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Effect of phosphate buffer on the kinetics of glycation of proteins†

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ABSTRACT: The glycation of γ-globulin is catalyzed by phosphate buffer, whereas that of human serum albumin (HSA) and ovalbumin is not. The observed rate constant of spontaneous glycation of HSA is twofold larger than ovalbumin and γ-globulin. When D-glucose-2-*h* is compared with D-glucose-2-*d*, the overall kinetic isotope effect for the buffer-independent rates is ${}^{H}k_0/{}^{D}k_0 = 4.43 \pm 0.06$ for HSA. The substrate isotope effect for the buffer-independent term excludes proton abstraction as the rate-determining step in the Amadori rearrangement by phosphate buffer. Catalysis by phosphate buffer of the glycation of γ-globulin indicates that phosphate is the abstracting base in the Amadori rearrangement. These results suggest that the phosphate buffer plays a fundamental role in the glycation of γ-globulin. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: glycation; proteins; catalysis; phosphate; γ-globulin; human serum albumin; ovalbumin

INTRODUCTION

A number of proteins undergo non-enzymatic alterations through the formation of covalent linkages with glucose. The chemical reactions involved are first the reversible formation of a Schiff base between the aldehydic functional group of glucose and the ε-amino groups of lysine residues in proteins, followed by a relatively slow, but essentially irreversible, Amadori rearrangement, with the formation of ketoamines, which cyclize to the hemiketal structure.[1–3] The reaction between glucose and free amino structures in proteins forms complex cyclic structures that are related to diabetes complications and aging. This process occurs in only a limited number of amino structures in the protein. Studies with a number of proteins indicate that glycation is not a random chemical modification of the amino groups in proteins. There is preferential glycation of hemoglobin[4] and albumin.[5]

The reaction between hemoglobin and glucose became the prototype for subsequent studies of non-enzymatic glycation. Several factors have been reported that can influence the rate of glycation of hemoglobin. The extent of glycation of hemoglobin is determined by the sum of the effects of a number of independently acting variables:[6] pH, temperature, glucose and protein concentration and incubation time.

Some progress has been made in identifying factors that affect the specificity of glycation of hemoglobin. The reaction is accelerated by phosphate,[7–9] carbonate and arsenate[10] buffers. In addition, other physiological factors have an influence on the rate of hemoglobin A_{1C} formation, including oxygen tension and 2,3-diphosphoglycerate, 3-phosphoglycerate and 2-phosphoglycerate levels.[11–15] Structural studies of glycated proteins also have emphasized that the reactivity of a particular amino group is highly dependent on its microenvironment within the protein.[16] The amino groups of a single protein react at different rates. Lys 525 of human serum albumin (HSA) reacts with glucose faster than all other amino groups[5] and Val 1 of the β-chain of hemoglobin reacts faster than Val 1 of the α-chain.[4]

There is relatively little information on the kinetics and factors that influence site specificity in the rate of glycation of other proteins. Few studies have quantitatively measured the complete kinetics of protein glycation owing to the slowness of the reaction with glucose. The differential reactivities of amino groups towards glycation are attributable to several factors which result in an enhanced rate of glycation. Mechanistic features are important to understand.

The purpose of this paper is to describe a procedure for determining the kinetic constants involved in the formation of glycated proteins and the effect of phosphate buffer for the glycation of HSA, ovalbumin and γ-globulin.

EXPERIMENTAL

HSA, ovalbumin, γ-globulin, glucose-2-*h*, glucose-2-*d* and a fructosamine kit (Sigma Kit 464) were obtained from Sigma Chemical. Sodium dihydrogenphosphate

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(NaH_2PO_4), disodium hydrogenphosphate ($Na_2HPO_4 \cdot 7H_2O$) and sodium chloride were purchased from Merck. Acrodisc filters (0.2 μm) were supplied by Gelman Sciences.

Glycation assay procedure

The extent of glycation was assayed by the fructosamine method. This method, devised by Johnson *et al.*,[17] is based on the ability of fructosamine to reduce nitroblue tetrazolium (NBT) in alkaline solutions. In the NBT assay, a protein sample is added to carbonate buffer (pH 10.8) at 37 °C containing NBT, which is subsequently reduced; the absorbance at 530 nm is measured after 10 and 15 min. (Sigma Kit 465). The fructosamine concentrations are then determined by comparing this absorbance change with that of a standard solution of glycated HSA. A modification of this routine procedure was made in this work. The procedure adopted for measuring fructosamine concentration was as follows: 0.1 ml of protein was added to 0.5 ml of carbonate buffer (0.1 M, pH 10.4 containing 0.25 mM NBT at 37 °C. The formation of the NBT reduction product was followed to up to 15 min; the absorbance at 530 nm was measured at 5 min intervals. Figure 1 shows the time course of the NBT reduction product as a typical experiment to illustrate the procedure. The solid lines were generated for a non-linear least-squares fitting procedure to the first-order equation. The derivative of the curve was calculated at 10 min for each protein and phosphate concentration, instead of the absorbance change between 10 and 15 min. A calibration curve was prepared, relating $(dA_{530}/dt)_{10\,min}$ with glycated HSA concentrations (Fig. 2).

Kinetics of glycation of proteins

In vitro glycation was performed by incubation of 0.3 mM HSA, ovalbumin or γ-globulin with 30 mM glucose in different concentrations of phosphate buffer (pH 7.3, 0.15 M NaCl), with 10 drops of toluene as microbial inhibitor agent. The final pH was 7.3 for all solutions. The reaction mixture (1 ml) was placed in sterile tubes. The tubes were removed at specific time intervals and glycated proteins were determined by fructosamine assay as described above. The reaction was carried out at 37 °C. All solutions were degassed and sterilized by ultra filtration through a 0.2 μm filter (Gelman) into sterile tubes. The first-order rate constants were calculated as the best-fit parameters obtained from a non-linear least squares fitting to the first-order equation

$$[F]_t = [F]_\infty + ([F]_0 - [F]_\infty)e^{-kt}$$

where $[F]_t$ = fructosamine concentration at time t, $[F]_\infty$ = fructosamine concentration at infinite time, $[F]_0$ = fructosamine concentration at zero time and k = first-order rate constant.

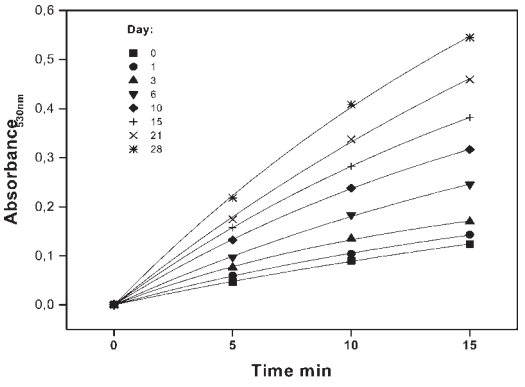

Figure 1. Plot of formation of the NBT reduction product as a function of time at 37 °C for the non-enzymatic glycation of γ-globulin in 50 mM phosphate buffer (pH 7.3), followed for 28 days

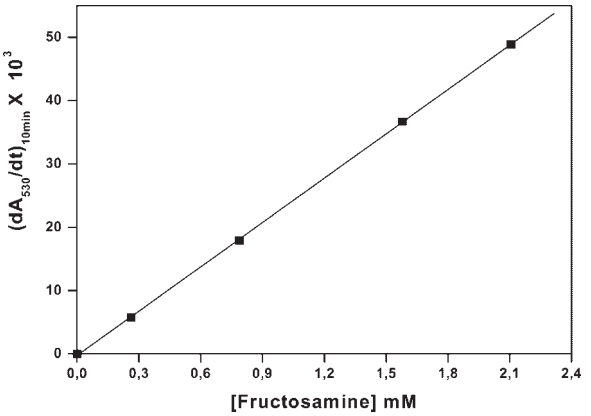

Figure 2. Calibration curve prepared from glycated human serum albumin

RESULTS AND DISCUSSION

Figure 3 shows the time course of glycation of human serum albumin as a function of phosphate buffer concentration (pH 7.3, 0.15 M NaCl). The formation of glycated HSA increases with time, but not with phosphate buffer concentration at fixed concentrations of 30 mM glucose and 0.3 mM HSA at 37 °C. Table 1 shows the observed first-order rate constants for the glycation of ovalbumin and HSA as a function of phosphate buffer concentration. The rates for HSA and ovalbumin are independent of phosphate buffer concentration. The statistical invariance of the rates as a function of phosphate buffer is indicative that the glycation of HSA and ovalbumin is not catalyzed by phosphate buffer. This result excludes proton abstraction by phosphate buffer. The kinetics exhibited only one term, the phosphate-independent or spontaneous glycation term k_0. This result

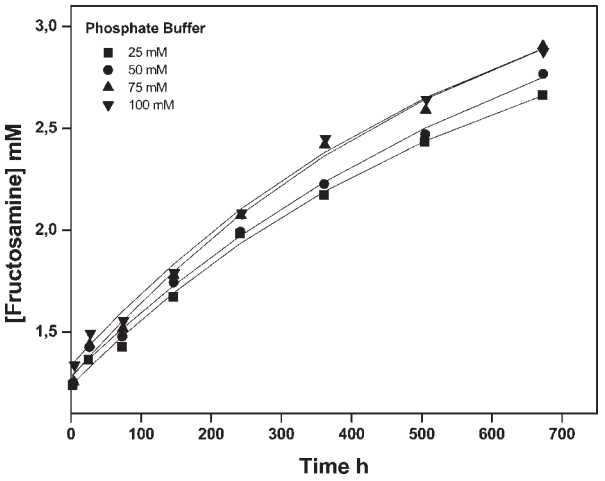

Figure 3. Formation of fructosamine as a function of time and phosphate buffer concentration (pH 7.3) at 37 °C for the glycation of human serum albumin

suggests that the proton abstraction step of the Amadori rearrangement may be rate determining. The proton-abstracting base could be hydroxide ion or a basic functional group of the protein. As noted in Table 1, the glycation of HSA is independent of phosphate buffer concentration with either glucose-2-*h* or glucose-2-*d* as reactant. The kinetics exhibited only a buffer-independent term with a large substrate isotope effect of 4.43 ± 0.06 (Table 2), showing that the proton abstraction does determine the rate. The abstracting base must not be subject to competition by external buffers such as phosphate. Studies of solvent isotope effects and substrate isotope effects are in progress in order to establish what the abstracting base in the Amadori rearrangement might be.

Figure 4 shows the time course of the glycation of γ-globulin as a function of phosphate buffer concentration (pH 7.3, 0.15 M NaCl). The formation of glycated γ-globulin

Table 1. Observed first-order rate constants for the non-enzymatic glycation of ovalbumin and human serum albumin as a function of phosphate buffer concentration[a]

Protein	[Buffer] (mM)	$10^7 k_{obs}$ (s^{-1})
$Ovalbumin_{Glucose\text{-}2\text{-}h}$	25	2.13 ± 0.11
	50	2.11 ± 0.08
	75	2.47 ± 0.05
	100	2.33 ± 0.10
	Average	2.26 ± 0.17
$HSA_{Glucose\text{-}2\text{-}h}$	25	4.58 ± 1.11
	50	4.00 ± 1.08
	75	4.75 ± 1.00
	100	4.56 ± 1.00
	Average	4.47 ± 0.33
$HSA_{Glucose\text{-}2\text{-}d}$	25	0.92 ± 0.06
	50	1.03 ± 0.04
	75	1.08 ± 0.05
	Average	1.01 ± 0.08

[a] At pH 7.30, 0.15 M NaCl with 30 mM glucose-2-*h* and glucose-2-*d* at 37 °C.

Table 2. Rate constants for the glycation of γ-globulin, ovalbumin and human serum albumin in phosphate buffer (pH 7.3) at 37 °C[a]

Protein	$k_0 \times 10^7 (s^{-1})$	$k_B \times 10^9$ ($mM^{-1} s^{-1}$)	${}^H k_0/{}^D k_0$
γ-Globulin	2.04 ± 0.19	2.04 ± 0.27	
Ovalbumin	2.26 ± 0.17	Not catalyzed	
HSA	4.47 ± 0.33	Not catalyzed	4.43 ± 0.06

[a] Phosphate buffer concentration: 25–100 mM.

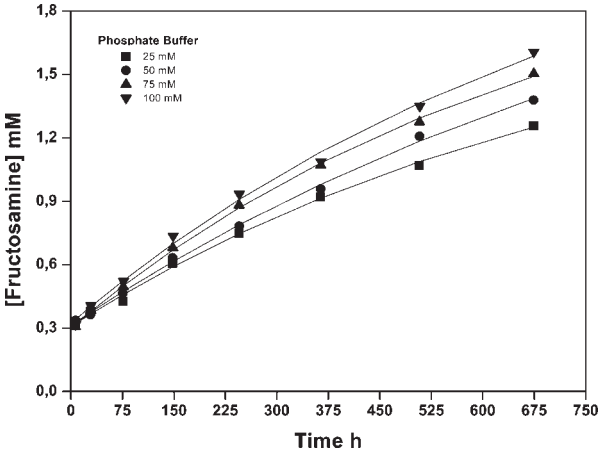

Figure 4. Formation of fructosamine as a function of time and phosphate buffer concentration (pH 7.3) at 37 °C for the glycation of -globulin

increases with time and phosphate buffer concentration at fixed concentrations of 30 mM glucose and 0.3 mM γ-globulin at 37 °C. Figure 5 shows the observed first-order rate constants for the glycation of γ-globulin as a function of phosphate buffer concentration. The increase in the apparent rate of glycation with phosphate concentration is indicative of catalysis by phosphate buffer. Site specificity is governed mainly by factors that 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

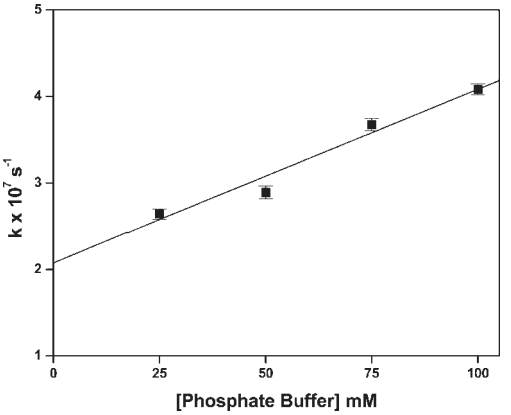

Figure 5. Plot of first-order rate constant as a function of phosphate buffer concentration (pH 7.3) at 37 °C for the glycation of γ-globulin

attached glucose of the aldimine. Catalysis by phosphate suggests, in the absence of other information, that the proton abstraction by phosphate is the rate-determining step in the non-enzymic glycation of γ-globulin. The kinetics exhibited two terms, one for the buffer-independent term (k_0) and one for the buffer-dependent term (k_B) (Table 2).

Table 2 shows that the rate of spontaneous glycation (k_0) of HSA is twofold larger than that of ovalbumin and γ-globulin. The main result of this study is the observation that the glycation of γ-globulin is catalyzed by phosphate buffer, but that of HSA and ovalbumin is not. Studies so far have dealt only with specific peptides in proteins and at one phosphate buffer concentration.

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