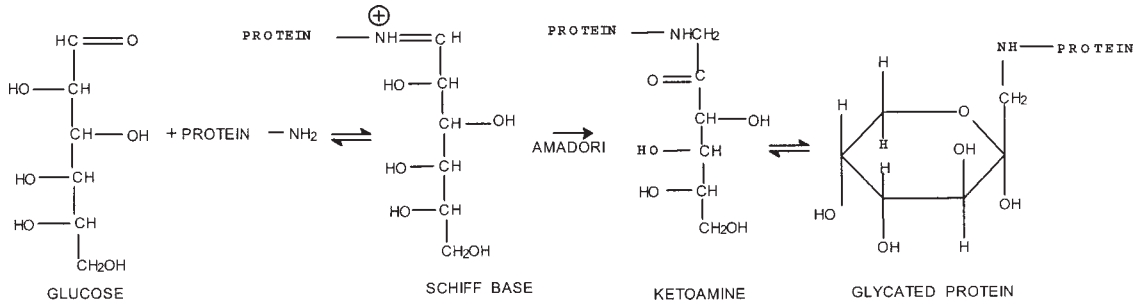

Scheme 1. Mechanism of the non-enzymic glycation of proteins

40 mM glucose at 0 °C. The reaction medium with different concentrations of buffer carbonate was 0.15 M NaCl, pH 8.78, at 37 °C. The final pH for arsenate buffer was 7.3 and for carbonate buffer 8.78 for all solutions. The reaction was started and maintained at 37 °C. All solutions were sterilized by ultrafiltration thorough a Gelman 0.2 μm filter (Acrodisc) in sterile plastic tubes.

The rates of glycation of hemoglobin under an atmosphere of air were measured by following the appearance of the glycated hemoglobin.[14,15] Glycohemoglobin HbA1C was eluted from a cation-exchange column and measured spectrophotometrically at 415 nm (Sigma Kit[14,15] No. 440). A hemoglobin concentration of 9.2×10^{-2} mM was employed with 40 mM glucose. The reaction media were 0.15 M NaCl, buffered at 37 °C with mixtures of sodium carbonate and bicarbonate at pH 8.78. The reactions in deuterium oxide were conducted at the corresponding pD[16] of 9.28. The reaction media were 0.15 M NaCl, buffered at 37 °C with mixtures of NaH_2AsO_4 and $Na_2HAsO_4{\cdot}7H_2O$ at pH 7.3. The reactions in deuterium oxide were conducted at the corresponding pD[16] of 7.8. The kinetics exhibited both a catalyst-independent term [reflecting reaction assisted by water, lyons (hydroxide or hydrogen ions) or protein functional groups] and a first-order term in buffer ($k_{obs} = k_0 + k_B$[Buffer]).

In the experiments to determine the solvent isotope effect for carbonate buffer in H_2O and D_2O, carbonate buffer was held constant at 14.0 mM, pH 8.78 and pD 9.28, at 37 °C under similar conditions of hemoglobin and glucose concentrations.

Proton inventory was carried out at fixed concentration of 40 mM glucose, 4.8 mM arsenate buffer, in different mixtures of H_2O and D_2O at 37 °C.

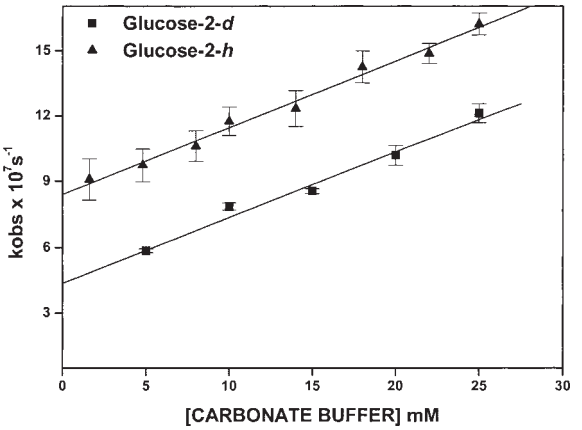

Figure 1. Plot of the first-order rate constants for the glycation of hemoglobin with 40 mM glucose-2-*h* and glucose-2-*d* as a function of the concentration at carbonate buffer, pH 8.78, at 37 °C

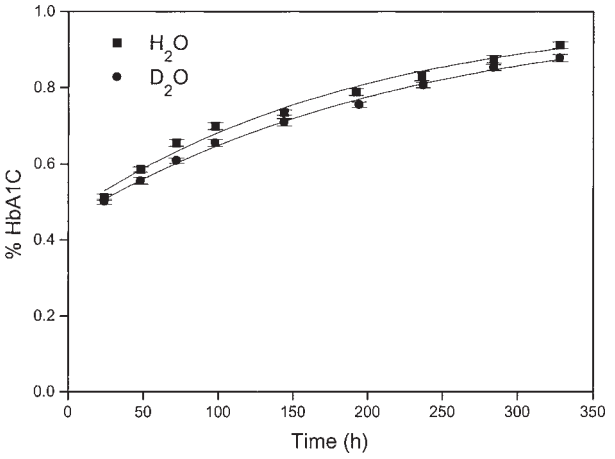

Figure 2. Plot of formation of HbA_{1C} as a function of time in 14.0 mM, carbonate buffer pH 8.78, pD 9.28, for the glycation of hemoglobin in H_2O and D_2O at 37 °C

RESULTS AND DISCUSSION

Figure 1 shows an increase in the first-order rate constants for the glycation of hemoglobin under similar conditions as a function of carbonate buffer concentrations, with 40 mM glucose-2-*h* and glucose-2-*d*. The primary deuterium isotope effects for the second-order rate constants (slope) and for the first-order rate constants (intercept) are ${}^{H}k_B/{}^{D}k_B = 1.06 \pm 0.07$ and ${}^{H}k_0/{}^{D}k_0 = 1.96 \pm 0.14$. The glycation of hemoglobin is catalyzed by carbonate buffer. In fact, general acid–base catalysis is expected for the proton-abstraction step of the Amadori rearrangement (Scheme 1). However, we find that the carbonate reaction occurs with identical rates ($k_{H_2O}/k_{D_2O} = 1.00 \pm 0.10$) in protium oxide and deuterium oxide (Fig. 2). The absence of a rate difference in isotopic water solvents shows that

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Table 1. First and second-order rate constants, solvent isotope effects, substrate isotope effects and proton inventory for the non-enzymic glycation of hemoglobin with different buffers

Buffer catalyst	k_0 (10^7 s^{-1})	k_B(10^8 mM^{-1} s^{-1})	k_{H_2O}/k_{D_2O}	$^{H}k_0/^{D}k_0$	$^{H}k_B/^{D}k_B$	Proton inventory
Carbonate	8.58 ± 0.39	2.81 ± 0.20	1.00 ± 0.10	1.96 ± 0.14	1.06 ± 0.07	
Arsenate	2.47 ± 0.18	5.99 ± 0.33	0.96 ± 0.23	2.11 ± 0.12	1.13 ± 0.06	Flat
Phosphate[8]	2.13 ± 0.04	2.38 ± 0.08	1.10 ± 0.10	2.17 ± 0.11	1.10 ± 0.10	Flat

proton donation from solvent, lyons (hydroxide or hydrogen ions) or buffer do not limit the rate. The solvent isotope effect can be predicted for each case.[13]

As noted in Fig. 1 the carbonate-dependent reaction occurs with identical rate constants with either glucose-2-*h* or glucose-2-*d*. This excludes proton abstraction as the rate-determining step by carbonate buffer. The absence of either a substrate isotope effect or solvent isotope effect in fact excludes as a rate-limiting step both proton-transfer steps of a general acid–base-catalyzed Amadori rearrangement because proton abstraction by carbonate buffer would show a substrate isotope effect and proton donation by buffer would show a solvent isotope effect. These results are indicative that a functional group on the hemoglobin is the proton-abstracting base in the Amadori rearrangement. Phosphate[8] and carbonate show the same behavior.

The catalytic constants (k_B) for phosphate[8] and carbonate are similar (Table 1), but the spontaneous reactions (k_0) are different, because the phosphate reaction was carried out at pH 7.30 and the carbonate reactions was carried out at pH 8.78 under similar conditions of hemoglobin and glucose concentration at 37 °C. The glycation of hemoglobin increases with pH,[4–6] hence the glycation of hemoglobin is greater in carbonate buffer than in phosphate buffer. This observation is consistent with the chemical prediction that only uncharged amino groups on the proteins can participate in this type of addition reaction with glucose. The interpretations of the pH dependences on rates would be complex, because the observed rates involve two terms, one for the buffer-independent term (k_0) and one for the buffer-dependent term (k_B). The former includes hydroxide ion as base, water as base and functional groups in the protein. The second term includes the carbonate dependence on the rate. k_0 increases with pH, but k_B apparently does not change.

Figure 3 shows an increase in the first-order rate constants for the glycation of hemoglobin under similar conditions as a function of arsenate buffer concentrations, with 40 mM glucose-2-*h* and glucose-2-*d*. The substrate isotope effects for the second-order rate constants (slope) and for the first-order rate constants (intercept) are $^{H}k_B/^{D}k_B = 1.13 \pm 0.06$ and $^{H}k_0/^{D}k_0 = 2.11 \pm 0.12$, respectively. The glycation of hemoglobin is catalyzed by arsenate buffer. General acid–base catalysis is expected for the proton-abstraction step of the Amadori rearrangement (Scheme 1). We find that the reaction occurs with identical rate constants in protium oxide and deuterium

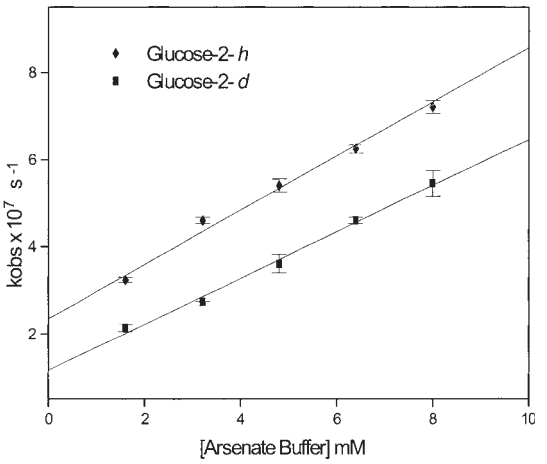

Figure 3. Plot of the first-order rate constants for the glycation of hemoglobin with 40 mM glucose-2-*h* and glucose-2-*d* as a function of arsenate buffer concentration, pH 7.3, at 37 °C

oxide (Table 1). The solvent isotope effect expected for the abstraction of the glucose-2-*h* on the Schiff base by any catalyst is 1, because this hydrogen does not exchange.

Catalysis by arsenate buffer, the observed solvent isotope effect of 1, the flat proton inventory (Fig. 4) and the substrate isotope effect of 2 in the arsenate-independent term are indicative that a functional group in the hemoglobin is the abstracting base in the Amadori

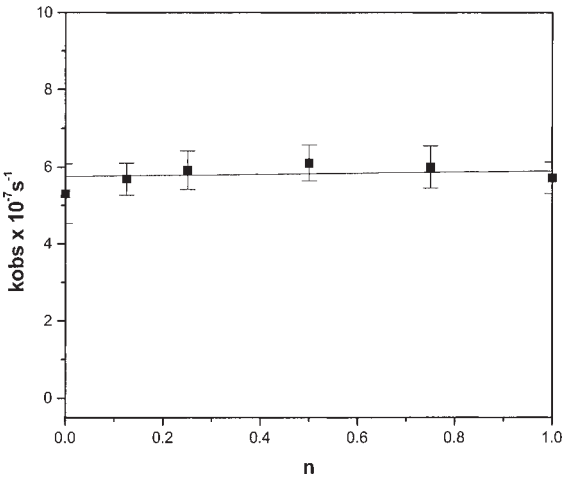

Figure 4. Overall isotope effect as a function of atom fraction *n* of deuterium in binary mixtures of protium and deuterium oxides for glycation of hemoglobin at 37 °C in 4.8 mM arsenate, 40 mM glucose

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rearrangement. As shown in Table 1, the catalytic constants (k_B) for carbonate and phosphate[8] buffer are the same. In contrast, there is about a twofold increase in the catalytic constant (k_B) in the glycation of hemoglobin for arsenate buffer versus phosphate[8] and carbonate buffer, but the spontaneous reaction (k_0) did not vary in arsenate buffer versus phosphate buffer at same pH of 7.3. Arsenate, which is anionic and similar in geometry and pK_a to phosphate, is more effective than phosphate as a catalyst of glycation of hemoglobin. Watkins *et al.*[7] reported a similar effect with RNase. They found that on incubation of RNase with 0.4 M glucose, pH 7.4, at 37 °C for 3 days in the various buffer systems, arsenate was a better catalyst than phosphate and TAPSO.[7] It is not clear whether these results from a difference in binding affinity or the slight, 0.4 unit decrease in the pK_a of arsenate versus phosphate can be the only factor enhancing the extent of glycation of hemoglobin and RNase.[7]

Hemoglobin is a highly complex molecule that functionally interacts with several other molecular species, including oxygen, CO_2, CO and 2,3-diphosphoglycerate (DPG). Hemoglobin binds oxygen at four sites which are distant from the β-chain amino terminus to which glucose adds to form HbA_{1C}. The binding of oxygen causes a series of conformational changes throughout the hemoglobin molecule, which include an alteration in the position of the two α-amino groups of the β-chains so that the distance between them increases from 16 to 20 Å.[17] This change in conformation is critical to the functional interaction of DPG with hemoglobin. This change in position could affect the rate of HbA_{1C} formation by altering the chemical environment of the α-amino group or by changing the accessibility of this group to glucose binding. The availability of this site for interaction with DPG is compromised when it is covalently linked to glucose.[18] DPG catalyzes the glycation of hemoglobin[12] by abstracting the proton in the Amadori rearrangement, but phosphate,[8] carbonate and arsenate may induce a conformational change that enhances the catalytic effectiveness of a basic functional group in hemoglobin.

Our results show a larger k_B for the arsenate-catalyzed reaction than for the case or either phosphate[8] or carbonate. The 3 pK_a unit difference between phosphate and carbonate causes no change in reactivity. On the other hand, the small 0.4 pK_a unit difference between phosphate and arsenate is clearly not the answer to the latter's catalytic potential.

The absence of structural data on anion–hemoglobin complexes at the atomic level makes a definite explanation for the higher catalytic potential of $HAsO_4^{2-}$ impossible. Nevertheless, a qualitative hypothesis can be advanced. Desolvation of strongly hydrated species such as HPO_4^{2-}, $HAsO_4^{2-}$ or CO_3^{2-} should make a contribution to the energy cost of formation of anion–hemoglobin complexes. Such an effect has been observed in the acetyl transfer reaction between *p*-nitrophenyl acetate and oxyanions.[19]

Aspartate-β-semialdehyde dehydrogenase (ASADH) catalyses the reversible reductive dephosphorylation of L-β-aspartyl phosphate to yield L-aspartate-β-semialdehyde. In the reverse direction, HPO_4^{2-} reacts with the acetyl enzyme formed by oxidation of the aldehyde. Kish and Viola[20] found that arsenate binds this enzyme with a K_M value half that measured for phosphate. They carried out calculations to determine the charge density on the peripheral oxygen atoms of different oxyanions. The result was -0.952 for HPO_4^{2-} and -0.892 for $HAsO_4^{2-}$. Our interpretation of their data is that there is a higher free-energy cost for the transfer of phosphate ion, from aqueous medium to the enzyme catalytic site. The oxyanion-mediated glycation of hemoglobin is a process that parallels ASADH.

Whereas phosphate and carbonate ($k_B = 2.4 \times 10^{-8}\ mM^{-1}\ s^{-1}$) show the same catalytic strength as $HAsO_4^{2-}$ will remain an open question until x-ray data on the anion–hemoglobin complexes become available.

Arsenate is a better catalyst than carbonate and phosphate[8] and the isotope effects are indicative that functional groups in the hemoglobin are the catalysts. In the absence of other information, the data suggest that proton abstraction by functional groups in the hemoglobin is the rate-determining step in the glycation of hemoglobin catalyzed by carbonate and arsenate. The role of carbonate and arsenate is the same as that of phosphate:[8] they induce hemoglobin to catalyze its own glycation.

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