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The initial noncovalent binding of glucose to human hemoglobin in nonenzymatic glycation

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Mechanisms for nonenzymatic protein glycation have been extensively studied albeit with an emphasis at the later stages that gives rise to advanced glycation end products. No detailed investigation of the initial, noncovalent binding of D-glucose to human hemoglobin A (HbA) exists in the literature. Although anionic molecules 2,3-bisphosphoglycerate (BPG), inorganic phosphate (Pi) and HCO_3^- have been implicated in the latter stages of glycation, their involvement at the initial binding of glucose to HbA has not yet been assessed. Results from this computational study involving crystal structures of HbA predict that the transient, ring-opened glucose isomer, assumed to be critical in the later stages of glycation, is not directly involved in initial binding to the β-chain of HbA. All the five structures of glucose generated upon mutorotation will undergo reversible, competitive and slow binding at multiple amino acid residues. The ring-opened structure is most likely generated from previously bound pyranoses that undergo mutarotation while bound. BPG, Pi and HCO_3^- also reversibly bind to HbA with similar energies as glucose isomers (∼3–5 kcal/mol) and share common binding sites with glucose isomers. However, there was modest amino acid residue selectivity for binding of certain anionic molecules (1–3 regions) but limited selectivity for glucose structures (≥7 regions). The clinical difference between average blood glucose and predicted HbA_{1c}, and the presence of unstable HbA-glucose complexes may be more fully explained by initial noncovalent binding interactions and different concentrations of BPG, Pi and HCO_3^- in serum vs. erythrocytes.

Keywords: bicarbonate / binding selectivity and specificity / glucose isomers / glycated hemoglobin / phosphate

Introduction

Nonenzymatic glycation describes a common post-translational process by which D-glucose interacts slowly with intracellular and extracellular proteins, resulting in glucose (in some form) being covalently bound to the protein (Watkins et al. 1985). Glycated human hemoglobin (HbA_{1C}) is the first example of an in vivo glycated protein (Bunn et al. 1975). HbA_{1C} is proportionately increased with persistent hyperglycemia and the measurement of HbA_{1C} has been a cornerstone in the monitoring and management of diabetes mellitus (Koenig et al. 1976).

Key determinants of the rate and extent of nonenzymatic glycation of proteins include the prevailing glucose concentration and protein life span. Normal levels of "stable" HbA_{1C} are ∼5–6% of total HbA and this value can increase to 15% or more in diabetic individuals (Trivelli et al. 1971). However, the presence of "labile" HbA_{1C}—which is formed during the early, reversible stages of the glycation process and reflects ambient vs. longer term glucose levels—may overestimate HbA_{1C} by up 2–3% in normal subjects and by 10% in subjects with diabetes (Nathan 1981). It is also noteworthy that the clinical measurement of HbA_{1C} is specific to the glycation of N-terminal valine of β-chains of the tetramer, although numerous lysine residues on the β-chains (Lys17, Lys59, Lys61, Lys65, Lys66, Lys82, Lys95, Lys120, Lys132 and Lys144) can also serve as glycation sites (Delpierre et al. 2004). As a result, the measurement of HbA_{1C} is likely not a comprehensive indicator of HbA glycation. In addition, it has been proposed that the presence of deglycating enzyme fructosamine 3-kinase [E.C. (Enzyme Commission) 2.7.1.171] within erythrocytes explains genetically determined differential glycation and the heritability of HbA_{1C} (Cohen et al. 2006). However, the finding that the activity of fructosamine 3-kinase is not related to HbA_{1C}, or the level of total glycated hemoglobin (Delpierre et al. 2006), reinforces the idea that early events in the glycation are important.

Despite an overall appreciation of the reaction between glucose and α- and ε-amino groups on proteins, a mechanistic understanding of the complete process whereby glucose is covalently bound to HbA is not fully developed. Extending from previous investigations and their suggestions (Holmquist and Schroeder 1966; Bunn et al. 1975; Koenig et al. 1977; Makita et al. 1992), our conception of the HbA glycation process is best described by the following sequential stages (Figure 1): (i) reversible, noncovalent binding of glucose to select sites on the protein, (ii) reaction between the bound electrophilic glucose and nucleophilic amine residues (typically the N-terminal (α-amino) valine and internal (ε-amino) lysines to generate a

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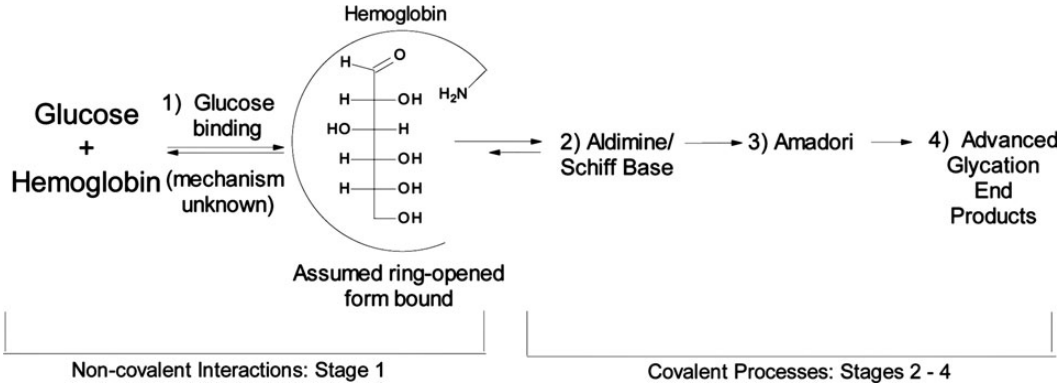

Fig. 1. Four-stage description of the nonenzymatic glycation of HbA. The equal length of the reaction arrows for the glucose-binding stage reflects a highly reversible interaction (Stage 1), whereas the reactions in Stages 2–4 are progressively less reversible.

covalently bound Schiff base or aldimine, (iii) rearrangement of the aldimine/Schiff base to a Amadori intermediate (amino-1-deoxyfructose) and (iv) the nonreversible formation of advanced glycation end products (AGE). The result of the process, the presence of various AGE, is thought to be linked to the chronic complications of diabetes mellitus and the normal aging process (Brownlee 1995).

A clinical prediction has been developed whereby average plasma glucose concentration is used to predict HbA_{1C} levels. This is, however, not an accurate predictor in many cases. To date, discordance between measured and predicted HbA_{1c} has been attributed to interindividual differences in the erythrocyte glucose concentration gradient (Khera et al. 2008) and erythrocyte survival time in the circulation (Cohen et al. 2008). However, there is increasing evidence that variation in HbA_{1C} is influenced by factors other than glucose concentration (reviewed in Hempe et al. 2012). The term "glycation (or glycosylation) gap" was developed to assess the difference between HbA_{1c} level and clinical measures of average plasma glucose in serum (e.g., fructosamine) (Cohen et al. 2003).

Most of the research dedicated to understanding the mechanism of HbA glycation has focused upon Stage 2 (McPherson et al. 1988), the transition from Stage 2 to Stage 3 (Furth 1988; Davis et al. 1989) or Stage 3 (Bunn and Higgins 1981; Nacharaju and Acharya 1992). Each of these investigations correlated overall glycation rate with some mechanistic factor and focuses on species or events after the initial binding. However, all species in Stages 2–4 that have been implicated as key factors associated with overall glycation rates must, by definition, have been provided to the protein (either directly or indirectly) through initial binding events. To the best of our knowledge, the effect that the initial binding interaction between glucose and HbA has on glycation has not yet been investigated.

A variable not fully addressed in previous investigations of HbA glycation is the presence of multiple structures (isomers and anomers) of glucose that are theoretically available to bind to the protein (Figure 1, Stage 1). Specifically, D-glucose undergoes reversible mutarotation in aqueous media whereby five different isomers interconvert, a pair of pyranose anomers as well as a pair of furanose anomers and a ring-opened glucose intermediate (Figure 2). Four of the structures are ring-closed: two five-membered furanose rings (α and β) and two six-membered pyranose rings (α and β). The central structure through which these isomers interconvert is a transient, ring-opened isomer that contains a free aldehyde group. The corresponding equilibrium distribution of the five glucose structures is: 35% α-pyranose, 64% β-pyranose, <1% for the sum of α- + β-furanoses (Baynes et al. 1989) and just 0.002–0.004% for the ring-opened isomer (Szwergold 2007).

Given the dynamic array of potential glucose structures available in aqueous solution, a fundamental and yet unanswered question is which of these structures are involved in initial binding event (Stage 1) with HbA within erythrocytes. It should be noted that in seminal papers pertaining to the glycation of HbA (Shapiro et al. 1980; Bunn 1981), the prevailing assumption is that a bound, ring-opened, aldehydic glucose is the singular reactive species that leads to aldimine/Schiff base (Stage 2). Whether mutarotation generating the ring-opened structure (that is reacted in Stage 2) takes place prior to initial glucose binding or after initial binding is not known.

Not only are there five structures of glucose available for the initial binding interactions with HbA; there are multiple forms of the targeted protein. HbA is a tetramer, possessing four heme groups, each of which can be oxygenated or deoxygenated with different secondary structures, giving rise to many interconverting HbA structures. Different HbA structures possess different stereo-chemical features and affinity for glucose (Lowrey et al. 1985; Yoshida and Nakashima 1990). The question of how various forms of HbA compare with one another in initial noncovalent binding with each of the five glucose structures (Stage 1) remains unanswered. A final mechanistic issue not previously investigated is the role that HbA-bound, anionic molecules (such as 2,3-bisphosphoglycerate (BPG), inorganic phosphate (Pi), or HCO_3^-) play in the initial binding of the glucose structures. When factoring in varying degrees of HbA oxygenation with the binding of anionic binding molecules, many HbA structures will exist in a dynamic equilibrium interacting with five glucose structures also in a dynamic equilibrium.

The focus of the current investigation is to assess how different HbA structures compare with one another in initial noncovalent binding (Stage 1) with each of the five glucose

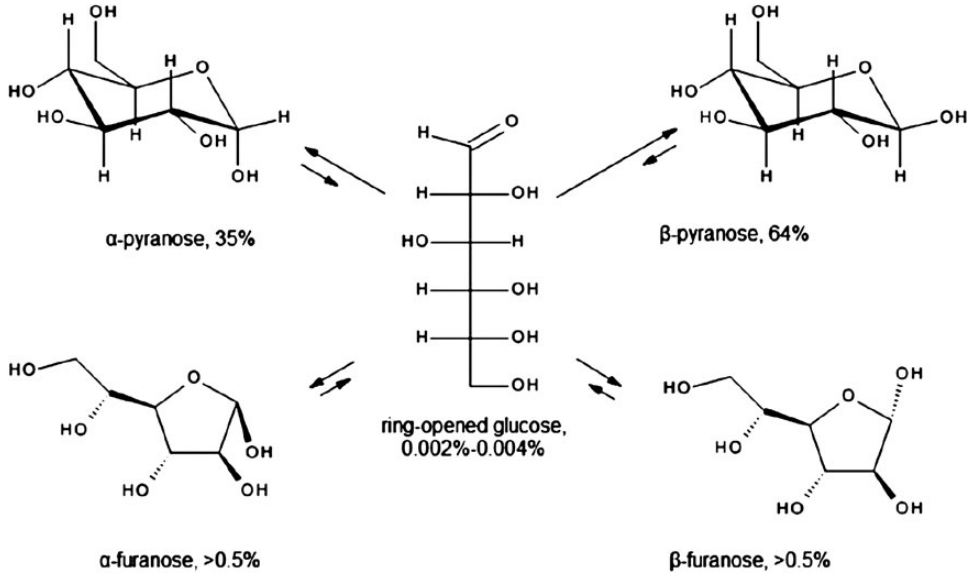

Fig. 2. Percent distribution of aqueous D-glucose structures at equilibrium when entering from the β-pyranose anomer (Baynes et al. 1989; Szwergold 2007). The reaction arrows reflect the extent and reversibility of interconversions between the ring-closed anomers and the ring-opened glucose.

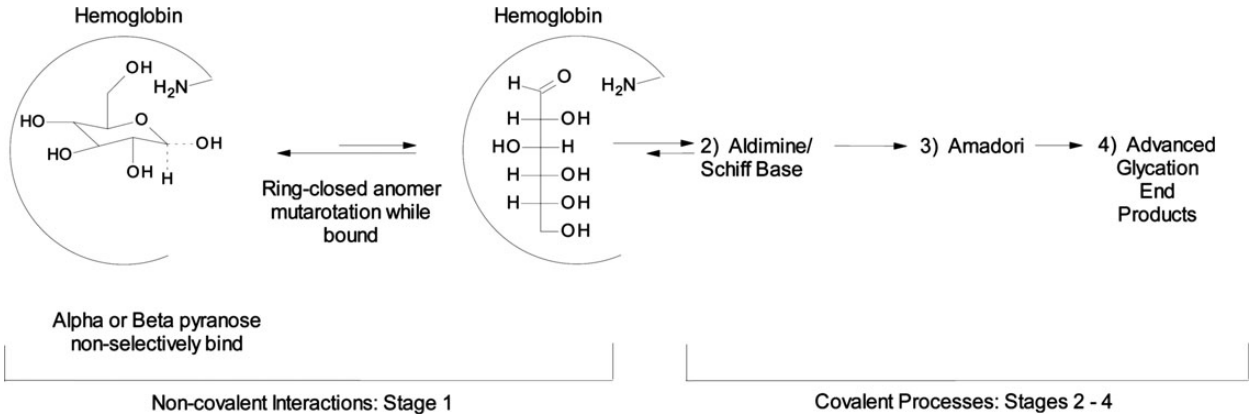

Fig. 3. Potential process for presenting ring-opened glucose to hemoglobin (Stage 1) and subsequent steps leading to glycation and production of AGE. Unlike previous models, this process involves nonselective binding of ring-closed anomers and mutarotation while bound. The reaction arrows reflect the reversibility of each interaction/reaction.

structures; both in the presence and in the absence of BPG, Pi and HCO_3^-. The goal is to determine at which stage in the four-stage glycation process (Figure 1) the ring-opened glucose is made available for reaction with HbA. Specifically, since glucose mutarotates in solution prior to binding, will the ring-opened structure selectively bind to HbA in the presence of the four ring-closed anomers of glucose? If selective binding of the ring-open structure does not occur and multiple forms of glucose bind HbA, can the ring-closed structures that do bind potentially mutarotate while bound and thus present the ring-opened form for further reaction (in follow-up stages, see Figure 3)? Further, the implications that Stage 1 noncovalent HbA binding interactions with glucose isomers in the presence of anionic species (BPG, Pi and HCO_3^-) have for the glycation gap are considered.

Results

Glucose docking computations with HbA: Amino acid residue selectivity

All of the results (unless otherwise stated) reflect binding computations involving an isolated β-chain of either fully oxygenated or fully deoxygenated HbA. Selectivity is an attribute of a glucose isomer. High selectivity is defined as binding to a single amino acid residue region of the protein, whereas low selectivity is binding at multiple amino acid residue regions. Binding of each of the five glucose structures to a β-chain of fully oxygenated HbA (in the absence of binding molecules) generates 20 combinations of glucose isomer to amino acid residue regions with binding exothermicities within 2 kcal/mol of each other (Table I). Of these combinations, 13 distinct regions bind a glucose isomer. In the binding of the same five

Table I. Summary of Autodock potentially productive binding interaction between glucose structures with the β-chain of oxygenated- and deoxygenated-human HbA organized by targeted amino acid residue

AA residues	Glucose isomers				
	α-pyranose	β-pyranose	Ring- opened	α-furanose	β-furanose
Val1	o, d	o, d	o, d	o	o
Lys8				d	o, d
Lys17	o		o	d	o, d
Lys59	o	o	o, d	o	d
Lys61		d	d	d	
Lys65					d
Lys66			d	o, d	o, d
Lys82	o, d	o, d	o, d	o, d	o, d
Lys117					d
Lys120					o

(i) The first column labeled "AA Residues" shows those amino acid residues that can theoretically be glycated as defined as having the nucleophilic nitrogen of that residue within 6 Å of the bound sugar. This is predicated upon the assumption that a 3 Å variation within the binding environment is possible and is consistent with the geometric parameters set forth by Ito et al. (2011). (ii) Docking to oxygenated (o) and deoxygenated (d) HbA. (iii) Sites identified from the assessment of data generated in triplicate.

glucose structures to deoxygenated HbA, there are 23 combinations of glucose isomer to amino acid residue regions constituting 7 distinct regions within the protein that bind an isomer. In view of the many regions binding various glucose structures, binding to HbA in the absence of binding molecules is predicted to be energetically favorable but will proceed with low selectivity for particular amino acid residues.

In contrast to the binding of the glucose isomers, BPG binds to a β-chain of oxygenated HbA in an amino acid residue selective manner, involving the N-terminal region with primary interactions at Val1, Leu78 and Lys82. When BPG is bound to the same β-chain of deoxygenated HbA, the binding occurs with less selectivity in three regions with primary interactions at Lys59, Lys65 or Lys82 and Leu81. When BPG is bound to oxygenated HbA first, followed by the binding of each of the five glucose structures, amino acid residue selectivity is limited for the glucose structures. In composite, glucose isomers are predicted to bind in 21 different combinations (within 1 kcal/mol of each other) of glucose isomer/amino acid residue regions, in which eight distinct amino acid regions are represented. In the sequential binding of BPG and glucose to a β-chain of deoxygenated HbA, 16 glucose/amino acid residue region combinations are observed in which seven distinct amino acid residue regions bind an isomer. Thus, if BPG binds to a β-chain of HbA first, and then glucose binding occurs (whether the HbA is oxygenated or deoxygenated), there will be low amino acid residue binding selectivity for glucose structures.

Pi, in two different forms, was also bound to one β-chain of both oxygenated HbA and deoxygenated HbA. When monobasic phosphate was bound to deoxygenated HbA, there are only two distinct binding regions involved (within 1 kcal/mol of each other), either the amino acid residues Val1/Lys82 or Lys59/Lys65/Lys66. When monobasic phosphate is bound to oxygenated HbA, binding involves primary interactions in two different regions with either Val1//Leu3/Lys8 or Val1/Leu81/Lys82. When dibasic phosphate binds to deoxygenated HbA, there are three distinct regions where binding occurs (within 0.5 kcal/mol of each other), involving Lys82/His143, Lys65 or Leu81/Lys82. When dibasic phosphate is bound to oxygenated HbA, binding takes place in three distinct regions, involving Leu3/Lys8, Val1/Leu81/Lys82 or Lys120. Overall, there are five distinct HbA binding regions on a β-chain associated with Pi, indicating low binding selectivity for these molecules.

The sequential binding of Pi (monobasic or dibasic) to a β-chain of oxygenated or deoxygenated HbA followed by the binding of each of the five glucose structures leads to the conclusion that amino acid residue selectivity is low for glucose. When monobasic phosphate is bound to oxygenated HbA followed by the binding of each of the five glucose structures, 16 distinct amino acid residue regions will bind a glucose. Of these regions, only five regions contain the N-terminal valine or a lysine residue capable of glycation. When monobasic phosphate is bound to deoxygenated HbA first followed by each of the five glucose structures, 18 distinct amino acid residue regions will bind glucose. Of these regions, only four contain the N-terminal valine or a lysine capable of glycating a sugar. When dibasic phosphate is bound to oxygenated HbA followed by the binding of each of the five glucose structures, 11 distinct amino acid residue regions will bind glucose while only 3 are capable of glycation. Overall, the prediction is that there will be limited amino acid residue selectivity for glucose binding.

When HCO_3^- is bound to a β-chain of oxygenated HbA (without any glucose), HCO_3^- binds to three regions (within 0.1 kcal/mol of each other):Val1/Leu3/Lys82, Lys20 or Lys82/His143. HCO_3^- binds to deoxygenated HbA in a single region involving Lys82/His143. In the sequential binding experiment where HCO_3^- is allowed to bind to oxygenated HbA and the HCO_3^--bound hemoglobin is then exposed to each of the five glucose structures, the glucose isomers exhibit little amino acid residue selectivity. Specifically, glucose binds in 18 different regions (within 1.5 kcal/mol of each other). In analogous binding experiments involving deoxygenated HbA, 24 different regions are targeted (within 1.5 kcal/mol of each other). Thus, consistent with the other anionic binding molecules tested, the data for sequential binding of HCO_3^- and glucose to a single β-chain of oxygenated or deoxygenated HbA lead to the conclusion that low amino acid residue selectivity exists for glucose structures.

The overall assessment of amino acid residue selectively identified 11 different amino acid residues that can theoretically be glycated through 74 different glucose-binding molecules/HbA binding combinations. These computational results lead to the conclusion that there will be limited amino acid residue selectivity for initial glucose binding to the β-chain of oxygenated- or deoxygenated HbA under any conditions, whether in the presence or in the absence of BPG, Pi or HCO_3^-.

Glucose docking computations with HbA: Isomer specificity

Specificity here is an attribute of HbA. If HbA were to bind to a single glucose isomer (among the five available), specificity occurs. Low specificity is where HbA binds multiple glucose isomers. The ring-closed α- and β-pyranose anomers of glucose bind more exothermically (with a more negative ΔG) to a β-chain of fully oxygenated HbA than does the ring-opened glucose isomer (Table II), albeit the thermodynamic values are within ∼1 kcal/mol of each other. With deoxygenated HbA, the

binding is more exothermic for the ring-closed pyranoses by ∼0.5 kcal/mol than for the ring-opened glucose (Table III). The exothermicities for the binding of the furanose anomers are less than those for the pyranose and ring-opened structures (∼1.5 and 0.5 kcal/mol less exothermic than the pyranose and ring-opened structures, respectively) whether the binding involves oxygenated or deoxygenated HbA. Overall, the binding of the five glucose structures to a β-chain of either deoxygenated or oxygenated HbA is not specific to any one structure. Thus, the initial selective binding of the ring-opened glucose to HbA in the presence of the ring-closed structures (α- and β-pyranose and α- and β-furanose) is unlikely.

BPG binds to a single β-chain of oxygenated and deoxygenated HbA with ∼1.0–2.5 kcal/mol greater binding exothermicities than do any of the glucose structures (Tables II and III). In the sequential binding of BPG, with either HbA, followed by the binding of either pyranose anomer, the α- and β-pyranoses bind within 0.4 kcal/mol of each other and bind with ∼1 kcal/mol greater exothermicity than the ring-opened glucose (Tables II and III). No ring-opened glucose specificity is observed with the sequential binding computations in the presence of BPG.

Pi in two different forms was also bound to a β-chain of both oxygenated and deoxygenated HbA. Both monobasic and dibasic phosphate binds to either HbA form with less exothermicity than the α- and β-pyranoses and with an exothermicity comparable with that of the α- and β-furanoses. In the binding of the Pi's with either HbA followed by the binding of glucose, the α- and β-pyranoses bind within 0.2 kcal/mol of each other and bind ∼1.2 kcal/mol more exothermic than the ring-opened glucose, while furanose binding is less exothermic (Tables II and III). Thus, no binding specificity for the ring-opened structure is observed in the presence of Pi, as the α-pyranose, β-pyranose and the ring-opened structures all bind with comparable energies (within 1 kcal/mol or less).

Glucose binding to either HbA form in the presence of HCO_3^- also results in binding exothermicities (Tables II and III) that are so similar as to make specific binding of the ring-opened structure under these conditions untenable.

In summary, there is no computational support for specific binding of the ring-opened glucose in the presence of ring-closed anomers under any conditions analyzed.

Discussion

At molecular level, the observed rates of nonenzymatic glycation of HbA have been correlated with an array of factors, each involving interactions after the initial, noncovalent binding of the sugar. For example, Bunn and Higgins (1981), in assessing

Table II. Binding exothermicities (ΔG in kcal/mol) of top 10 conformations generated upon docking glucose isomers and binding molecules (as independent species and in sequential docking) to the β-chain of fully oxygenated HbA

First molecule	Second molecule (sequential binding)					
	None	α-pyranose	β-pyranose	Ring-opened	α-furanose	β-furanose
α-pyranose	−4.6					
β-pyranose	−4.8					
Ring-opened	−3.5					
α-furanose	−3.0					
β-furanose	−2.7					
BPG	−5.4	−4.3	−4.2	−3.3	−2.9	−2.9
$H_2PO_3^-$	−3.1	−3.5	−3.7	−2.4	−1.0	−0.8
HPO_3^{-2}	−3.4	−3.7	−3.7	−2.5	−0.7	−0.9
HCO_3^-	−3.2	−4.6	−4.8	−3.6	−2.9	−2.5

(i) BPG, 2,3-bisphosphoglycerate. (ii) All energetic values reported are from triplicate analysis with all reported values within±5% relative measurement. (iii) All energies reported in sequential docking reflects the exothermicity of the second species in its binding.

Table III. Binding exothermicities (ΔG in kcal/mol) of top 10 conformations generated upon docking glucose isomers and binding molecules (as independent species and in sequential docking) to the β-chain of deoxygenated HbA

First molecule	Second molecule (sequential binding)					
	None	α-pyranose	β-pyranose	Ring-opened	α-furanose	β-furanose
α-pyranose	−4.4					
β-pyranose	−4.5					
Ring-opened	−3.7					
α-furanose	−3.3					
β-furanose	−3.0					
BPG	−5.5	−4.7	−4.7	−3.7	−3.1	−3.3
$H_2PO_3^-$	−3.3	−3.7	−3.3	−2.1	−0.9	−1.0
HPO_3^{-2}	−3.4	−3.5	−3.4	−2.6	−1.5	−1.1
HCO_3^-	−3.3	−4.4	−4.5	−3.9	−2.7	−3.1

(i) BPG (2,3-bisphosphoglycerate). (ii) All energetic values reported are from triplicate analysis with all reported values within ±5% relative measurement. (iii) All energies reported in sequential docking reflects the exothermicity of the second species in its binding.

Amadori product formation (Figure 1, Stage 3), asserted that glycation rate tracks the quantity of open-chain aldehyde (carbonyl group) in the sugar. This seminal paper is the primary basis for the longstanding assumption that the singular reactive form of the sugar is the ring-opened structure. However, McPherson et al. (1988) suggested that glycation rate is most critically correlated with generation of an aldimine/Schiff base (Stage 2), which is itself very dependent upon protein structure. Individual proteins have distinct sites for catalyzing the formation of the aldimine/Schiff base, and the nucleophilicity of the amino acid residues in the vicinity of the bound sugar is asserted to be the rate-determining factor. In later work, Nacharaju and Acharya (1992) asserted that accommodating a suitable pocket within the protein to facilitate Amadori formation (our Stage 3 and what is referred to as the Amadori rearrangement potential of the protein) is as significant as is the catalysis of aldimine/Schiff base formation. Davis et al. (1989) also posit that the reactivity of the aldimine/Schiff base formed (Stages 2–3 transition) is the rate-determining factor in glycation. Furthermore, Furth (1988) proposed that the reactivity of the aldimine/Schiff base is determined by the extent to which the covalently bound aldimine/Schiff base resides in a ring-opened form vs. in a cyclic form. Building upon these previous studies, we addressed two unanswered mechanistic questions. First, does the ring-opened glucose generated upon mutarotation in the erythrocyte specifically bind to HbA in the presence of the four ring-closed anomers of glucose? Secondly, can the ring-closed anomers that do bind potentially mutarotate while bound and thus present the ring-opened form for further reaction (Figure 3)?

There are two requirements for a bound sugar to proceed past the noncovalent stage of the glycation process onto the covalent stages of the glycation process (Figure 1). Glucose must first noncovalently bind (Stage 1) and, while bound, present a suitable electrophilic anomeric carbon to a nucleophilic nitrogen of the protein for reaction to an aldimine/Schiff base to occur (Stage 2). If the ring-opened glucose in the erythrocyte binds specifically to HbA in the presence of the four ring-closed glucose structures, then the ring-opened glucose must bind better than do the ring-closed anomers. Our results focusing on a single β-chain do not reveal isomeric specificity, whether the ring-opened structure is noncovalently bound to oxygenated HbA or deoxygenated HbA in the presence or in the absence of anions BPG, Pi or HCO_3^-. In fact, the ring-opened structure, while exothermically binding in every case, binds with less exothermicity than do the ring-closed pyranose anomers (Table II), suggesting a binding preference for the ring-closed anomers. In addition, the ring-opened glucose is likely generated within the erythrocyte at an exceedingly low concentration (both absolute and relative to the ring-closed glucose anomers). In water, ring-opened glucose is generated via mutorotation at a relative concentration of just 0.002–0.004% of total structures (Szwergold 2007). Given that human erythrocytes possess a mutarotase (E.C. 5.1.3.3, aldose 1-epimerase, Sacks 1967), which catalyzes the interconversion of ring-closed glucose anomers, we cannot be sure of the relative concentrations of α- and β-pyranoses in the erythrocyte. That said, the activity of this enzyme should not increase the relative concentration of the ring-open glucose. It is likely that the glucose structures will interconvert in the cytosol of the erythrocyte at a lower rate and reach an equilibrium concentration of ring-opened glucose that is lower than is the case for mutarotation in pure water (Levy and Cook 1954). The lower rate and resulting lower concentration of ring-opened glucose in intracellular water is because the aqueous media is nonideal (much less than unity) due to the high concentration of macromolecules and elevated osmolarity (Cayley et al. 1991). Some researchers even posit that spontaneous intracellular mutarotation in the erythrocyte is so unfavorable as to mandate a requirement for an enzyme in vivo to support anomeric interconversions (Timson and Reece 2003). Thus, the likelihood that the ring-opened structure in the erythrocyte would be available to bind to HbA is exceeding (perhaps diminishingly) low. The conclusions drawn from the binding data, when coupled with the higher relative concentrations of the α- and β-pyranoses to the ring-opened glucose (at a molar ratio of ∼50,000: 1), makes specific binding of the ring-opened form untenable.

While it is clear that the ring-opened structure is not the singular glucose species that binds HbA within erythrocytes under our experimental conditions, it may well be the only species that, while bound, reacts and proceeds on to Stage 2 of the glycation process. That is, the ring-closed anomers might be unreactive while bound and revert back to the erythrocyte cytosol. If this is the case, the amino acid residue regions that bind to the ring-opened structure must be consistent with the amino acid residues that are known to glycate HbA. The ring-opened glucose is predicted to undergo binding with the proper proximity to react with Val1, Lys17, Lys59, Lys61, Lys66 and Lys82 (Table I). These are all known glycation sites (Bunn et al. 1975; Shapiro et al. 1980; Delpierre et al. 2004; Ito et al. 2011). Moreover, no false positives were observed (i.e., predictions of glycation at amino acids that experimentally not known to glycate) in the current study. These data are consistent with the premise that ring-opened glucose is generated upon mutarotation in the erythrocyte (albeit very low in concentration). While not undergoing isomer-specific initial binding, the ring-opened glucose generated upon mutarotation from the bound ring-closed isomers may be the singular source of reactive glucose in Stage 2 of the glycation process (if the ring-closed glucose isomers that bind preferentially are unreactive). If one asserts that initially bound pyranose and furanose anomers can noncovalently bind and then mutarotate while bound so as to generate the ring-opened structure (Figure 3), then the number and identity of amino acid residue regions that bind the ring-closed anomers must also be considered. It is also necessary that the region that binds the ring-closed anomers must also accommodate the ring-opened structure. The ring-closed anomers of glucose bind to the β-chain of HbA (when taken in composite over all conditions, specifically HbA in the presence of binding molecules) such that Val1, Lys17, Lys59, Lys61, Lys66 and Lys82 can be theoretically glycated. These amino acid residues are all known glycation sites and no false positives were observed. The α- and β-pyranoses generally bind in common regions, both relative to each other and relative to the ring-opened glucose (Table I). As a result, shared binding regions between the α- and β-pyranoses and the ring-opened structure can facilitate an appropriate environment for binding and mutarotation of α- and β-pyranose anomers (while bound)

to the ring-opened glucose. Further, the mutarotation of the α- and β-pyranose anomers to the ring-opened structure is thermodynamically more favorable for glucose structures while bound to HbA than is mutarotation of the structures in aqueous media (where mutarotation is known to take place). Specifically, when we determine the thermodynamic differences (ΔΔH) between the respective structures in solution utilizing ab initio computational methods at the density functional theory B3LYP-level with a 6-31G* basis set, the ring-opened glucose is 4.2 kcal/mol endothermic from the α-pyranose anomer and 6.9 kcal/mol endothermic from the β-pyranose anomer. From our binding computations, the bound ring-opened structure is 1.1 kcal and 1.3 kcal/mol endothermic from the bound α- and β-pyranose anomers, respectively. It is noteworthy that α- and β-furanoses also share some HbA binding regions on the β-chain with the ring-opened glucose. However, the furanose anomers do not have as much amino acid residue overlap with the ring-open structure as do the α- and β-pyranose anomers (Table I). That said, the mutarotation from the bound furanoses to the bound ring-opened glucose is exothermic. While this finding does not take into consideration kinetic effects, it does make mutarotation of the bound furanoses to generate bound ring-opened structure thermodynamically favorable.

In summary, the most plausible means of presenting a ring-opened glucose to the amino acid residues for HbA glycation (in Stages 2–4) is the initial binding of the α- and β-pyranoses (and, to a lesser extent, the α- and β-furanoses) followed by the mutorotation of these ring-closed sugars while bound (Figure 3). Multiple lines of evidence support this mechanistic assertion. First, mutarotation of glucose while bound within a protein is reasonable to posit because this is precisely what occurs in the enzymatic action of aldose 1-epimerase. This enzyme initially binds a β-pyranose ring of glucose that then mutarotates, while bound, to generate a transient ring-opened glucose (Hucho and Wallenfels 1971). The ring-opened glucose is within an active site that does not have either an N-terminal valine or a lysine and, as such, no nucleophilic nitrogen is available to react with the ring-opened glucose (Thodin et al. 2004). The ring-opened structure then ring closes to the α-pyranose anomer that is ultimately expelled as product. In the case of initial binding of the α- and β-pyranose anomers to a β-chain of HbA, α-and β-pyranose rings bind with about the same affinity (within 0.2 kcal/mol of each other) and, moreover, the N-terminal valine and/or internal lysine nucleophiles are available for capture of the transient ring-opened structure so as to generate Schiff base/aldimine. Secondly, it has been shown that two isomeric cyclic glycosylamine intermediates are involved in the conversion of the aldimine/Schiff base to the Amadori intermediate (Stage 2 of the HbA glycation process, Furth 1988). These two cyclic glycosylamine intermediates arise via the mutarotation of the bound acyclic aldimine/Schiff base within HbA. Finally, it is known that at Stage 3, the Amadori intermediate is in fact three species, a ring-opened glucose and two cyclic anomers that arise via mutarotation of the bound acyclic glucose (Gil et al. 2004). That both the bound aldimine/Schiff base and the bound Amadori intermediates undergo mutarotation in the same amino acid environment as the initially bound α-and β-pyranose/furanose rings (Stage 1) provides further support for initially bound cyclic glucose mutarotating on HbA once bound.

That the ring-opened structure of glucose is presented for further glycation stages via mutarotation from a previously bound, cyclic glucose anomer does not make the glucose mutarotation in the erythrocyte inconsequential. The small subset of ring-opened glucose that is generated in the erythrocyte that does bind to HbA may also contribute to the glycation process, albeit to a very minor extent based upon both concentration and thermodynamics.

There are multiple reasons why the initial, noncovalent binding of glucose to HbA should affect the overall rate of HbA glycation. First, unproductive binding events will slow down the glycation process. If a potentially productive binding event occurs only when the glucose structure resides within a suitable distance to react with a nucleophilic N-terminal valine or internal lysine, then a nonproductive binding event occurs. The data presented in Table I address only those interactions whereby binding is potentially productive. Over 15 different nonproductive amino acid residues on a β-chain of HbA can bind the various glucose structures. The percent of unproductive initial binding for the glucose across both forms of HbA and binding molecules are: 39% for α-pyranose, 45% for β-pyranose, 53% for the ring-opened structure and 74 and 71% for the α- and β-furanoses, respectively. Secondly, the binding events that are potentially productive (and those that are not) are almost certainly reversible. Interactions at HbA with binding exothermicities <5 kcal/mol are expected to be reversible. Thus, the reversibility of the initial binding of glucose has implications even for potentially productive events. In particular, the nucleophilic attack of a valine or lysine within HbA must occur within the brief lifetime of the bound ring-opened glucose before it either mutarotates while bound back to a ring-closed bound anomer or detaches from the protein entirely. Thirdly, a glucose structure competes with other glucose structures and with water for H-bond donating/H-bond accepting sites on HbA. Via our computations, water binds HbA with less exothermicity (∼2 kcal/mol) than do the glucose structures, but is known to exist in much higher concentration than glucose. Specifically, erythrocyte water is at 39.7 M concentration (Dill et al. 1937; Hald and Eisenman 1937) relative to hemoglobin at 5 mM (Drabkin 1945, assuming a molecular weight of 68,000) and the glucose structures at 4 mM (Sikaris 2009). Thus, a ratio of nearly 10,000 water molecules exist for every glucose molecule, and there is evidence for extensive water of hydration associated with hemoglobin aggregates (2.3–3.4 g H_2O/g dry mass) in human erythrocytes (Cameron et al. 1988). Thus, when factoring water competition with the low statistical likelihood of productive binding events, the rate of initial binding is very low.

A further mechanistic issue not previously investigated is the role that HbA-bound, anionic molecules play in the initial binding of glucose to HbA. It is well known that these anions do affect glycation rates. Specifically, (i) phosphate enhances the rate of glucose mutarotation in solution (Bailey et al. 1970); (ii) phosphorylated sugars undergo significantly higher rates of HbA glycation than does glucose (by up to several orders of magnitude, Stevens et al. 1977) and (iii) erythrocyte BPG levels are positively correlated with glycated HbA (Cauchie

et al. 1992). BPG is present in the erythrocyte at concentrations of ~6 mM (Goodman and Bessman 1975), is known to bind to HbA (Benesch et al. 1968) and can increase HbA_{1C} formation (Smith et al. 1982; Lowrey et al. 1985). Moreover, Gould et al. (1997) reported higher intra-erythrocyte concentrations of BPG in high glycators ($N = 7$, 5.61 ± 0.26 mM) vs. low glycators ($N = 5$, 4.81 ± 0.24 mM). For comparison, the concentration of glucose (pyranose rings) in the erythrocyte is ~4 mM in fasting, nondiabetic humans (Sikaris 2009), whereas the concentration of fructose (furanose rings) in plasma from fasting humans is just 31 µM (Aloia 1973). Inorganic Pi [H_3PO_4, and the related potassium and sodium salts of mono- and di-basic phosphate ($H_2PO_4^-$ and HPO_4^{-2}, respectively)] are all present in the erythrocyte in a pH-dependent equilibrium at a composite concentration of 1.8 mM (Goodman and Bessman 1975) and can theoretically bind HbA as well. Finally, normal bicarbonate (HCO_3^-) concentrations in human erythrocytes are 15.4 and 17.4 mM in arterial and venous blood, respectively (Westen and Prange 2003), making HCO_3^- participation in HbA glycation a major point of consideration.

Pi, BPG and HCO_3^- each bind with exothermicities comparable with or exceeding that of the glucose isomers (Tables II and III) and can compete against and/or complement the binding of glucose to HbA. These anions bind in similar locations within HbA (Table IV) and can bind near known glycation sites in HbA.

Our ongoing research focuses on elucidating the effect that these anions have on glucose isomer distribution and on the chemical mechanism whereby the aldimine/Schiff base is generated (Stages 1–2 transition). That these anions bind in similar HbA regions as the glucose isomers and can modify the chemistry and lead to differing glycation rates may have implications for an unanswered clinical question. Specifically, our results point to a potential contributor to the explanation for the glycation gap based upon the importance of differences of available anions in the differing chemical environments within the erythrocyte vs. serum.

In conclusion, all the five glucose structures generated upon mutorotation within the erythrocyte will undergo reversible, competitive binding to both the fully oxygenated and deoxygenated HbA (and, presumably, each of the intermediate hemoglobin structures that reside between the two ends of the oxygenation continuum) with low amino acid residue selectivity. The most plausible means of presenting a ring-opened glucose to the nucleophilic amino acid residues in the later stages (Stages 2–4) of the glycation process for HbA is the nonspecific binding of the α- and β-pyranoses (and, to a lesser extent, the α- and β-furanoses) followed by the mutorotation of these ring-closed sugars while bound (Figure 3). BPG, Pi and HCO_3^- also reversibly bind to HbA with similar energies and common binding sites with the glucose isomers. Finally, the clinical difference between average blood glucose and predicted HbA_{1C}, referred to as the "glycation gap" and the presence of unstable HbA-glucose complexes may be more fully explained by (i) initial, noncovalent glucose binding that is not productive, (ii) binding that is potentially productive but reverts back to free glucose before aldimine/Schiff base formation and (iii) different concentrations of BPG, Pi and HCO_3^- in serum vs. erythrocytes.

Table IV. Exothermic interactions of anionic binding molecules with HbA amino acid (AA) residues in the absence of glucose isomers

AA Residues	Anionic binding molecules			
	BPG	$H_2PO_3^-$	HPO_3^{-2}	HCO_3^-
Val1	o	o	o	
Lys8	o		o	o
Lys59	d			
Lys61	d			
Lys65	d	d	d	
Lys82	o, d	o, d	o, d	o, d
Lys120		o	o	o

(i) The first column labeled "AA Residues" has those amino acid residues that can theoretically be glycated as defined as having the nucleophilic nitrogen of that residue within 6 Å of the anion. This is predicated upon the assumption that a 3 Å variation within the binding environment is possible and is consistent with the geometric parameters set forth by Ito et al. (2011). (ii) BPG (2,3-bisphosphoglycerate). (iii) Noncovalent binding to oxygenated (o) and deoxygenated (d) HbA. (iv) These binding molecules do not interact with the following glycation sites: Lys17,66, 117,132 and 144.

Materials and methods

All binding data were obtained via computational methodologies. The structures utilized in the docking computations were obtained as follows: (i) the HbA crystal tetramers, 2DN2 deoxygenated (Park et al. 2006) and 3B75 oxygenated (Saraswathi et al.), were obtained from the RCSB Protein Data Bank (http://www.pdb.org/pdb/home/home.do), (ii) β- chains of both the fully deoxygenated HbA and fully oxygenated HbA were isolated from the tetramer using Deep View-Swiss PDB Viewer (WINE, Guex et al. 1999, http://spdv.vital-it.ch/wine.html), (iii) the α- (glc) and β- pyranose (bgc) anomers, HCO_3^- (bct) and BPG (dg2) were obtained from Hetero-compound Information Centre Uppsala (three-letter compound designations) (HIC-UP, Kleywegt Jones 1998), http://xray.bmc.uu.se/hicup/) and (iv) the α- and β-furanose anomers, the ring-opened form and the dibasic phosphate were constructed utilizing The GlycoBioChem PRODRG2 Server (Schüttelkopf and van Aalten 2004, http://davapc1.bioch.dundee.ac.uk/prodrg/index.html) while the monobasic phosphate was built with The PyMOL Molecular Graphics System (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC, http://www.pymol.org/). It is important to note that the HbA structures, the α-(glc) and β-pyranose (bgc) anomers, HCO_3^- (bct) and BPG structures obtained from HIC-UP are static structures. The α- and β-furanose anomers, the ring-opened glucose isomer and the dibasic phosphate were constructed utilizing The GlycoBioChem PRODRG2 Server and are geometry-optimized dynamic structures.

Binding with various molecules was performed on only one β-chain of HbA, in part, because of computational simplicity in view of spatial resolution. Moreover, the clinically relevant glycation site, specifically Val1 for HbA_{1C}, is on the β-chain where this residue accounts for ~30% of total glycation sites (Zhang et al. 2001). Although focusing on one β-chain will not account for inter-chain interactions, this simplified approach is consistent with the scope of this initial investigation. Once the β-chain was isolated, the resulting file (filename.pdb) was imported into the AUTODOCK program (Morris et al. 1998, Vs 4.2.5.1)

to be used as the docking protein. An AUTODOCK calculation is a two-step process in which the interactions between the atom types in the glucose and/or binding molecules and the target HbA structure are pre-calculated in a three-dimensional grid surrounding the binding region (our grid calculation was set at a spacing of 1 Å, with the *X*, *Y* and *Z* coordinates set at 60, 70 and 60, respectively (Forli and Olson 2012). The default settings within AUTODOCK were utilized throughout all computations. The resulting docking file produced by AUTODOCK (filename.pdbqt) was subsequently saved for sequential docking, whereby computations from the file were used as a starting structure to dock a second structure or binding molecule. This process does not provide for competitive binding; rather, it does give insight into how HbA accommodates consecutive binding of multiple molecules within the same region. In sequential docking, the glucose structure and specific binding molecule must also be within 6 Å of each other and within 6 Å of the amino acid residue for direct participation of the binding molecule in the facilitation of glycation. Each docking computation was replicated in triplicate to assess reproducibility and all docking computations reported are within ±5% relative measurement.

Each AUTODOCK analysis produces the 10 most energetically favorable (exothermic) conformations of the binding. The readout includes atoms in H-bonds, H-bonds formed and the Gibbs free energy (ΔG) associated with each overall conformation. Docked molecules were visualized with the program JMol (2012, ver. 12.3, http://jmol.sourceforge.net/), which allows selection and visualization of salient regions of the molecule, including distances between structures and/or binding molecules and specific amino acid atoms in the protein sequence.

Glucose binding selectivity for amino acid residues of the β-chain of HbA was assessed by determining how many nucleophilic amino acid residues have a noncovalently bound glucose with geometry suitable for a potential reaction to proceed from Stage 1 to Stage 2. That distance is 6 Å, assuming a 1–3 Å distortion enabling a ∼3 Å interaction (Ito et al. 2011). The question of whether the ring-opened glucose structure specifically binds to HbA in the presence of the four ring-closed anomers was evaluated by comparing binding exothermicities of each of the five structures.

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Conflict of interest

None declared.

Abbreviations

BPG, 2,3-bisphosphoglycerate; HbA, human hemoglobin A; HIC-UP, Hetero-compound Information Centre Uppsala Pi, inorganic phosphate.

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