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Catalysis by organic phosphates of the glycation of human hemoglobin†

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ABSTRACT: The non-enzymic glycation of hemoglobin is catalyzed by 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate, but not by sodium propionate. Solvent isotope effects were determined (k_{H_2O}/k_{D_2O}). Catalysis by 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate, the observer solvent isotope effect of unity, and flat proton inventory tend to suggest that the proton abstraction step of the Amadori rearrangement by 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate is the rate-determining step in the non-enzymatic glycation of hemoglobin. The solvent isotope effect of unity, and a flat proton inventory for sodium propionate are indicative that a functional group on the hemoglobin is the proton-abstracting base in the Amadori rearrangement. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: glycation; hemoglobin; catalysis; organic phosphates; isotope effects

INTRODUCTION

The reaction of glucose with amino groups in proteins occurs non-enzymically *in vivo*.[1–3] It has long been recognized that glucose can react with α- and ε-amino groups on proteins *in vitro*.[4–6] An *in vivo* occurrence of this reaction was demonstrated with the discovery of a naturally existing minor human hemoglobin component, HbA_{1C},[2,7] the best understood example of such a modification. This intrinsically very slow process appears to be critical in the pathogenesis of various secondary complications associated with diabetes mellitus[8–10] and in the process of aging,[11] so that it is important to understand its mechanistic features. The paradigmatic reaction of glucose with normal hemoglobin (HbA_0) has been studied[12] and is known to occur most rapidly at the N-terminal valine of the β-subunit. An initial, rapid aldimine formation is succeeded by a slower Amadori rearrangement to give the amino ketone[13] (Scheme 1).

Some progress has been made in identifying factors affecting the specificity of glycation of hemoglobin. Shapiro *et al.*[14] noted an increased glycation of lysine residues adjacent to acidic amino acids in hemoglobin, suggesting that the carboxyl groups could serve as a catalyst for the Amadori rearrangement. The reaction is accelerated by buffer phosphate.[15–17] In fact, general acid–base catalysis by buffer is expected for the proton-abstraction and proton-donation steps at the Amadori rearrangement. However, the phosphate-dependent reaction occurs with identical rate constants in protium oxide and deuterium oxide.[16] The solvent isotope effect expected for abstraction of the glucose-2-*h* on the Schiff base by phosphate is 1, because this hydrogen does not exchange. The solvent isotope effect expected for proton donation is in the range 2–8.[18] The phosphate-dependent reaction occurs with identical rate constants with either glucose-2-*h* or glucose-2-*d* as reactant.[16] This excludes proton abstraction as the rate-determining step. Absence of either a solvent or substrate isotope effect excludes as a rate-limiting step both proton-transfer steps of the general acid–base-catalyzed Amadori rearrangement[16,17] because proton abstraction by phosphate buffer would show a substrate isotope effect, and proton donation by phosphate buffer would show a solvent isotope effect. The buffer acceleration of the rate is not a classical, proteolytic general acid–base catalysis. Thus, a kinetic event other than proton transfer must limit the rate. The most straightforward hypothesis is that a protein structural change before or during the Amadori rearrangement is the rate-limiting step for the phosphate-accelerated process.[16,17]

The rate of formation of HbA_{1C} was increased by increasing the concentration of 2,3-diphosphoglycerate[19,20] (DPG). For the DPG-dependent term, the absence of a solvent isotope effect and the flat proton inventory exclude proton donation from DPG as the rate-limiting step. The substrate isotope effect of ~2 for the DPG kinetic term indicates that the proton abstraction

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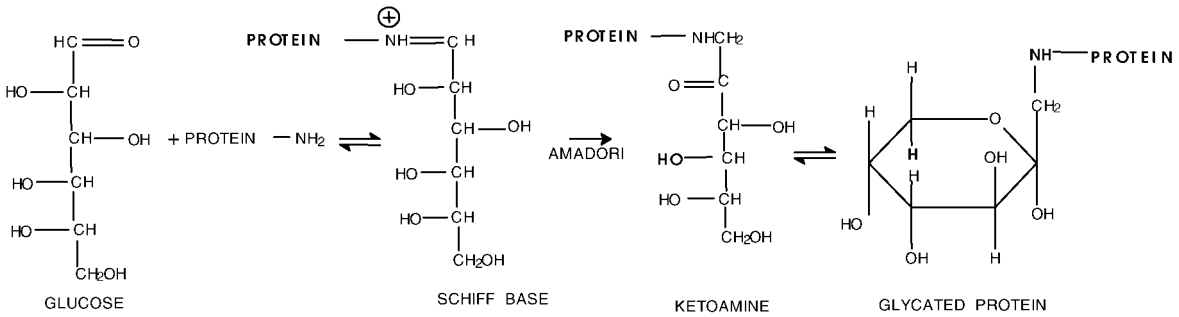


Scheme 1. Mechanistic scheme for the non-enzymic glycation of proteins

step of the Amadori rearrangement by DPG is wholly or partially rate-limiting for this reaction.[20] This finding may suggest that the reactivity of the N-terminal valine of the β-chain is influenced by the conformation of the proteins and/or by local shifts in the charge.

Inorganic and organic phosphates may play an important role in determining the kinetics and specificity of glycation of hemoglobin in the red cell. In order to have a much clearer understanding of the chemical mechanism for the formation of HbA_{1C}, we performed a kinetic analysis of 3-phosphoglycerate, 2-phosphoglycerate, 2-glycerolphosphate and sodium propionate in order to establish which groups (phosphate and/or carboxylate) on the 2,3-diphosphoglycerate catalyze the non-enzymic glycation of hemoglobin.

EXPERIMENTAL

Materials and methods

Human hemoglobin, 3-phosphoglycerate, 2-phosphoglycerate, 2-glycerolphosphate, sodium propionate and Tris buffer were obtained from Sigma Chemical and deuterium oxide from Aldrich Chemical. Acrodisc filters (0.2 μm) were purchased from Gelman Sciences. Tris buffer solutions at pH 7.3 were prepared by mixing appropriate volumes of 10 mM trizma base and 10 mM trizma hydrochloride, 0.15 M NaCl. Buffers in D_2O were prepared similarly. Hemoglobin solutions were prepared by dissolving a weighed sample of hemoglobin in known volumes of 10 mM Tris buffer, pH 7.3, 0.15 M NaCl, 40 mM glucose. The reaction mixtures were prepared by mixing known volumes of hemoglobin with different concentrations of 3-phosphoglycerate, 2-phosphoglycerate, 2-glycerolphosphate and sodium propionate in 10 mM Tris buffer, 0.15 M NaCl, pH 7.3, 40 mM glucose at 0 °C. The final pH was 7.3 for all solutions. The reaction was started and maintained at 37 °C. All solutions were sterilized by ultrafiltration thorough a 0.2 μm Acrodisc filter 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.[1,3] Glycohemoglobin HbA_{1C} was eluted from a cation-exchange column and measured spectrophotometrically at 415 nm (Sigma Kit[1,3] No. 440). A hemoglobin concentration of 9.2×10^{-2} mM was employed with 40 mM glucose. The reaction media were 0.15 M sodium chloride, 10 mM Tris buffer (pH 7.3) at 37 °C and different concentrations (1.6–8 mM) of 3-phosphoglycerate, 2-phosphoglycerate, 2-glycerolphosphate and sodium propionate (0.01–1 mM). The reactions in deuterium oxide were conducted at the corresponding pD[21] of 7.8. The kinetics exhibited both a catalyst-independent term (reflecting reaction assisted by water, lyons or protein functional groups) and a first-order term in catalyst ($k_{obs} = k_0 + k_B$[catalyst]).

Proton inventories were carried out at fixed concentration of 40 mM glucose, 10 mM Tris buffer, 3.2 mM 3-phosphoglycerate, 8 mM 2-phosphoglycerate and 8 mM sodium propionate in different mixtures of H_2O and D_2O at 37 °C.

RESULTS AND DISCUSSION

Figure 1 shows the first-order rate constants for the glycation of hemoglobin as a function of 3-phosphoglycerate and 2-glycerolphosphate concentrations. The increase in the rate constants with increase in 3-phosphoglycerate and 2-phosphoglycerate concentration is indicative of catalysis by 3-phosphoglycerate and 2-glycerolphosphate. The solid lines were generated from the best-fit parameter obtained by linear regression. The kinetics exhibited both a 3-phosphoglycerate- and 2-phosphoglycerate-independent term, reflecting that the reaction assisted by water, lyons (hydroxide or hydronium ions) or protein functional groups and a first-order term in 3-phosphoglycerate and 2-phosphoglycerate ($k_{obs} = k_0 + k_B$[catalyst]). The second-order rate constants (k_B) calculated from the slopes and the first-order rate constant (k_0) for the spontaneous reaction from the intercepts are given in Table 1.

Glycation of hemoglobin is catalyzed by 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate (Fig. 1, Table 1). In fact, general acid–base catalysis by 3-

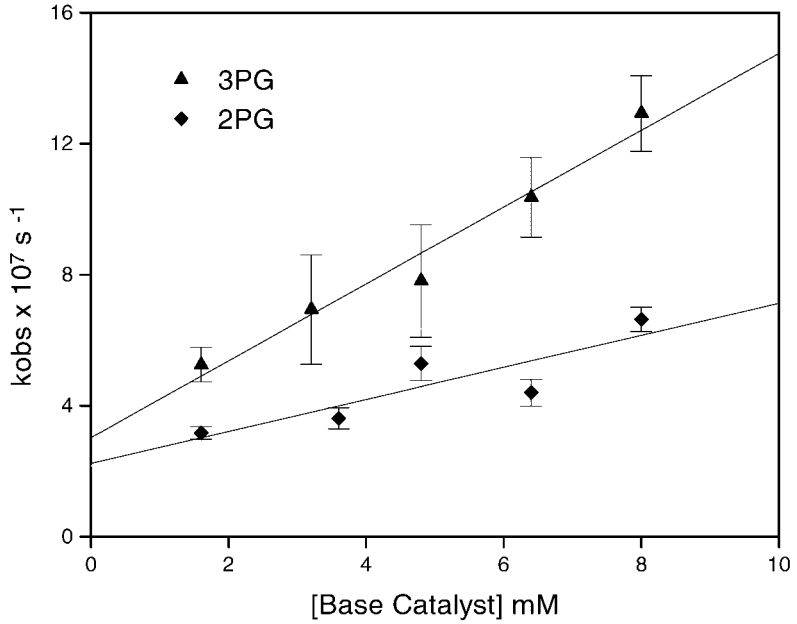


Figure 1. Plot of the first-order rate constants for non-enzymatic glycation of hemoglobin with 40 mM glucose as a function of (▲) 3-phosphoglycerate and (◆) 2-phosphoglycerate concentration in 10 mM Tris buffer, pH 7.3, 0.15 M NaCl at 37 °C

phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate is expected for the proton-abstraction and proton-donation steps of the Amadori rearrangement (Scheme 2). However, we find that the reaction occurs with identical rate constants in protium oxide and deuterium 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. The solvent isotope effect expected for the proton donation is in the range 2–8.[18] Catalysis by 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate, the observed solvent isotope effect of 1 (Table 1) and the flat proton inventory (Fig. 2 and Table 1) tend to suggest that the proton abstraction by 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate is the rate-determining step in the glycation of hemoglobin.

Figure 3 shows the first-order rate constants for the glycation of hemoglobin as a function of sodium propionate concentration. The non-enzymic glycation of hemoglobin is not catalyzed by sodium propionate. The kinetics exhibited only one term, the propionate-independent term (k_0), and the observed solvent isotope effect of 1 and the flat proton inventory (Fig. 4) tend to suggest that proton abstraction step of the Amadori rearrangement at least partially determines the rate. The proton-abstracting base could in principle be water, hydroxide ion or a basic functional group of the protein.

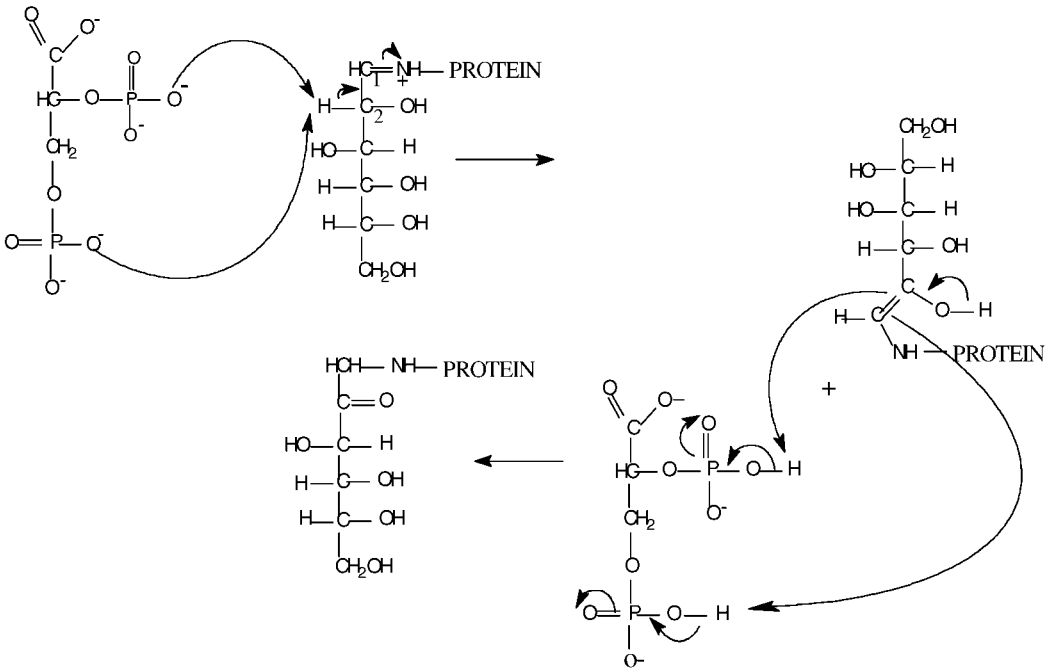
The solvent isotope effect can be predicted for each case.[22,23] Taking water as a base, the transition state L_2O^+—H···S (L = H, D) will give $k_{H_2O}/k_{D_2O} = 2.1$ as the solvent isotope effect (late transition state), while the transition state L_2O···H—S will give 1.0 (early transition state). For hydroxide ion as a base, the transition state LO—H···S will give 0.5 (late transition state) while LO^-···H—S will give 1.0 (early transition state). A protein functional group should give $k_{H_2O}/k_{D_2O} = 1.0$, since the use of the corresponding pD will cause the fractional ionization to be the same in two solvents.[18] The solvent isotope effect is $k_{H_2O}/k_{D_2O} = 0.96 \pm 0.15$. Although this value is not decisive, it is consistent with any base if the transition state is early. The data are most readily reconciled with a protein functional group acting as the proton-abstracting base.

Site specificity is governed mainly by factors that

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

Base catalyst	$k_0 \times 10^7$ (s^{-1})	$k_B \times 10^8$ (mM^{-1} s^{-1})	k_{H_2O}/k_{D_2O}	Proton inventory
3-Phosphoglycerate	2.94 ± 0.23	11.83 ± 0.43	0.96 ± 0.23	Flat
2-Phosphoglycerate	2.25 ± 0.28	4.92 ± 0.72	0.99 ± 0.11	Flat
2-Glycerolphosphate	2.99 ± 0.63	2.63 ± 0.43	1.01 ± 0.10	
Sodium propionate	2.85 ± 0.14	No catalysis	0.96 ± 0.15	Flat

[a] 10 mM Tris buffer, pH 7.3, pD 7.8, 0.15 M NaCl, 40 mM glucose at 37 °C.



Scheme 2

affect the second stage of glycation, the Amadori rearrangement. In fact, in this rate-controlling step, a base catalyst is required to remove proton 2 of the attached glucose of the aldimine (Scheme 2). Both stages of glycation include tautomerizations which are subject to acid or base catalysis. This implies that, for the glycation to occur at a significant rate, the carbohydrate residue of the Schiff base must be close to a base catalyst. The non-enzymatic glycation of hemoglobin is catalyzed by DPG,[20] 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate. The role of all these organic phosphates is the abstraction of the proton at the Amadori

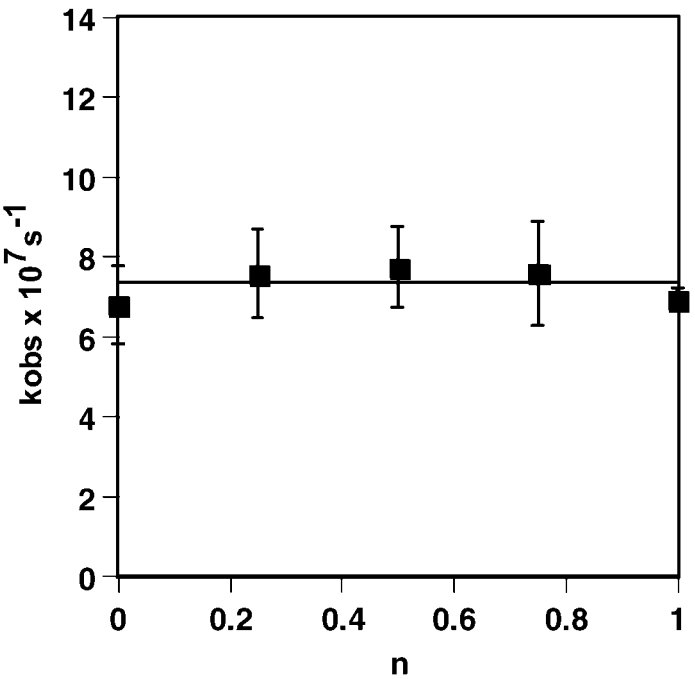


Figure 2. Overall isotope effect as a function of atom fraction *n* of deuterium in binary mixtures of protium and deuterium oxides for glycation of hemoglobin at 37 °C in 3.2 mM 3-phosphoglycerate, 40 mM glucose

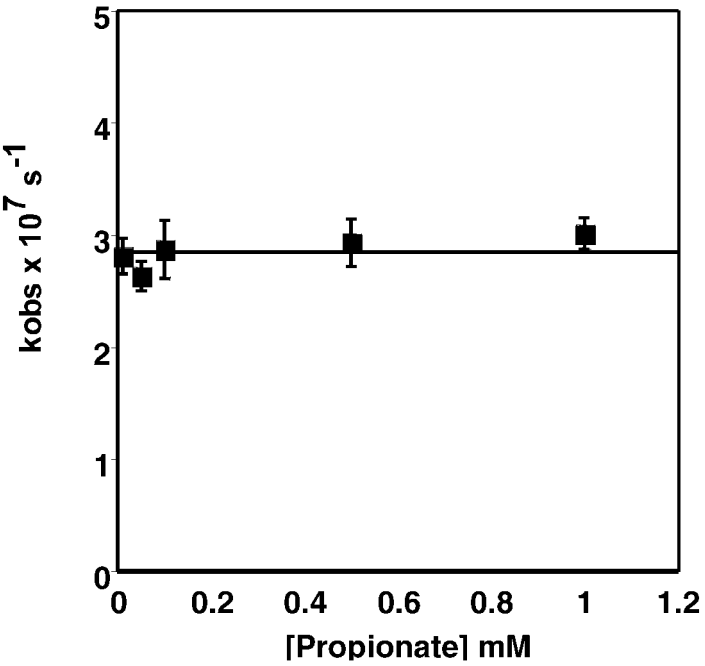


Figure 3. Plot of the first-order rate constants for non-enzymatic glycation of hemoglobin with 40 mM glucose as a function of sodium propionate in 10 mM Tris buffer, pH 7.3, 0.15 M NaCl at 37 °C

rearrangement. These facts indicate that both, 2- and 3-phosphates on DPG catalyze the reaction. The phosphates at positions 2 and 3 on the DPG act as a base catalyst of the Amadori rearrangement (Scheme 2). 3-Phosphoglycerate is a better catalyst that 2-phosphoglycerate (Fig. 1). 2-Phosphoglycerate is a better catalyst that 2-glycerolphosphate, probably owing to the carboxyl group (Table 1). The lack of catalysis by sodium propionate indicates that the carboxylate group on the DPG does not catalyze the glycation of hemoglobin.

This study, together with our previous[16,17,20] studies on *in vitro* glycation of hemoglobin, provides interesting

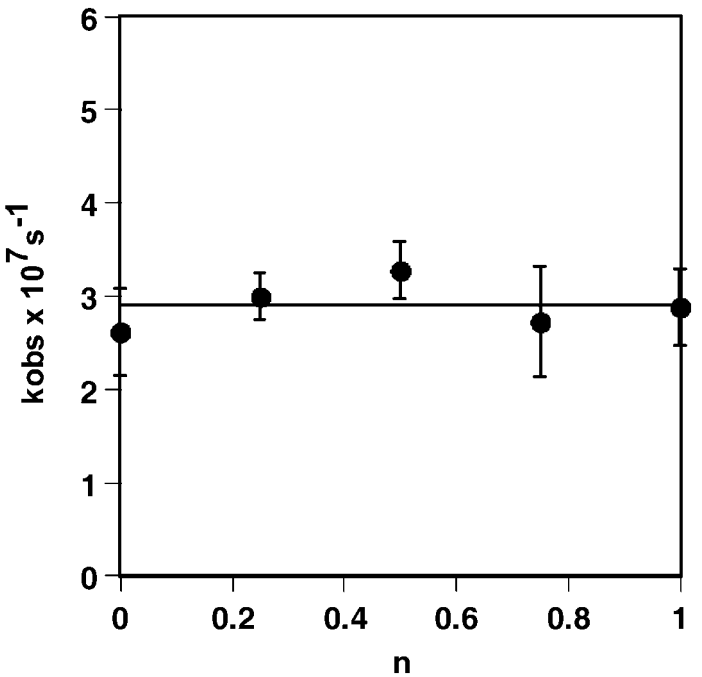


Figure 4. Overall isotope effect as a function of atom fraction *n* of deuterium in binary mixtures of protium and deuterium oxides for glycation of hemoglobin at 37 °C in 8 mM sodium propionate, 40 mM glucose

features of the effect that different compounds such as inorganic phosphate, 2,3-diphosphoglycerate, 3-phosphoglycerate, 2-phosphoglycerate, 2-glycerolphosphate and sodium propionate have on enhancing the extent of glycation on hemoglobin. This catalysis, or similar effects by other species present in the biological environment, may lead to a doubling of the rate of protein damage at the aggregate concentration of several millimolar expected for such species.

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