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Non-Enzymatic Glycation: Investigations into the Initial Non-Covalent Binding of Monosaccharides to Intra- and Extracellular Proteins

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A research paper submitted in partial fulfillment of the requirements for the degree of Master of Science Biomedical and Pharmaceutical Sciences, specializing in Medicinal Chemistry

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To the Graduate Faculty:

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Chapter 2. The initial non-covalent binding of glucose to human hemoglobin in non-enzymatic glycation

Abstract

Non-enzymatic glycation describes the process by which a monosaccharide becomes covalently bound to a protein. This process is extremely important to the pathology of diabetes. This thesis focuses upon the very first interaction between the monosaccharide and the protein, the initial non-covalent binding. In investigating this initial binding interaction eight primary questions were asked: 1) which of the five glucose isomers are initially non-covalently bound to HbA, 2) how is the Schiff base generated from initially bound glucose isomer/s, 3) is the initial binding event limited to the participation of the sugar and the protein or are physiological anions that are known to be present in the body involved in the initial binding event as well, and 4) if physiological anions are involved, what role do they play in the mechanism, 5) will other reducing sugars (fructose, xylulose, and galactose) exhibit the same binding characteristic as glucose, 6) which of the five glucose isomers are initially non-covalently bound to HSA and how does this compare to the binding event for HbA, 7) is Schiff base formation in HSA the same or different than Schiff base formation in HbA, and 8) what are the clinical implications associated with the answers to the preceding questions?

Chapter 1: Unifying Introduction

Diabetes mellitus is currently considered to be a global epidemic. According to the World Health Organization over 347 million people worldwide currently are diagnosed with diabetes (*1*). In 2010 an estimated 3.4 million deaths were attributed to this disease (*2*). According to the Center for Disease Control and Prevention (CDC) in the United States alone in 2012 there were 28.9 million adults, aged 20 or older, diagnosed with diabetes (*3*). As of 2012 in the U.S. it is believed that close to 86 million people fit under the classification of pre-diabetics (*3*). The total costs of diagnosed diabetes in the U.S. were a staggering 245 billion in 2012 (*3*).

There exist multiple types of diabetes; most pointedly Type I and Type II diabetes. Diabetic type is categorized by issues associated with the protein hormone insulin. A primary function of insulin is the regulation of the level of sugar circulating in the blood. Type I diabetes is classified based on the absence of sufficient insulin to regulate the level of sugar in the blood. Type I diabetes is generally attributed to a genetic autoimmune disease which attacks cells that are responsible for producing insulin (β-cells in the pancreas). Consequently, Type I patients must inject insulin on a daily basis (*3*). Type II diabetes is more prevalent than Type I and is classified based on the presence of excess insulin within the body. Due to the presence of excess insulin, the body develops a type of resistance to insulin and can no longer use it efficiently (*3*). However, Type II diabetes also occurs in the absence of adequate insulin and these individual must

also supplement their circulating insulin (*2-4*). The cause of Type II diabetes is not well understood although excess weight and lack of physical activity have been identified as possible contributing factors (*4*).

When the insulin system no longer works efficiently at regulating sugar in the bloodstream for extended periods of time, the patient becomes at risk for developing diabetes and long-term vascular complications. These complications include coronary disease, heart attack, stroke, kidney failure, gangrene, and many others (*5-6*).

The molecular basis for the development of diabetic complications is not fully understood. That said, a process called protein glycation is certainly a contributor to diabetic complications. Protein glycation is an overarching term used to describe the chemical binding, both covalent and non-covalent, of sugars (predominantly glucose) to a protein (*7*). Glucose is the primary sugar in the human body, although other sugars such as fructose and galactose exist in both intracellular and extracellular compartments. Glucose is not a single chemical structure but is rather a manifold of interconverting isomers. The predominant forms of glucose are: four ring-closed isomers; two six member rings (α-glucopyranose and β-glucopyranose) and two five member rings (α-glucofuranose and β-glucofuranose). These ring-closed isomers interconvert by means of a fifth isomer, a ring-opened structure (Fig. 1).

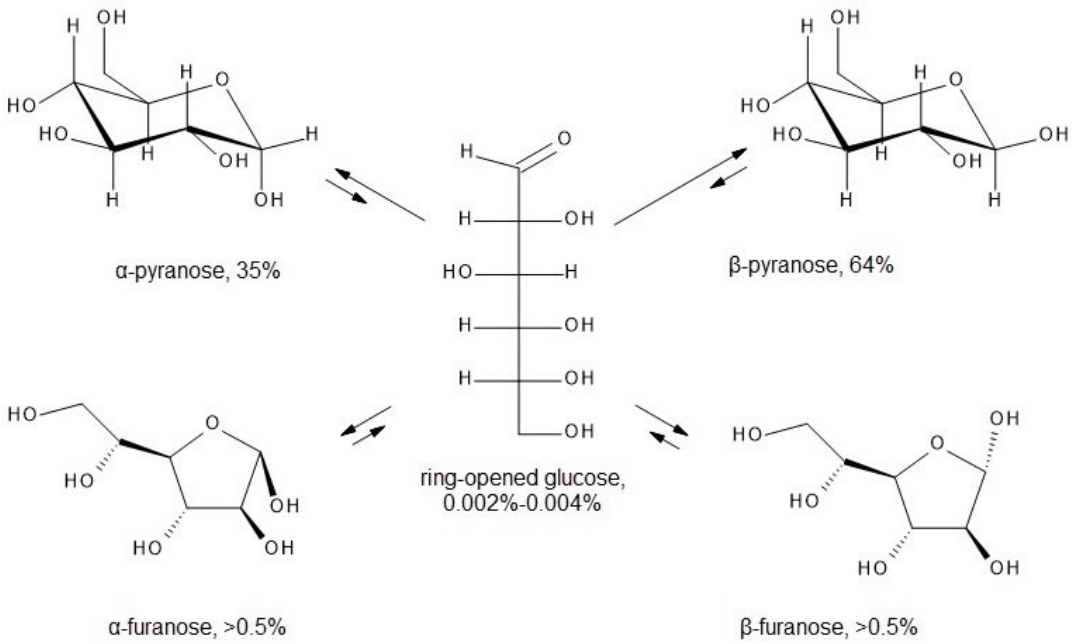

Figure 1. The five isomers of glucose with respective solution equilibrium concentrations (*8-9*).

Consequently glycation that involves glucose will proceed via the non-enzymatic binding (non-covalent and covalent) and subsequent chemistry of five distinct glucose isomers with a protein.

The glycation of proteins is a normal, time-dependent process that is extremely important for the pathology of diabetes. The human body contains thousands of different proteins (both intra- and extracellular) that can possibly interact with glucose and alter protein function. Hemoglobin is a critical intracellular protein in red blood cells that is responsible for transferring oxygen molecules throughout the body. Each hemoglobin protein has a tetrameric structure that consists of two α subunits and two β subunits all linked at the center of the molecule (Fig. 2).

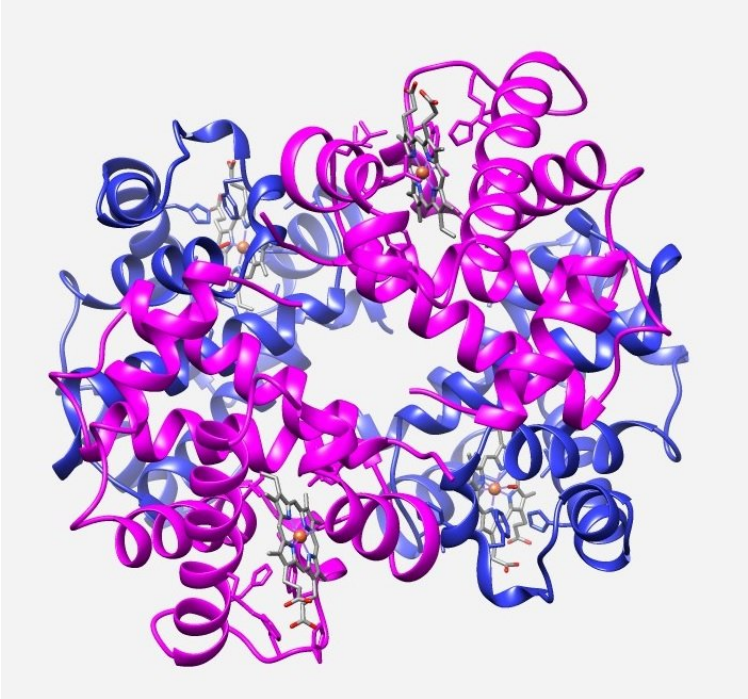

Figure 2. The entire oxygenated Hemoglobin tetramer. Blue indicates the α subunits while magenta indicates the β subunits.

In diabetic patients, the primary intracellular protein of interest to clinicians is Hemoglobin A (HbA). Glycated HbA is known as HbA_{1C}. The amount of measured HbA_{1C} is used clinically to represent the average level of glucose in the blood over a two-month period (*10*). This measurement is considered to be a more reliable blood glucose measure than simply taking a single sample of glucose in the blood. The reason behind this is that throughout the day the average concentration of blood glucose in the body will change depending on meal time, exercise, and many different factors unique to each individual. Because HbA is glycated over the entire lifetime of the red blood cell, approximately 120 days, the extent of glycation to generate HbA_{1C} is a good measure of time-averaged glucose concentration (*11*). Clinically, a healthy human has an HbA_{1C} around 4-5.9% (*12-13*). As of 2010, the American Diabetes Association established diagnosis criterion for diabetes as having an HbA_{1C} level $\geq$ 6.5% (*14*).

The primary extracellular protein of clinical interest is human albumin, HSA. Albumin circulates in the blood as a dimer that consists of two identical subunits, an α subunit and a β subunit (Fig. 3). The clinical measure involving HSA is referred to as fructosamine level. Fructosamine level is a measure of glycated extracellular (serum) proteins, predominantly HSA, and, like the clinical measure of HbA_{1C}, is used to represent average blood glucose level over time (*15*). Normal glycated albumin values for a healthy non-diabetic patient are

generally found between 6-15%. Values >18% are considered to be in the diabetic range (*16*).

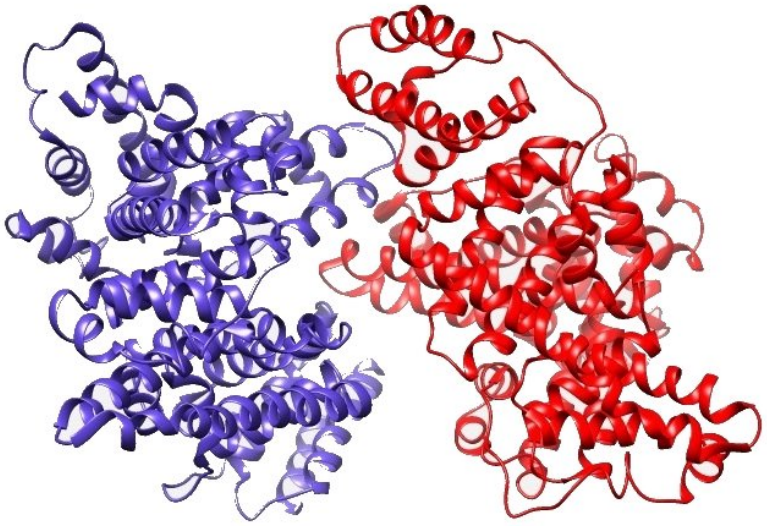

Figure 3. The human serum albumin dimer (HSA). The α subunit is indicated in red while the β subunit is indicated by blue.

The initial interaction between a sugar molecule (glucose isomers or other sugar molecules) and a protein (intracellular or extracellular) will be an electrostatic non-covalent binding interaction that is reversible. During the lifetime of the bound sugar within a particular protein cavity, a reaction must occur to generate a covalent bond. The first species generated upon such a reaction is an aldimine or Schiff Base (*17*). The Schiff Base will continue to react to form a structure known as an Amadori intermediate, which will further react to create any of an array of Advanced Glycation End products (AGE) (*18-21*). Once the Amadori intermediate and/or any of the AGE products form(s), the process becomes irreversible. As such, the protein will remain glycated until the end of its lifetime. Once glycated, the secondary structure of the protein will be modified and may no longer function as it was originally intended to. Specifically these AGE products have been linked to some of the most devastating effects of diabetes; blindness, loss of limbs, nephropathy, and others (*22*).

The general scheme for the glycation of proteins can be broken down into related sequential stages. By dividing the process into stages this facilitates the assessment of the bond-making/bond-breaking chemical mechanisms present within each stage. Previous research has represented this process in two or three stages, with the first stage being the aldimine/Schiff Base (Fig. 4) (*17-21*).

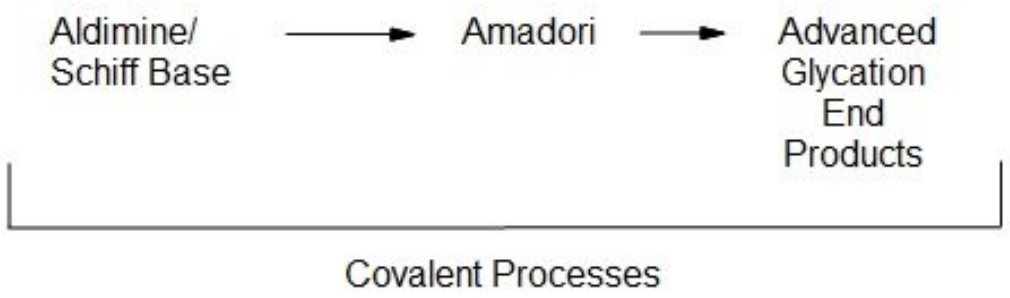

Figure 4. Flow chart of the covalent processes in glycation based on the most important stages as asserted by the literature.

This classic perspective does not account for initial electrostatic non-covalent binding and is limited only to covalent processes. This position assumes that any non-covalent process is likely non-rate determining and thus not critical to the pathology of diabetes. Thus, the majority of research on this disease has focused on Amadori intermediate and AGE product formation as the only chemically relevant processes in the pathology of diabetes. However Gil et al. performed kinetic analyses on the formation of the Amadori intermediate (*23-25*). The studies by Gil et al. showed that the rate determining step must be *prior* to the formation of the Amadori structure (*23-25*). Based on this landmark finding, we set out to start at the very beginning of the process to better understand the dynamic relationship between sugars and proteins. As such, non-covalent interactions between sugars and proteins are the primary focus of our research. Specifically, we forward a four stage scheme for glycation whereby we insert an initial stage that entails non-covalent binding interactions (Figure 5).

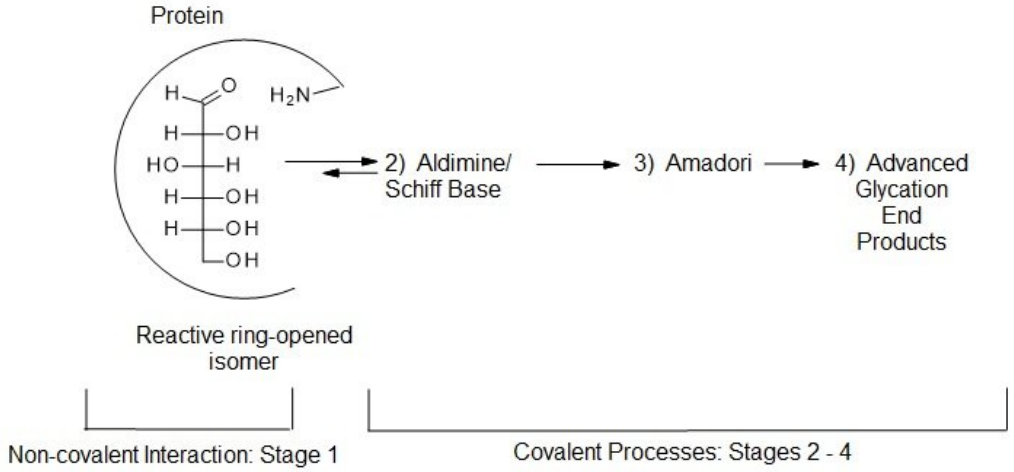

Figure 5. The glycation mechanism based on our assertion that the non-covalent interactions play a significant role in the overall glycation process.

The research described within this thesis is divided into five chapters. Chapter 1 is this unifying introduction. Chapters 4 and 5 are the unifying methods and conclusions, respectively. Chapters 2 and 3 are two independent yet related investigations into the nature of the non-covalent interactions between sugars and proteins in the glycation process with relevance to the pathology of diabetes. In each of these two chapters, a more specific and detailed introduction as well as results and discussion are featured. Specifically Chapter 2 is entitled *The initial non-covalent binding of glucose to human hemoglobin in non-enzymatic glycation*. The primary questions addressed in Chapter 2 are: a) which of the five glucose isomers are initially non-covalently bound to HbA, b) how is the Schiff base generated from initially bound glucose isomer/s, c) is the initial binding event limited to the participation of the sugar and the protein or are physiological anions that are known to be present in the body involved in the initial binding event as well, and d) if physiological anions are involved, what role do they play in the mechanism? Chapter 3 is entitled *A comparison of the initial non-covalent binding of D-glucose and D-fructose with hemoglobin and albumin in non-enzymatic glycation*. The primary questions addressed in this chapter are: a) will other reducing sugars (fructose, xylulose, and galactose) exhibit the same binding characteristic as glucose, b) which of the five glucose isomers are initially non-covalently bound to HSA and how does this compare to the binding event for HbA, c) is Schiff base formation in HSA the same or different than Schiff base formation in HbA, and d) what are the clinical implications associated with the answers to the preceding questions? The methods utilized in Chapters 2 and 3

include experimental Nuclear Magnetic Resonance Spectroscopy (NMR) and theoretical computations.

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Chapter 2. The initial non-covalent binding of glucose to human hemoglobin in non-enzymatic glycation

2.1 Introduction

Glycated human hemoglobin (HbA_{1C}) is the first example of a non-enzymatically glycated protein *in vivo* (*1*). 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 (*2*).

Key determinants of the rate and extent of non-enzymatic glycation of proteins include the prevailing glucose concentration and protein life span. Normal levels of "stable" HbA_{1C} are approximately 5-6% of total HbA and this value can increase to 15% or more in diabetic individuals (*3*). However, the presence of "labile" HbA_{1C} – which is formed during the early, reversible stages of the glycation process and reflects ambient versus longer term glucose levels - may overestimate HbA_{1C} by up 2-3% in normal subjects and 10% in subjects with diabetes (*4*). It is also noteworthy that the clinical measurement of HbA_{1C} is specific for 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 (*5*). As a result, the measurement of HbA_{1C} is likely not a comprehensive indicator of HbA glycation. In addition, the presence of deglycating enzyme fructosamine 3-kinase (EC 2.7.1.171)

within erythrocytes was proposed to explain genetically determined differential glycation and the heritability of HbA_{1C} (*6*). However, the finding that the activity of fructosamine 3-kinase is not related to HbA_{1C}, or the level of total glycated hemoglobin (*7*), reinforces the idea that early events in glycation are important.

Despite an overall appreciation of the potential reactions 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 (*1, 8-10*), our conception of the HbA glycation process is best described by the following sequential stages (Fig. 1): 1) reversible, non-covalent binding of glucose to select sites on the protein, 2) reaction between the bound electrophilic glucose and nucleophilic amine residues (typically the N-terminal (α-amino) Valine and internal (ε-amino) Lysine residues to generate a covalently-bound Schiff base or aldimine, 3) rearrangement of the aldimine/Schiff base to a Amadori intermediate (amino-1-deoxyfructose) and 4) the non-reversible formation of advanced glycation end products (AGE). The result of the process, the presence of various AGE products, is thought to be linked to the chronic complications of diabetes mellitus and the normal aging process (*11*).

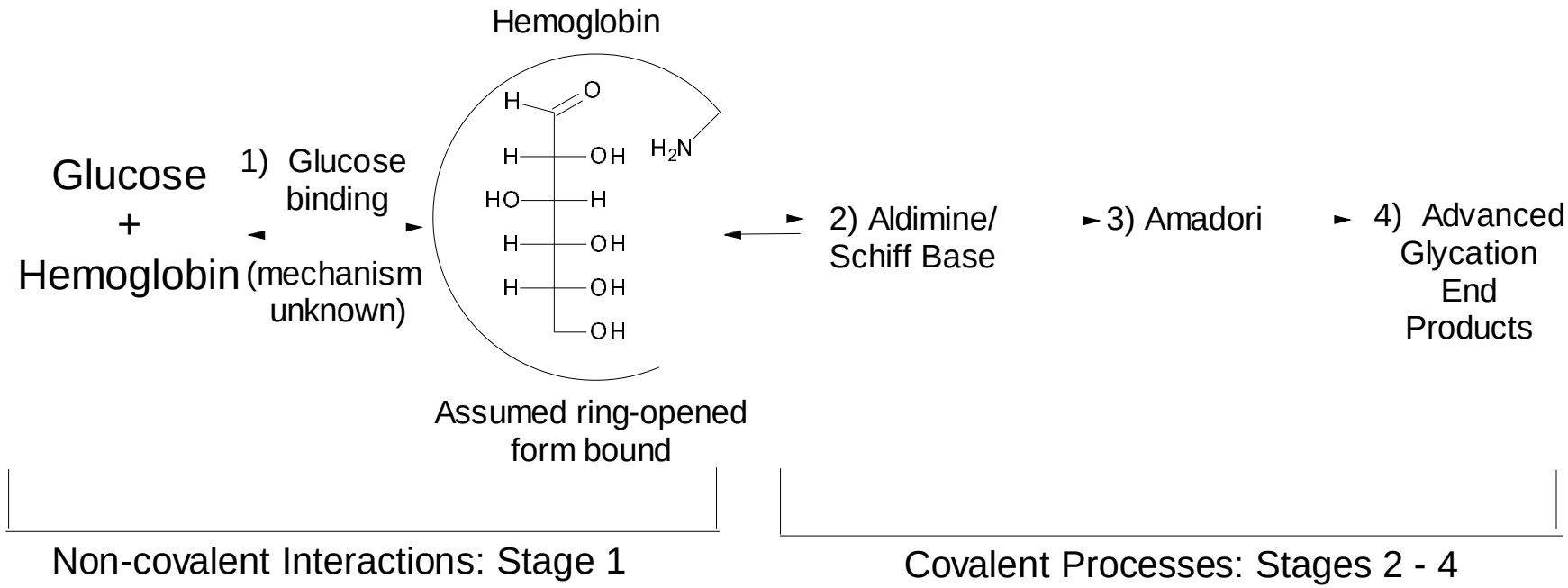


Figure 1. Four-stage description of the non-enzymatic glycation of human hemoglobin A. The equal length of the reaction arrows for the glucose binding stage reflects a highly reversible interaction (Stage 1), whereas the reactions in Stages 2-4 are progressively less reversible.

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 inter-individual differences in the erythrocyte glucose concentration and erythrocyte survival time in circulation (*12-13*). However, there is increasing evidence that variation in HbA_{1C} is influenced by factors other than glucose concentration (reviewed in Hempe et al.) (*14*). 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) (*15*).

Most of the research dedicated to understanding the mechanism of HbA glycation has focused upon Stage 2 (*16*), the transition from Stage 2 to Stage 3 (*17-18*), or Stage 3 (*19*-20). Each of these investigations correlated overall glycation rate to some mechanistic factor and focuses on species or events after the initial binding. However, all reactive species 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 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 (Fig. 1, Stage 1). Specifically, D-glucose undergoes reversible mutarotation in aqueous solution

whereby five different isomers interconvert, a pair of pyranose anomers as well as a pair of furanose anomers and a ring-opened glucose intermediate (Fig. 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, less than 1% for the sum of α- + β-furanoses (*21*), and just 0.002%-0.004% for the ring-opened isomer (*22*).

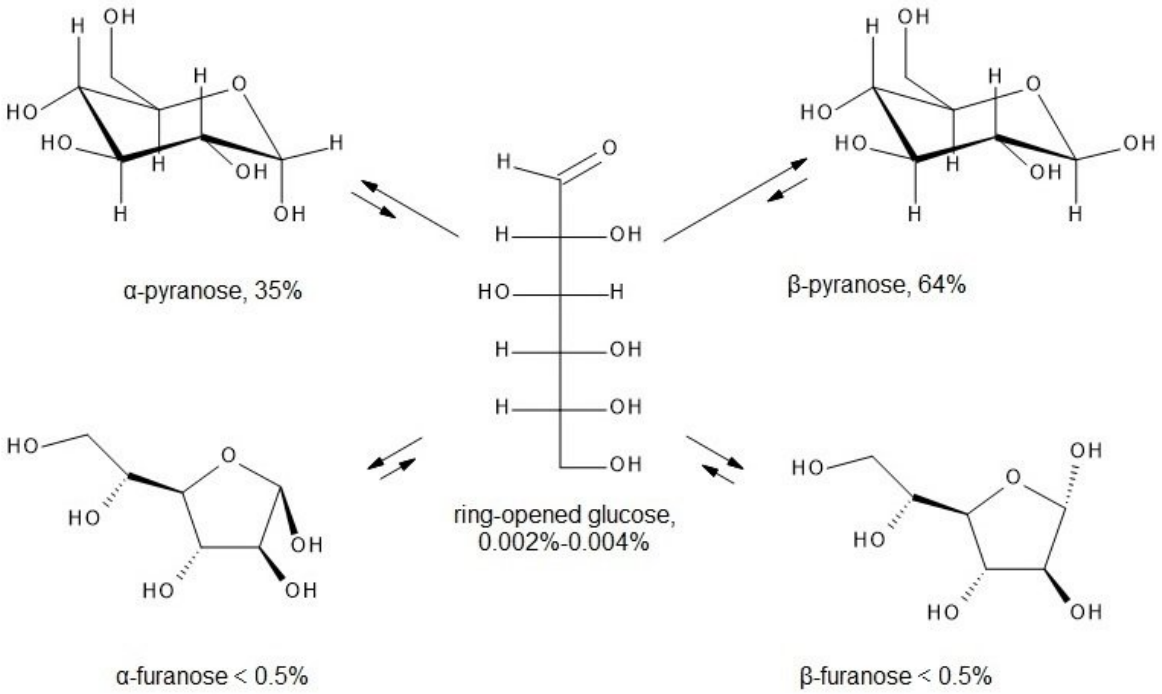


Figure 2. Percent distribution of aqueous D-glucose structures at equilibrium when entering from the β-pyranose anomer (*21-22*). The reaction arrows reflect the extent and reversibility of interconversions between the ring-closed anomers and the ring-opened glucose.

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 the initial binding event (Stage 1) of HbA within erythrocytes? It should be noted that in seminal papers pertaining to the glycation of HbA (*23-24*), 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 interaction 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 inter-converting HbA structures. Different HbA structures possess different stereo-chemical features and affinity for glucose (*25-26*). The question of how various forms of HbA compare to one another in initial non-covalent 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 bicarbonate (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 research reported upon in this chapter is to assess how different HbA structures compare to one another in initial non-covalent binding (Stage 1) with each of the five glucose structures, both in the presence and in the absence of BPG, Pi, and HCO_3^-, with the goal being to determine at which stage in the four-stage glycation process (Fig. 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 Fig. 3)?

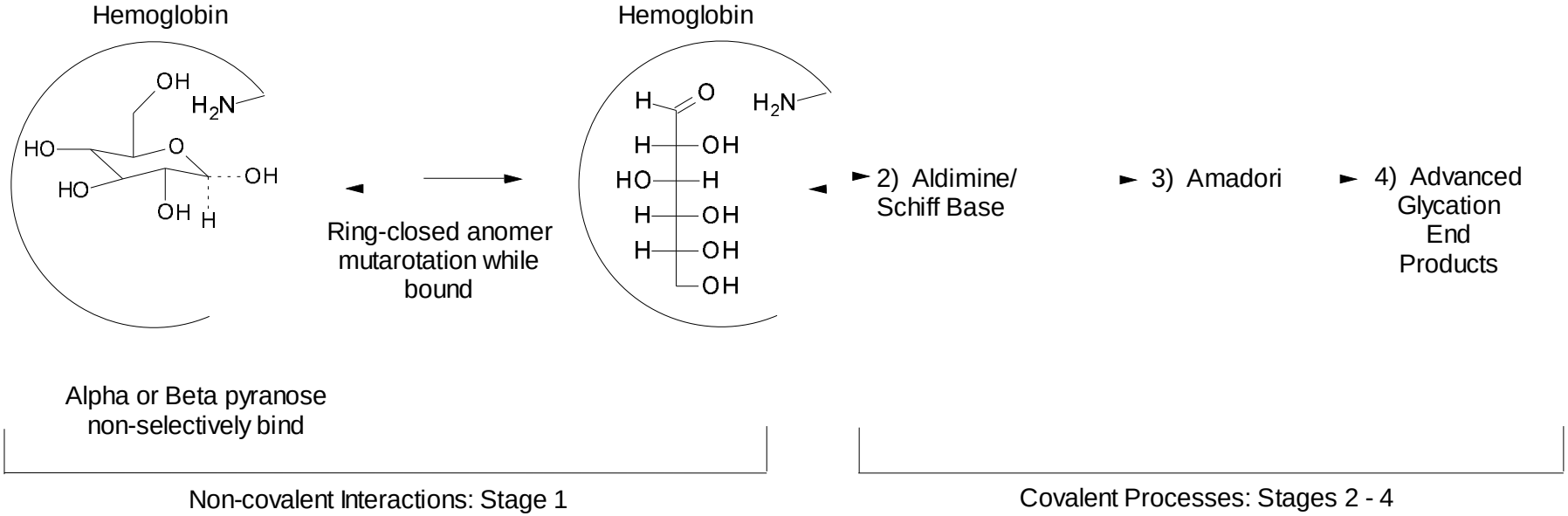


Figure 3. Potential process for presenting reactive ring-opened glucose to hemoglobin (Stage 1) and subsequent steps leading to glycation and production of advanced glycation end products. Unlike previous models, this process involves non-selective binding of ring-closed anomers and mutarotation while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

2.2 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. 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 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.

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
Lys120					o

i: The first column labeled "AA Residues" are those amino acid residues that can theoretically be glycated as defined as having the nucleophilic nitrogen of that residue within 6Å of the bound sugar. This is predicated upon the assumption that a 3Å variation within the binding environment is possible and is consistent with the geometric parameters set forth by Ito (*27*).
ii**:** Docking to oxygenated (o) and deoxygenated (d) HbA.
iii: Sites identified from the assessment of data generated in triplicate

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 (Table I). 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 8 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 7 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 (Table I). 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 either Lys82/His143, or Lys65, or Leu81/Lys82. When dibasic phosphate is bound to oxygenated HbA, binding takes place in three distinct regions, involving Leu3/Lys8, or 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 5 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 4 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 (Table I). 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 leads to the conclusion that low amino acid residue selectivity exists for glucose structures.

The overall assessment of amino acid residue selectivity 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. 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 ca. 1 kcal/mol of each other. With deoxygenated HbA, the binding is more exothermic for the ring-closed pyranoses by ca. 0.5 kcal/mol than for the ring-opened glucose (Table III). The exothermicities for the binding of the furanose anomers are less than that for the pyranose and ring-opened structures (ca. 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 for 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 ca. 1.0 – 2.5 kcal/mol greater binding exothermicities than do any of the glucose structures (Tables II & 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 ca. 1 kcal/mol greater exothermicity than the ring-opened glucose (Tables II & III). No ring-opened glucose specificity is observed with the sequential binding computations in the presence of BPG.

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.

1st Molecule	2nd 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.
ii: all energies reported in sequential docking reflects the exothermicity of the second species in its binding

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 comparable exothermicity to the α- and β-furanoses. In the binding of the inorganic phosphates 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 ca. 1.2 kcal/mol more exothermic than the ring-opened glucose, while furanose binding is less exothermic (Tables II & 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 & 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.

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 deoxygenated HbA.

1st Molecule	2nd 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.

HbA Glycation: Potential Stage 1and Stage 1-Stage 2 transition mechanisms involving only protein amino acid residues. A computational investigation was undertaken to assess what available HbA β-chain amino acid residues are within reacting distance of a bound α- or a bound β-pyranose isomer (Tables IV and V). The bound pyranose isomers were chosen because they constitute 99.99% of the initially bound glucose molecules. These ring-closed species must ring open, while bound, to generate a transient electrophilic ring-opened glucose that then must react with a nucleophilic lysine or terminal valine to proceed in the glycation process. Because the ring opening will be facilitated by either acid or base catalysis and must react with a nucleophile, we assess which acidic, which basic, and which nucleophilic amino acid residues are within reacting distance. The criteria that constitutes reacting distance differs for nucleophile/electrophile and acid/base reactions between bound glucose and the proximate amino acid residues is 9Å, this is predicated upon the assumption that a 5Å variation within the binding environment is possible and is consistent with the geometric parameters set forth by Ito (*27*) which assumes a 6Å reacting distance for sugar to amino acid residue with and extra 3Å reacting distance to accommodate a physiological anion as set for by Bobadilla (*28*).

Table IV. Summary of Autodock binding glycation sites (on the β-chain of oxygenated human HbA) organized by binding cavity, proximate amino acid residues, and potential mechanisms for facilitated bound β- pyranose ring opening.

Glycation Site	HbA Cavity (kcal/mol)	Nucleophilic AA Residues (distance in Å)	Acidic AA Residues (distance in Å)	Basic AA Residues (distance in Å)	Potential Mechanism
Val1	-3.9	Val1; Lys132		Lys132	Base Only
	-3.9	Lys8; Val1	Glu7; Asp79	Lys8; Val1; His2	Acid/Base or Acid or Base
	-4.2	Lys82;Val1	Asp79	Lys82; His143; His146	Acid/Base or Acid or Base
Lys8	-3.9	Lys8; Val1	Glu7; Asp79	Lys8; Val1; His2	Acid/Base or Acid or Base
Lys17	-3.4	Lys17	Glu121	Lys17	Acid Only
Lys59	-4.3	Lys59; Lys66		His63; Lys59; Lys66	Base Only
	-3.8	Lys66; Lys59		His63; Lys66	Base Only
Lys65	-3.6	Lys61; Lys65	Glu22; Asp21	Lys61	Acid Only
	-3.5	Lys65	Asp21; Glu22; Asp73	Lys65	Acid Only
Lys66	-3.8	Lys66; Lys59		His63; Lys66	Base Only
	-2.9	Lys95; Lys66	Glu90	Lys95; Lys66; His63	Acid/Base or Acid or Base
	-4.3	Lys59; Lys66		His63; Lys59; Lys66	Base Only
Lys82	-4.2	Lys82; Val1	Asp79	Lys82; His143; His146	Acid/Base or Acid or Base
	-3.4	Lys144; Lys82		Lys144; Lys82; His143; His146	Base Only
Lys95	-2.9	Lys95; Lys66	Glu90	Lys95; Lys66; His63	Acid/Base or Acid or Base
Lys144	-3.4	Lys144; Lys82		Lys144; Lys82; His143; His146	Base Only

i: The first column labeled "glycation site" is organized by nucleophilic lysine or valine amino acid residue that can theoretically be glycated within the listed cavity(ies) as defined as having the reactive centers within 9Å (*27-28*). The energy values are binding exothermicities are reported as ΔG in kcal/mol for the most stable conformation generated upon docking glucose hydrates to the β-chain of fully-oxygenated HbA

ii**:** The data is organized by nucleophilic residues (with distance between nucleophilic AA residue nitrogen and electrophilic glucose anomeric carbon reported), acidic residues (distance between AA acidic proton and basic hemiacetal oxygen on glucose reported), and basic residues (distance between AA basic nitrogen and acidic proton on the glucose anomeric hydroxyl reported)

iii: Sites identified from the assessment of data generated in triplicate

Table V. Summary of Autodock binding glycation sites (on the β-chain of oxygenated human HbA) organized by binding cavity, proximate amino acid residues, and potential mechanisms for facilitated bound α - pyranose ring opening.

Glycation Site	HbA Cavity (kcal/mol)	Nucleophilic AA Residues (distance in Å)	Acidic AA Residues (distance in Å)	Basic AA Residues (distance in Å)	Potential Mechanism
Val1	-5.0	Val1; Lys82	Asp79	Val1; Lys82; His143; His146	Acid/Base or Acid or Base
	-4.6	Lys82; Val1		Lys82; Val1; His143; His146	Base Only
	-4.0	Lys8; Val1	Asp79	Lys8; Val1; His77	Acid/Base or Acid or Base
	-3.6	Val1; Lys132		Val1; Lys132; His2	Base Only
Lys8	-3.7	Lys8; Val1	Asp79	Lys8; Val1; His77	Acid/Base or Acid or Base
Lys17	-3.8	Lys17	Glu121	His117; His116	Acid/Base or Acid or Base
Lys59	-4.5	Lys59; Lys66		Lys66; Lys59; His63	Base Only
Lys61	-3.6	Lys65; Lys61	Asp21; Glu22	Lys65; Lys61	Acid/Base or Acid or Base
Lys65	-3.6	Lys65; Lys61	Asp21; Glu22	Lys65; Lys61	Acid/Base or Acid or Base
	-3.1	Lys65; Lys66	Asp21	Lys65; Lys66; His63	Acid/Base or Acid or Base
Lys66	-4.5	Lys59; Lys66		Lys66; Lys59; His63	Acid/Base or Acid or Base
	-3.1	Lys65; Lys66	Asp21	Lys65; Lys66; His63	Acid/Base or Acid or Base
	-2.9	Lys95; Lys66		Lys95; Lys66	Base Only
Lys82	-4.6	Lys82; Val1		Lys82; Val1; His143; His146	Base Only
	-5.0	Val1; Lys82	Asp79	Val1; Lys82; His143; His146	Acid/Base or Acid or Base
	-3.2	Lys144; Lys82	Asp94; Glu90	Lys82; Lys144; His143	Acid/Base or Acid or Base
Lys95	-2.9	Lys95; Lys66		Lys95; Lys66	Base Only
Lys132	-3.6	Val1; Lys132		Val1; Lys132; His2	Base Only
Lys144	-3.2	Lys144; Lys82	Asp94; Glu90	Lys82; Lys144; His143	Acid/Base or Acid or Base

i: The first column labeled "glycation site" is organized by nucleophilic lysine or valine amino acid residue that can theoretically be glycated within the listed cavity(ies) as defined as having the reactive centers within 9Å (*27-28*). The energy values are binding exothermicities are reported as ΔG in kcal/mol for the most stable conformation generated upon docking glucose hydrates to the β-chain of fully-oxygenated HbA

ii**:** The data is organized by nucleophilic residues (with distance between nucleophilic AA residue nitrogen and electrophilic glucose anomeric carbon reported), acidic residues (distance between AA acidic proton and basic hemiacetal oxygen on glucose reported), and basic residues (distance between AA basic nitrogen and acidic proton on the glucose anomeric hydroxyl reported)

iii: Sites identified from the assessment of data generated in triplicate.

Based upon the proximity of reacting amino acids relative to initially bound α-pyranose (Table IV), eleven protein cavities have been identified that can theoretically lead to glycation. Each protein cavity possesses a nucleophilic amino acid that will react with the bound glucose. These are Val1, Lys8, Lys17, Lys59, Lys61, Lys65, Lys66, Lys82, Lys95, Lyus132, and Lys144. Within each of these cavities, in order for the glycation to proceed other amino acid residues must act as an acid, a base, or both. Val1, Lys8, Lys17, Lys61, Lys65, Lys66, Lys82, and Lys144 can each theoretically undergo acid, base, or simultaneous acid/base catalysis in facilitated glucose ring opening. No residues involving bound α-pyranose are limited to just acid catalysis, while Lys59, Lys95 and Lys132 are limited to base catalysis.

For the β-pyranose, a total of nine protein cavities can theoretically lead to glycation (Val1, Lys8, Lys17, Lys59, Lys65, Lys66, Lys82, Lys95, and Lys144; Table V). The amino acid residues Val1, Lys8, Lys 66, Lys82, and Lys95 can all theoretically undergo acid, base, or simultaneous acid/base catalysis in facilitated glucose ring opening. In contrast, Lys17 and Lys65 can undergo acid catalysis only, while Lys59and Lys144 are limited to base catalysis. Notably, when the β-pyranose binds near Lys17, if the lysine nitrogen lone pair functions better as a base rather than as a nucleophile, then no glycation will be possible

HbA Glycation: Potential contribution from physiological anions in Stage 1 and/or the Stage 1/Stage 2 transition. A computational investigation was undertaken to assess whether the ring-closed pyranose anomers and/or the ring-opened glucose isomers and a single physiological anion (Pi or HCO_3^-) can bind non-covalently to common cavities within HbA. HbA cavities that would enable glycation of Val1 and Lys82 on the β-chain can concurrently accommodate each of the glucose anomers/isomers and either Pi or bicarbonate anions (Table VI). The exothermicity of non-covalently binding both species in the same cavity is +/- 1.0 kcal/mol relative to the binding of the individual species in that same cavity (Table VI).

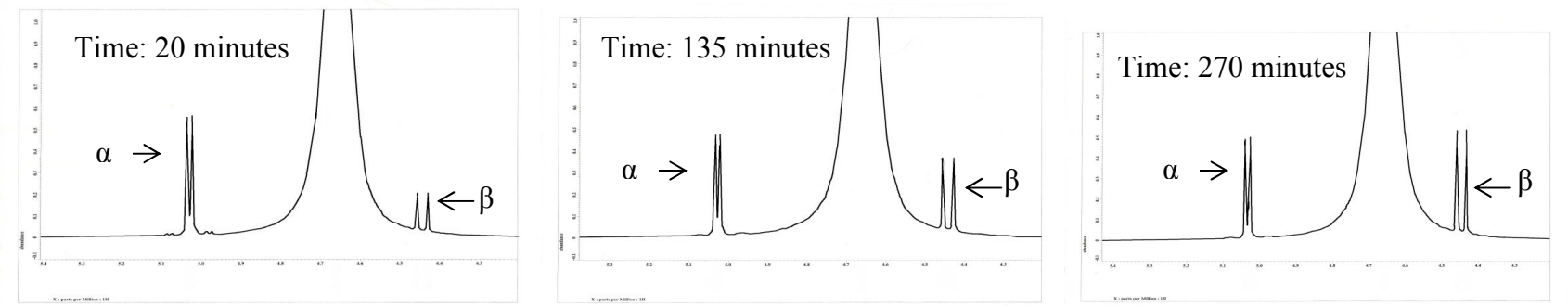
Table VI. Summary of Autodock binding of the α-and β- pyranose anomers and the ring-opened isomer of glucose within the β-chain of oxygenated human HbA.

Sugar	Anion	Binding ΔG in kcal/mol	Potentially glycated residues
α_6	Dibasic Pi	-3.8	Lys17, Val1, Lys82, Lys8, Lys61
β_6	Dibasic Pi	-3.9	Lys82, Val1, Lys8, Lys144
RO	Dibasic Pi	-4.4	Val1, Lys82, Lys66, Lys120, Lys132, Lys61
α_6	Bicarbonate	-4.0	Val1, Lys82, Lys120, Lys59, Lys17
β_6	Bicarbonate	-5.5	Val1, Lys82, Lys120
RO	Bicarbonate	-4.5	Val1, Lys82, Lys132, Lys120

i: Binding exothermicities are reported as ΔG in kcal/mol for the most stable conformation generated upon docking glucose and a physiological anion to the β-chain of fully-oxygenated HbA and are reported as a range.
ii: The fourth column designates the nucleophilic lysine or valine amino acid residue that can theoretically be glycated within that cavity as defined as having the nucleophilic nitrogen of that residue within 9Å of the bound sugar (*27-28*).

HbA Glycation: Potential implications of bound physiological anions on bound glucose ring opening. To test for anion effects on glucose ring opening, pure α-pyranose was placed in an aqueous (D_2O) solution at a pH in the physiological range (6.4-7.2) and its interconversion (a reversible process) with the β-pyranose anomer at room temperature (~20°C) was followed as a function of time by ^{1}H NMR. The extent of α-pyranose to- β-pyranose interconversion was measured and expressed in terms of the time required for inflection to occur (inflection time). Inflection time is defined as the time required for the integration of the ^{1}H NMR signal of the anomeric C-H of the α-pyranose (which is diminishing with time) to equal the integration of the ^{1}H NMR signal of the

anomeric C-H of the β –pyranose (which is increasing with time), equating to a 50:50 mixture of α and β anomers.



i. The ^{1}H NMR of pure α-pyranose in D_2O at 20°C was followed as a function of time. Three representative spectra are shown as the α-pyranose isomer converted to the β-pyranose isomer. The initial time point was measured ca. 8 minutes and followed the conversion across a time course of ca. 350 minutes (equilibrium point).

ii. The doublet at 5.06 PPM is assigned to the proton on the anomeric carbon of the α-pyranose isomer. The doublet at 4.47 PPM is assigned to the proton on the anomeric carbon of the β-pyranose isomer. The large, broad singlet at 4.66 PPM is water contamination in the D_2O solvent.

iii. The bottom spectrum is the 270 minute point designated as the inflection point defined as that point where the α- and -β-pyranose isomers are found in equal concentration.

Figure 4. ^{1}H Nuclear Magnetic Resonance Spectroscopy (NMR) of pure α-D-glucose over ca. 350 minutes

Table VII. Time-dependent ^{1}H NMR data for α- β Glucose interconversion in D_2O in the absence and in the presence of physiological anions.

Sugar	Sugar Equivalency	Binding Molecule	Physiological Anion Equivalency	Inflection Time (minutes)	Relative Rate Enhancement/Decrease
α-Glucose	ref	-	-	270	Ref
α-Glucose	1	Na_2PO_4H	1	9	30
α-Glucose	1	K_2PO_4H	1	9	30
α-Glucose	5	Na_2PO_4H	1	12	22.5
α-Glucose	5	K_2PO_4H	1	12	22.5
α-Glucose	20	Na_2PO_4H	1	23	11.7
α-Glucose	20	K_2PO_4H	1	25	10.8
α-G6P	ref	-	-	<5	-
α-Glucose	1	G6P	1	270	1
α-Glucose	1	G1P	1	27	10
α-Glucose	1	$NaHCO_3$	1	28	9.6
α-Glucose	1	$NaHCO_3$	5	20	13.5

i: Inflection time is defined as the time required for the integration of the ^{1}H NMR signal of the anomeric C-H of the α-pyranose (which is diminishing with time as the α-pyranose interconversion to the β-pyranose) to equal the integration of the ^{1}H NMR signal for the anomeric C-H of the β –pyranose (which is emerging with time).

An inflection time of 270 min is observed for α- to- β-pyranose inflection in D_2O (Table VII). This serves as a reference for inflection in the absence of physiological anions. The inflection time for α- to- β-pyranose in the presence of di-basic phosphate (either the sodium or potassium salts of HPO_4^{-2}) is just 9-12 min, an approximate 30-fold decrease relative α- to- β-pyranose inflection in water (Table VII). Further support for phosphate participation leading to enhanced ring opening comes from NMR experiments involving α-glucose with G6P and with G1P. G6P is readily formed in the human body via cellular glycolysis and is a structure that is used to transport phosphate for energy. For G6P the glucose is phosphorylated at the carbon 6 location. G1P is formed when glucose is cleaved from a glycogen polymer and is the precursor for G6P. The G1P structure can exist in either the α or β isomeric form phosphorylated at the anomeric carbon 1. When a 1:1 α-pyranose:G6P solution was prepared and the inflection for α- to- β-pyranose was followed by ^1H NMR, the inflection time was identical to that of the benchmark (270 min, like that of α-pyranose in water alone, Table VII). When a 1:1 α-pyranose:G6P was prepared and analyzed by ^{31}P NMR, the chemical shift for the G6P phosphate group was identical to that for G6P in the absence of glucose, consistent with there being no bimolecular interaction between the phosphate group on G6P and the α-pyranose anomer of glucose. Because the phosphate of the G6P is not available to interact with ring-closed glucose, no facilitation of α- to- β-pyranose was observed. In contrast, when a 1:1 α-pyranose:G1P mixture was prepared and analyzed by ^{31}P NMR, the

chemical shift for the G1P phosphate group in the presence of glucose differs from that for G1P alone (changes from 2.73 ppm alone to 2.94 ppm in the presence of glucose). This is consistent with a bimolecular interaction between G1P and the α-pyranose anomer of glucose. As such, phosphate participation from G1P should enhance α- to- β-pyranose conversion. Accordingly, the inflection time for α- to- β-pyranose in a 1:1 α-pyranose: G1P was a factor of 10 shorter than that of the benchmark (270 min, Table VII). The inflection time for α- to- β-pyranose in a 1:1 α-pyranose: bicarbonate mixture in water is 28 min, an approximate order of magnitude facilitation relative to α- to- β-pyranose inflection in water alone.

In an effort to attain further mechanistic insight regarding anion-facilitated α- to- β-pyranose interconversion, three additional experiments were performed. First, solutions at near neutral pH with glucose:Pi molar ratios at 5:1 and 20:1 were generated and their inflection times measured. The resulting α- to- β-pyranose inflection times were 22 and 12 times faster than benchmark (α- to- β-pyranose in the absence of phosphate), respectively (Table VII). That the inflection times for α- to- β-pyranose interconversion are so much shorter than benchmark, even when the HPO_4^{-2} anion concentration is $1/20^{th}$ that of the sugar, is consistent with a rapid, reversible pyranose/Pi interaction whereby interconversion is facilitated within the lifetime of a transient interaction. Second, the ^{31}P chemical shift for the Na_2HPO_4 in D_2O (in the absence of the sugar) is 2.78ppm, while that for

NaH_2PO_4 is 0.47ppm. When Na_2HPO_4 is placed in a 1:1 molar ratio with α-pyranose, a single broadened phosphorous signal at 1.77 ppm is observed. At a 1:5 ratio, the single broadened signal is at 1.66ppm, while at a 5:1 ratio the signal, again broadened, and is at 2.13ppm. Third, ^{1}H and ^{13}C NMR experiments are also consistent with rapid reversible interactions between α-pyranose and the bicarbonate anions. The enhanced basicity of each of these physiological anions relative to water and the presence of the Na^{+} cation to stabilize the leaving group is what likely facilitates enhanced interconversion relative to water. As the basicity of the physiological anion increases (bicarbonate, pKa = 6.4 => phosphate, pKa = 7.2) the extent of facilitation of α- to- β-pyranose interconversion increases accordingly.

HbA Glycation: Potential contribution from glucose co-binding with multiple physiological anions in Stage 1 and/or the Stage 1/Stage 2 transition. A computational investigation was undertaken to assess whether the ring-closed pyranose anomers and two physiological anions (in every combination of Pi and bicarbonate) can concomitantly non-covalently bind in common cavities within the β-chain of HbA (Table VIII). The ring-closed α- and β-pyranose anomers can concomitantly bind in many combinations of any two of the three physiological anions evaluated at Val1, Lys17, Lys59, and Lys82 (Table VIII). Those cavities where glucose binds and where glycation is known to occur that *cannot* accommodate concomitant binding of sugar with two physiological anions are those involving Lys61, Lys65, Lys66, Lys95, and Lys144.

Table VIII. Summary of Autodock binding of the α-and β- pyranose anomers of glucose with multiple physiological anions within the β-chain of oxygenated human HbA.

Physiological Anions	β-glucose	α-glucose
Pi/Pi	Lys82	Val1, Lys17
Pi/Bicarbonate	Lys82	Val1, Lys17
Bicarbonate/Bicarbonate	Val1, Lys82	Val1, Lys17, Lys59

i: The amino acid residues listed possess binding exothermicities (ΔG) ranging from -1.3 to -3.7 kcal/mol (for the binding of the second anion).
ii: The amino acid residues listed have the nucleophilic nitrogen of that residue within 9Å of the bound sugar (*27-28*).

2.3 Discussion

At the molecular level, the observed rates of non-enzymatic glycation of HbA have been correlated to an array of factors, each involving interactions after the initial, non-covalent binding of the sugar. For example, Bunn and Higgins (*19*), in assessing Amadori product formation (Fig. 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 and colleagues (*16*) suggested that glycation rate is most critically correlated to 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 (*20*) 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 and colleagues (*18*) also posit that the reactivity of the aldimine/Schiff base formed (Stage 2-Stage 3 transition) is the rate determining factor in glycation. Furthermore, Furth (*17*) 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 versus 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? Second, can the ring-closed anomers that do bind potentially mutarotate while bound and thus present the reactive ring-opened form for further reaction (Fig. 3)?

Glucose Docking Computations with HbA: Isomer Specificity. There are two requirements for a bound sugar to proceed past the non-covalent stage of the glycation process onto the covalent stages of the glycation process (Fig. 1). Glucose must first non-covalently 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 does not reveal isomeric specificity, whether the ring-opened structure is non-covalently 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

(*22*). Given that human erythrocytes possess a mutarotase (E.C. 5.1.3.3, aldose 1-epimerase) (*29*), 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 slower rate and reach an equilibrium concentration of ring-opened glucose that is lower than is the case for mutarotation in pure water (*30*). The slower rate and resulting lower concentration of ring-opened glucose in intracellular water is because the aqueous media is non ideal (much less than unity) due to the high concentration of macromolecules and elevated osmolarity (*31*). 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 (*32*). Thus, the likelihood that the ring-opened structure in the erythrocyte would be available to bind to HbA is exceedingly 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 ca. 50,000: 1), makes specific binding of the ring-opened form untenable.

Glucose Docking Computations with HbA: Amino Acid Residue Selectivity. 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 (*1, 5, 23, and 27*). 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, can be the singular source of reactive glucose within the glycation process (if the ring-closed glucose isomers that bind preferentially are unreactive). The data, however, does not preclude the option of bound ring-closed anomers undergoing mutarotation while bound.

If one asserts that initially-bound pyranose and furanose anomers can non-covalently bind and then mutarotate while bound so as to generate the reactive ring-opened structure (Fig. 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 reactive 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 DFT 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 to present a reactive 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 (Fig. 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 (*33*). 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 (*34*). 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. Second, 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) (*17*). 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 (*35*). 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, non-covalent 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 non-productive binding event occurs. The data presented in Table I addresses only those interactions whereby binding is potentially productive. Over 15 different non-productive 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. Second, the binding events that are potentially productive (and those that are not) are almost certainly reversible. Interactions at HbA with binding exothermicities less than 5 kcal/mol are expected to be reversible. Thus, the reversibility of the initial binding of glucose has implications even on 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. Third, 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 (ca. 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 (*36*-*37*) relative to hemoglobin at 5 mM (assuming a molecular weight of 68,000) (*38*) and the glucose structures at 4 mM (*39*). 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 (*40*). 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, (1) phosphate

enhances the rate of glucose mutarotation in solution (*41*); (2) phosphorylated sugars undergo significantly higher rates of HbA glycation than does glucose (by up to several orders of magnitude) (*42*); and (3) erythrocyte BPG levels are positively correlated with glycated HbA (*43*). BPG is present in the erythrocyte (cell) at concentrations of ~6 mM (*44*), is known to bind to HbA (*45*), and can increase HbA_{1C} formation (*25, 46*). Moreover, Gould et al. (*47*) reported higher intra-cellular concentrations of BPG in high glycators (N = 7, 5.61 ± 0.26 mM) versus low glycators (N = 5, 4.81 ± 0.24 mM). For comparison, the concentration of glucose (pyranose rings) in the erythrocyte (cell) is ~4 mM in fasting, non-diabetic humans (*39*), whereas the concentration of fructose (furanose rings) in plasma from fasting humans is just 31 μM (*48*). 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 (*44*) 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 (*49*), making HCO_3^- participation in HbA glycation a major point of consideration.

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

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

	Anionic Binding Molecules			
AA Residues	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" are those amino acid residues that can theoretically be glycated as defined as having the nucleophilic nitrogen of that residue within 6Å of the anion. This is predicated upon the assumption that a 3Å variation within the binding environment is possible and is consistent with the geometric parameters set forth by Ito (*27*).
ii. BPG (2,3-bisphosphoglycerate)
iii. Non-covalent 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.

The question that now arises is this: what is the effect that these anions have on the chemical mechanism whereby the aldimine/Schiff base is generated (Stage 1-Stage 2 transition).

HbA Glycation: Acid and/or base catalysis facilitation of bound glucose ring opening by amino acid residues only. In the absence of concomitant binding of facilitating reagents, the only potential acid and/or base catalysts available to facilitate ring opening of glucose are proximate amino acid residues. The amino acid residues within HbA that can potentially

serve as acid catalysts towards bound glucose ring opening are Asp and Glu, while those suitable for base catalysis are Lys, Arg, and His. The identity of the acidic and basic amino acid residues and the relative geometries for the residues relative to the bound glucose differ both from cavity to cavity for the same initially-bound isomer (Table IV) and also differ based upon the identity of the initially-bound isomer (α- vs. β-pyranose; Tables IV and V). Further, the charge state of the acid and base residues and their respective pKa values also vary. Hence, if any of the mechanism at this stage of the overall glycation process is rate determining, then in the absence of concomitantly-bound facilitating reagent, the rate determining step may differ for differing cavities within the same protein and even differ based upon which initially-bound ring-closed glucose anomer binds.

HbA Glycation: Acid and/or base catalysis facilitation of bound glucose ring opening by amino acid residues in conjunction with a single physiological anion. The physiological anions of interest for glycation of HbA in the current study are Pi and bicarbonate. These anions are termed physiological based upon their abundance relative to glucose (present at ca. 4-5 mM) in a physiological setting (i.e., the cytosol of the erythrocyte). Inorganic phosphate, Pi, consists of H_3PO_4 and the related potassium and sodium salts of mono- and di-basic phosphate ($H_2PO_4^-$ and HPO_4^{-2}), respectively. These various forms of Pi are all present in the human erythrocyte in a pH-dependent dynamic equilibrium at a composite

concentration of 1.8 mM (*44*). Bicarbonate (HCO_3^-) exists in concentrations in human erythrocytes of 15.4 and 17.4 mM in arterial and venous blood, respectively (*49*).

HbA cavities that would enable glycation of Val1 and Lys82 on the β-chain (among other sites known to be glycated) can concurrently bind each of the sugar anomers/isomers and either Pi or bicarbonate (Table VI). As such, the sugar isomer and the physiological anion can theoretically reside in the same cavity *at the same time*. In assessing the relative extent of enhanced glucose anomer ring-opening that results from interaction with each of the three physiological anions, it was determined that Pi-facilitated α- to β-pyranose transformation is ca. 30 times faster than benchmark (the rate in the absence of any added facilitating reagent; Table VII). This observed enhancement reflects the equilibrium composite interchange between ring opening ($k_{ro\alpha}$ and $k_{ro\beta}$) and ring closure ($k_{rc\alpha}$ and $k_{rc\beta}$). Because $k_{ro\alpha}$ and $k_{ro\beta}$ are ca. 1000 times slower than $k_{rc\alpha}$ and $k_{rc\beta}$ (*50*), the primary effect of the anion is most likely the facilitation of ring opening. The time to transform pure α-pyranose to a 50/50 mixture of α- and- β-pyranose in a 1:1 α-pyranose: bicarbonate mixture in water is ten times shorter that of benchmark (Table VII). Thus, as the basicity of the facilitating agent (physiological anion) is increased the rate of glucose ring opening in aqueous media (with no protein involved) is increased. Thus, the predominant facilitation in aqueous media is via the facilitating agent acting as a base.

If a physiological anion binds in a cavity that also has a bound glucose (either the α- or the β-pyranose isomer), then the anion can, theoretically, serve as an acid catalyst, a base catalyst, or both. Five potential mechanisms for physiological anion-facilitation towards glycation are proposed (Figure 5), each involving the anion *directly* increasing the extent of ring-opening of the protein-bound pyranose isomer.

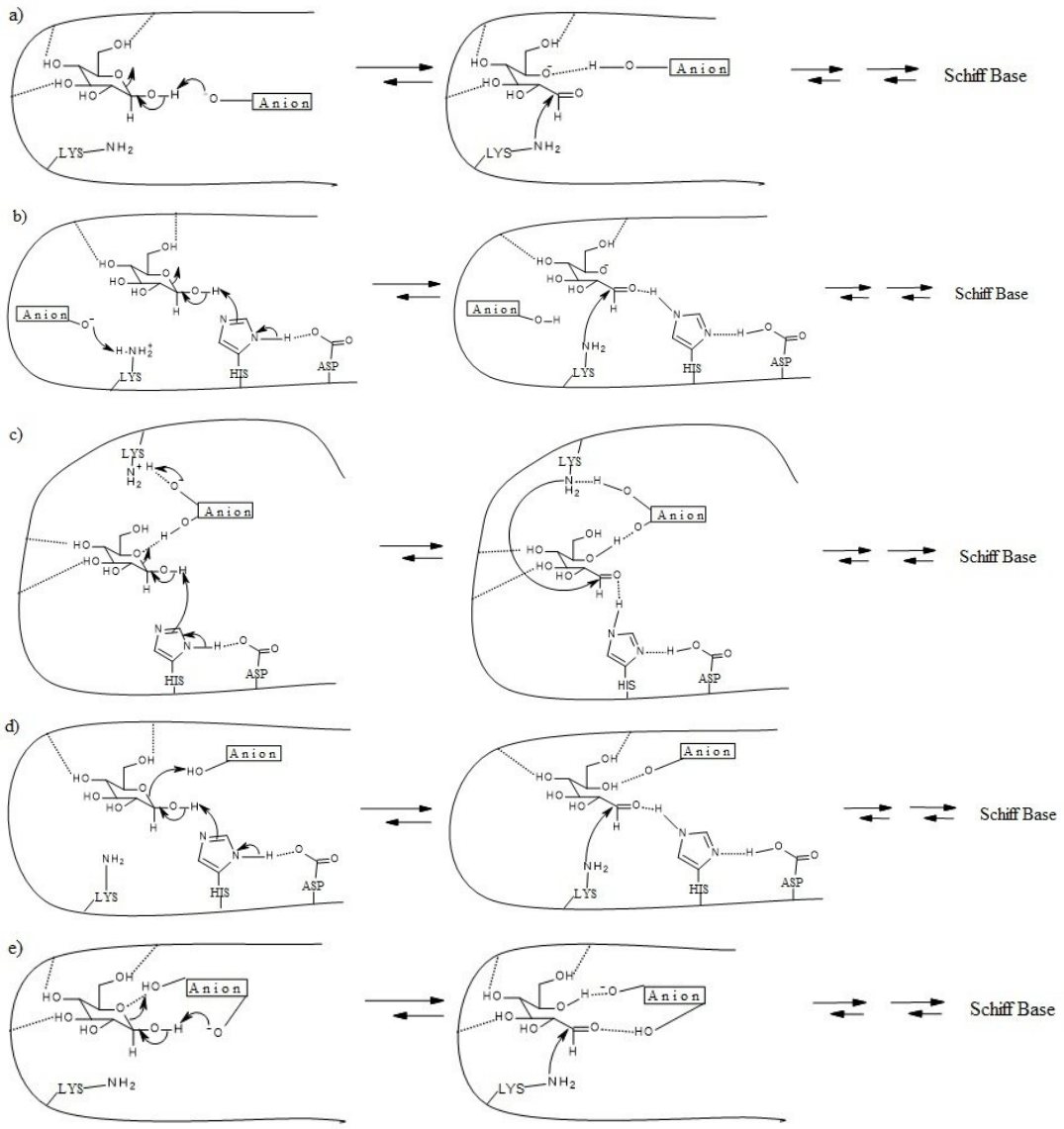


Figure 5. Potential mechanisms for ring opening of a bound pyranose ring in the presence of one physiological anion based upon the data from Tables IV-VIII.

First, the anion can act as a base alone deprotonating the pyranose hydroxyl at C1 with vastly greater basicity than water (Figure 5, process a). For this process to result in facilitated nucleophilic attack leading to Schiff base/imine the Lys/Val1 must be deprotonated and stabilization of the O5 anion by an amino acid residue, by water or by the anion is necessary. Second (Figure 5, process b), the physiological anion can act as a base alone in deprotonating the previously protonated Lys/Val1, making the previously non-nucleophilic amine nucleophilic. For this process to result in facilitated Schiff base/imine formation an amino acid residue (e.g. histidine) must act as the base on the hydroxyl on C1 of the pyranose and stabilization of the O5 anion by an amino acid residue or water is also necessary. This process, invoked here at the pre-Amadori stage, is analogous to the "phosphate triangle" mechanism proposed by Ito to explain Amadori formation in HbA (*51*). A third direct role of the physiological anion (Figure 5, process c) is to bridge between the Lys/Val1 and the O5 of the pyranose and act as both a base (to deprotonate the previously protonated Lys/Val1) and as an H-bond donor with the incipient O5 anion, facilitating ring opening that must also involve an amino acid residue acting as a base to deprotonate the C1 hydroxyl. Alternatively, if the Lys/Val1 is already deprotonated and an amino acid residue (e.g. histidine) deprotonates the C1 hydroxyl, then the physiological anion can facilitate pyranose ring opening by H-bond stabilization of the

incipient O5 anion (Figure 5, process d). Finally, the physiological anion may form a bridge with the pyranose ring (Figure 5, process e) similar to the water bridging observed in aqueous media (*52-54*). If the anion bridges on the glucose, it can serve as both base (deprotonating the C1 hydroxyl) and acid (stabilizing the incipient O5 anion). Such an interaction is plausible because the probability of tight ion-pair formation, bridging interactions (*55*) and asynchronous overlapping acid/base interactions is increased in proteins relative to aqueous solution (*56-57*). Ab Initio calculations at the HF-631G*B3LYP were conducted to see if stable bridged adducts between glucose and the physiological anions can form in the absence of water. While such adducts are unfavorable in polar aqueous media owing to a vast molar excess of accessible water molecules that compete as H-bond donors/acceptors, the likelihood of bridging adduct formation in a protein environment is much higher in the vastly less polar environment involving little or no water. A β-pyranose/monobasic Pi bridging adduct is calculated to be ca. 29.88 kcal/mol stabilized relative to isolated starting materials in the gas phase. The bicarbonate (HCO_3^-) anion is computationally predicted to bridge with β-pyranose with an exothermicity of 26.05 kcal/mol.

HbA Glycation: Acid and/or base catalysis facilitation of bound glucose ring opening by amino acid residues in conjunction with multiple physiological anions. The ring-closed α- and β-pyranose anomers can concomitantly bind in many combinations with any two of the three

physiological anions; specifically at Val1, Lys17, Lys59, and Lys82 (Table VIII). The multiple physiological anions in these binding scenarios can function in multiple mechanistic roles (combinations of the mechanistic possibilities depicted in Figure 5). Any combination of these reactive outlets is possible, and any combination likely would enhance ring opening of bound glucose and potentially have overall rate implications. Such is not the case for those cavities on the β-chain of HbA where glucose binds and where glycation is known to occur that *cannot* accommodate concomitant binding of glucose anomers with two physiological anions. These cavities are those involving Lys61, Lys65, Lys66, Lys95, and Lys144 (Table VIII). Thus, the contribution from glucose co-binding with multiple physiological anions is variable from cavity-to-cavity, is manifest likely with a diversity of acid/base mechanisms, and the resulting rate implications are as well quite variable.

2.4 Conclusion

In conclusion, all 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 specificity. The most plausible means to present a reactive ring-opened glucose to the nucleophilic amino acid residues for HbA glycation is the non-specific 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 (Fig. 3). BPG, Pi, and

HCO_3^- also reversibly bind to HbA with similar energies and common binding sites with the glucose isomers. Based on the results of the experiments regarding the physiological anions and the different roles these anions could possibly play in the glycation mechanism more experiments are needed to further our understanding of these specific anions; however, we believe that there is no one singular glycation mechanism and that the facilitation of these physiological anions may be both site and anion specific.

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Chapter 3. A comparison of the initial noncovalent binding of D-glucose and D-fructose with hemoglobin and albumin in nonenzymatic glycation.

3.1 Introduction

In this and in all previous work, the prevailing assumption is that a bound ring-opened glucose is the *singular* reactive structure that leads to the formation of an aldimine/Schiff base (*1-2*). The basis for this assumption is that the ring-opened glucose is the most electrophilic isomer because it possesses a carbonyl group and should therefore be the most reactive species. Interpretations from our computational study involving Stage 1 substrate binding to crystal structures of HbA (reported upon in Chapter 2) reveal that the transient, ring-opened glucose is likely not directly involved in initial binding to the β-chain of HbA (*3*). The most plausible means to present a ring-opened glucose to the amino acid residues for HbA glycation is the initial binding of the α- and β-pyranoses and, to a lesser extent, the α- and β-furanoses (Figure 1) followed by ring opening to generate the ring-opened glucose while bound. This transient non-covalently bound ring-opened electrophilic glucose can then proceed to a covalently-bound Schiff base/aldimine (Stage 1 to Stage 2 transition) via reaction with a deprotonated N-terminal Valine (α-amino group) and/or internal Lysine (ε-amino group) acting as the nucleophile.

A comprehensive description for the HbA glycation process, factoring together our recent interpretations (*3*) with previous investigations and their suggestions (*4-7*), is a multi-step process in the following sequential stages: 1)

reversible, non-covalent binding of a ring-closed glucose isomer that reversibly mutarotates to a transient ring-opened isomer while bound to select sites on the protein, 2) reaction between the bound transient ring-opened electrophilic glucose isomer and one of several deprotonated nucleophilic amine residues to generate a covalently-bound Schiff base/aldimine, 3) rearrangement of the Schiff base/aldimine to a Amadori intermediate (amino-1-deoxyfructose) and 4) the non-reversible formation of AGE.

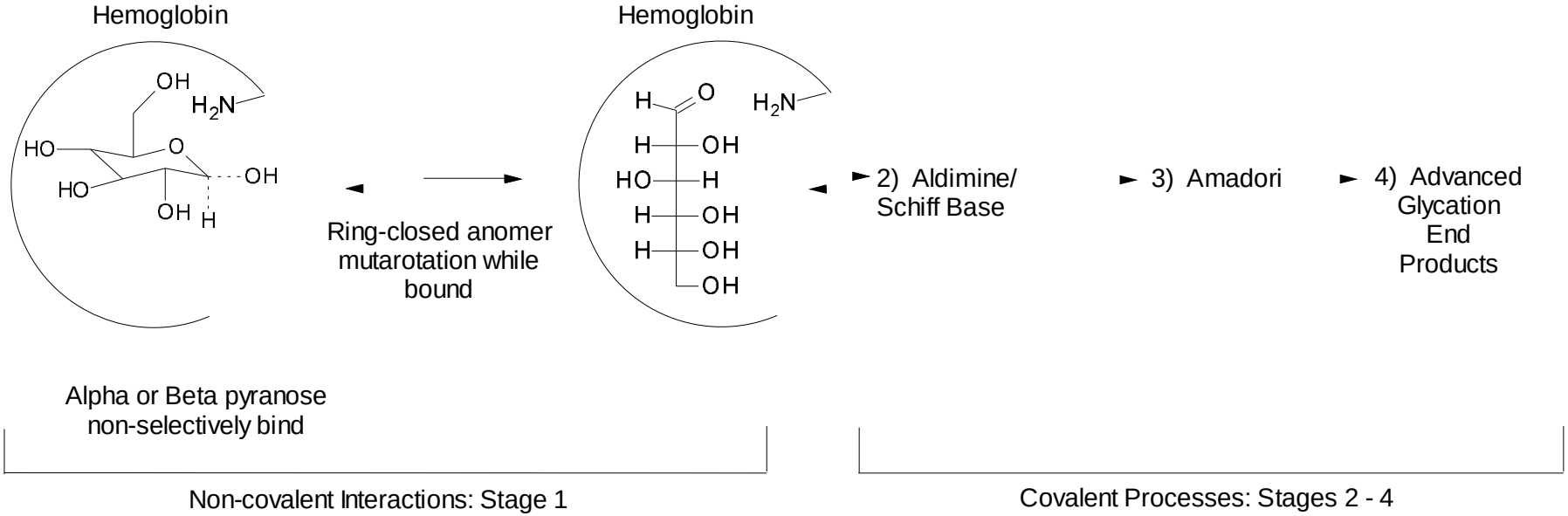

Figure 1. Potential process for presenting reactive ring-opened glucose to hemoglobin (Stage 1) and subsequent steps leading to glycation and production of advanced glycation end products. Unlike previous models, this process involves non-selective binding of ring-closed anomers and mutarotation while bound. The reaction arrows reflect the reversibility of each interaction/reaction.

The first objective for the research reported upon in this chapter is to determine whether other monosaccharides share the same initial binding characteristics as glucose; namely that they undergo reversible initial HbA binding with low amino acid residue selectivity and bind as ring-closed species that then ring opens while bound. Fructose, an increasingly important sugar, and galactose (another reducing sugar similar to glucose) and xylulose are models for assessing this question. Our second research objective is to test whether proteins other than HbA, such as human albumin (HSA), also undergo initial reversible monosaccharide binding with low isomer specificity and low amino acid residue selectivity. HSA was chosen as a model protein because it is extracellular rather than intracellular (like HbA) and is a major component of fructosamine measurements for monitoring of long-term glycemia in diabetes mellitus. The third objective for the research reported upon here is a clinical rather than mechanistic. A major clinical goal with relevance to monitoring glycemia in patients is to predict the long-term average blood glucose levels. Two methods can be applied towards this end. Intracellular levels of HbA1c can be measured and used as a predictor of average serum glucose concentration. Extracellular glycated HSA (fructosamine) concentrations can also be measured and used to predict average serum glucose concentration. If the two clinical measures predict the same serum glucose concentration, then that patient does not possess a "glycation gap." If the two predictors are disparate, that patient has a glycation gap (proportional to the difference in the two predicted levels). The

glycation gap has been shown clinically to correlate directly to the pathology of nephropathy *(8)*. Two prevailing explanations for why a glycation gap exists for certain patients have been forwarded: a) inter-individual differences in the glucose gradient across the red cell membrane and b) differences in protein life span (*9-10*). Thus, the third and final research objective for the work in this chapter is to assess whether physiological anions can affect HSA glycation.

3.2 Results

Glucose Docking Computations with HbA: Isomer Specificity. Isomer specificity is a physicochemical attribute of a protein (in this case, HbA) in relation to interaction with a manifold of isomers (in this case the 5 glucose isomers). If only a single glucose isomer was to bind to HbA, specificity would be high. Low specificity occurs when multiple sugar isomers bind. The ring-closed α- and β-pyranose isomers 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 I). That said, the thermodynamic values are within ca. 1 kcal/mol of each other. The exothermicities for the binding of the glucofuranose anomers are less than that for the pyranose and ring-opened structures (ca. 1.5 and 0.5 kcal/mol less exothermic than the pyranose and ring-opened structures, respectively). Overall, the binding of the five glucose structures to a β-chain of HbA is not specific for any one isomer. Thus, the initial specific 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.

Table I: Summary of Autodock binding glycation sites (on the β-chain of oxygenated human HbA) organized by sugar bound, binding exothermicity and potentially glycated amino acid residue.

Bound Sugar Isomer	Energy	Potentially productive amino acid residues*
α-glucopyranose	-5.1 to -4.1	Val1; Lys17; Lys59; Lys66; Lys82; Lys120, Lys144
β-glucopyranose	-5.3 to -4.2	
α-glucofuranose	-4.0 to -2.1	
β-glucofuranose	-3.5 to -1.8	
Ring-Opened Glucose	-4.3 to -2.7	
α-fructopyranose	-3.5 to -1.9	Val1; Lys17; Lys66; Lys82, Lys120,
β-fructopyranose	-5.0 to -4.3	
α-fructofuranose	-3.6 to -2.1	
β-fructofuranose	-3.2 to -2.0	
Ring-Opened Fructose	-4.3 to -3.4	
α-galactopyranose	-2.8 to -0.8	Val1; Lys17; Lys59; Lys66; Lys82; Lys120
β-galactopyranose	-2.8 to -1.3	
α-galactofuranose	-1.7 to -0.1	
β-galactofuranose	-1.7 to -0.3	
Ring-Opened Galactose	-0.4 to +1.0	
α-xylulose	-2.4 to -1.1	Val1; Lys17; Lys59; Lys66; Lys82; Lys120
β-xylulose	-2.6 to -1.1	
Ring-Opened Xylulose	-1.5 to +0.3	

i: The first column labeled "sugar" is organized by sugar isomer bound

ii. The energy values are binding exothermicities reported as ΔG in kcal/mol for the range of stable conformations for the listed potentially productive amino acid residues listed in column 3.

iii. The third column lists those nucleophilic valine or lysine amino acid residues that can theoretically be glycated within the listed cavity(ies) as defined as having the reactive centers within 9Å (*11-12*)

iv. *These amino acid residues represent a composite of all the potentially productive residues across all of the assessed isomers/anomers.

Fructose Docking Computations with HbA: Isomer Specificity. Each of the five fructose isomers (ring-closed α- and β-pyranose, ring-closed α- and β-furanose, and the ring-opened isomer) bind to a β-chain of HbA with exothermicities within 1.5 kcal/mol of each other. Overall, the binding of the five fructose isomers is likely not specific for any one isomer and is certainly not specific for the ring-opened structure (-4.3 kcal/mol for the ring- opened isomer vs. -5.0 kcal/mol for the β-pyranose). Similar to the binding of the glucose isomers (exothermicities -5.3 to -1.8 kcal/mol), binding exothermicities for the fructose isomers ranges from -5.0 kcal/mol and -1.9 kcal/mol. Thus, like glucose binding, the initial binding of the fructose isomers is predicted to be non-isomer specific.

Glucose Docking Computations with HbA: Amino Acid Residue Selectivity. Binding of each of the five glucose structures to a β-chain of fully-oxygenated HbA results in low selectivity binding in multiple cavities leading to the potential glycation of seven different amino acid residues (Val1; Lys17; Lys59; Lys66; Lys82; Lys120, Lys144; Table I) of the 12 amino acid residues theoretically able to be glycated (11 lysines and the terminal valine).

Fructose Docking Computations with HbA: Amino Acid Residue Selectivity. The fructose isomers bind to a β-chain of oxygenated HbA with slightly more selectivity than do the glucose isomers (five potential glycation residues for fructose vs. seven for glucose; Val1; Lys17; Lys66; Lys82, Lys120). That said, because five different amino acid residues can be

glycated, the initial binding of the fructose isomers must still be considered to proceed with low amino acid residue selectivity.

Galactose and Xylulose Docking Computations with HbA. To test the generality of these observations, two additional monosaccharides (galactose and xylulose) were tested for isomer specificity and amino acid residue selectivity in initial binding to HbA (Table I). Neither of these monosaccharides bind as well as do either glucose or fructose. Specifically, the isomers of galactose bind to HbA with a range of -2.8 kcal/mol to an endothermic value of +1.0 kcal/mol. The xylulose isomers bind to HbA with a range of -2.6 kcal/mol to an endothermic value of +0.3 kcal/mol. Both of these monosaccharides also exhibit low isomer specificity. The ring-closed isomers of galactose and xylulose bind with exothermic values (-2.8 to -0.3 kcal/mol) while the ring-opened isomers begin with exothermic values and end with endothermic values (-1.5 to +1.0 kcal/mol). While neither galactose nor xylulose generally bind as well as either fructose or glucose, like the other monosaccharides, the binding will occur with low amino acid selectivity. The galactose isomers and the xylulose isomers bind to the same array of HbA cavities, those that can lead to the potential glycation of Val1; Lys17; Lys59; Lys66; Lys82; and Lys120. With the exception of Lys144, these are the identical amino acid residues potentially glycated by the glucose isomers. With the exception of Lys59, these are also the identical amino acid

residues potentially glycated as those observed for the binding of the fructose isomers (Table I).

Glucose Docking Computations with HSA: Isomer Specificity.

The ring-closed α- and β-pyranose isomers of glucose bind more exothermically to HSA than does the ring-opened glucose isomer (-5.7, -5.5, and -4.8 kcal/mol, respectively; Table II), albeit the thermodynamic values are within ca. 1 kcal/mol of each other. The exothermicities for the binding of the glucofuranose anomers are less negative than that for the pyranose and ring-opened structures (ca. 1.5 and 0.5 kcal/mol, respectively). As such, no selective binding of ring-opened glucose is predicted.

Table II: Summary of Autodock binding exothermicity for each of the glucose isomers with human serum albumin (HSA) proteins.

Monosaccharide	Energy	Potential glycated Lysine residues
α-glucopyranose	-5.7 to -4.5	199, 432, 436, 524, 545
β-glucopyranose	-5.5 to -4.5	64, 73, 106, 432, 436, 536, 538, 541, 545
α-glucofuranose	-4.4 to -1.5	20, 64, 73, 106, 159, 174, 190, 195, 199, 212, 274, 276, 281, 286, 323, 351, 413, 414, 432, 436, 466, 500, 524, 534, 536, 538, 541, 545
β-glucofuranose	-4.2 to -1.3	20, 93, 106, 136, 137, 159, 174, 195, 205, 212, 233, 286, 313, 317, 323, 402, 414, 432, 436, 475, 519, 524, 525, 534, 536, 538, 557, 560, 564
Ring-Opened Glucose	-4.8 to -3.3	64, 73, 190, 195, 199, 205, 402, 432, 436, 519, 524, 536, 538, 541, 545

i: The energy values are binding exothermicities reported as ΔG in kcal/mol for the range of the most stable conformations that could lead to potential glycation.

ii: Potential glycation is defined here as those conformations in which a nucleophilic lysine amino acid residue can theoretically be glycated within the binding cavity having the reactive centers within 9Å (*11-12*).

Glucose Docking Computations with HSA: Amino Acid Residue Selectivity. Binding of each of the five glucose isomers to HSA is predicted to occur with exceedingly low amino acid residue selectivity. Composite binding of the five glucose isomers is predicted to involve cavities leading to the potential glycation of 43 different amino acid residues (out of 59 possible nucleophilic lysine amino acid residues in HSA; Table II). That said, the glucopyranoses bind with greater (yet still low) site selectivity (11 sites) than do the other isomers.

Fructose Docking Computations with HSA: Isomer Specificity. The ring-closed β-pyranose isomer of fructose binds with similar exothermicity to HSA than does the ring-opened fructose isomer (-5.7 vs. -5.6 kcal/mol; Table III), which binds better than α-fructopyranose (-4.4 kcal/mol), followed by the fructofuranoses (-4.0 and -4.1 kcal/mol for the α- and β-furanoses, respectively).

Fructose Docking Computations with HSA: Amino Acid Residue Selectivity. Binding of each of the five fructose structures to HSA is predicted to occur with exceedingly low amino acid residue selectivity. Independent binding of the five fructose isomers is predicted to involve cavities leading to the potential glycation of 39 different amino acid residues (out of 59 possible nucleophilic lysine amino acid residues in HSA; Table III). The β-pyranose isomer of fructose binds with greater site selectivity (10 sites) than do the other isomers (that range from 15 and 31 sites).

Table III: Summary of Autodock binding exothermicity for each of the fructose isomers with human serum albumin (HSA) proteins.

Sugar	Energy	Potential glycated Lysine residues
α-fructopyranose	-4.4 to -1.5	12, 20, 106, 136, 137, 181, 190, 195, 199, 205, 212, 274, 313, 317, 323, 351, 402, 413, 414, 432, 436, 500, 519, 525, 534, 536, 538, 541
β-fructopyranose	-5.7 to -4.1	106, 190, 199, 205, 317, 432, 436, 524, 536, 545
α-fructofuranose	-4.0 to -1.2	20, 65, 73, 106,174, 181, 190, 195, 205, 225, 276, 313, 317, 323, 402, 413, 432, 436, 524, 534, 536, 538, 545
β-fructofuranose	-4.1 to -1.8	20, 51, 93, 106, 162, 174, 181, 190, 195, 199, 212, 225, 313, 317, 323, 413, 414, 432, 436, 524, 534, 536, 538, 541, 545
Ring-Opened Fructose	-5.6 to -3.0	159, 190, 195, 199, 317, 413, 414, 432, 436, 519, 524, 534, 536, 541, 545

i: The energy values are binding exothermicities reported as ΔG in kcal/mol for the range of the most stable conformations that could lead to potential glycation.

ii: Potential glycation is defined here as those conformations in which a nucleophilic lysine amino acid residue can theoretically be glycated within the binding cavity having the reactive centers within 9Å (*11-12*).

Glucose/Physiological Anion Adduct Docking Computations with HSA: A computational investigation was undertaken to assess whether the ring-closed pyranose anomers and/or the ring-opened glucose isomers and a single physiological anion (Pi or HCO_3^-) can bind non-covalently to common cavities within HSA. Based on the exothermicities obtained there is no selective binding of the ring-open isomer adducts (Table IV). Amongst the three different types of physiological anion adducts there is no greater than a 1.5 kcal/mol difference amongst the glucose isomers. Similar to glucose binding to HSA alone there is no observed amino acid residue selectivity. Amongst the three types of physiological anion adducts there are 42 different Lysine residues (out of a possible 59 glycatable residues) that will accommodate the binding of both a glucose isomer as well as a physiological anion.

Table IV. Summary of Autodock binding of the α-and β- pyranose anomers and the ring-opened isomer of glucose within the β-chain of oxygenated human HSA.

Sugar	Anion	Binding ΔG in kcal/mol	Potentially glycated residues
α_6	Dibasic Pi	-5.9	137, 162, 190, 195, 199, 212, 240, 274, 281, 378, 389, 402, 413, 414, 432, 436, 444, 475, 500, 519, 524, 525, 534, 536, 541, 545, 557, 564,573, 574
β_6	Dibasic Pi	-6.0	12, 20, 159, 162, 190, 195, 199, 225, 351, 359, 402, 413, 414, 432, 436, 444, 519, 524, 534, 541, 542, 545, 557, 573, 574
RO	Dibasic Pi	-5.9	64, 159, 190, 195, 199, 225, 286, 323, 372, 378, 402, 413, 414, 432, 436, 519, 524, 534, 536, 545, 557, 560, 573, 574
α_6	Bicarbonate	-7.6	190, 195, 199, 351, 359, 402, 413, 414, 432, 436, 500, 519, 534, 545, 557, 560
β_6	Bicarbonate	-7.2	190, 195, 199, 359, 372, 402, 413, 414, 432, 436, 500, 519, 534, 541, 545, 573, 574
RO	Bicarbonate	-6.3	190, 195, 199, 225, 276, 359, 372, 402, 413, 414, 432, 436, 439, 500, 519, 534, 541, 545, 557, 560, 574

i: Binding exothermicities are reported as ΔG in kcal/mol for the most stable conformation generated upon docking glucose and a physiological anion to the β-chain of fully-oxygenated HbA and are reported as a range.

ii: The fourth column designates the nucleophilic lysine or valine amino acid residue that can theoretically be glycated within that cavity as defined as having the nucleophilic nitrogen of that residue within 9Å of the bound sugar (*11-12*).

3.3 Discussion

Monosaccharides Compared: Glucose, Fructose, Galactose, and Xylulose. Fructose, galactose, and xylulose were compared with glucose to test for the generality of monosaccharide noncovalent binding to HbA. The predominant dietary simple carbohydrates include the disaccharides sucrose, maltose, and lactose and the monosaccharides fructose and glucose (*13*). Following intestinal digestion and absorption of the simple carbohydrates, glucose, fructose, and galactose are the predominant species generated. The fructose and galactose will largely be converted to glucose or glycolytic intermediates in the liver, yet these monosaccharides can be made available to proteins prior to conversion. The concentration of fructose and galactose in plasma averages just 31 μM (*14*) and 1.5 μM (*15*), respectively, in nondiabetic subjects. D-fructose is of particular interest based upon acute metabolic and hemodynamic responses to this monosaccharide (*16*), and the possibility that excessive fructose intake contributes to several chronic health disorders including obesity, diabetes, and cardiovascular disease (*17-18*). The majority of dietary fructose comes from two sweeteners, disaccharide sucrose (consisting of 50% fructose and 50% glucose) and high-fructose corn syrup (HFCS). HFCS exists in multiple formulations, each of which arises from corn syrups that have undergone enzymatic processing. These formulations contain anywhere from 30-55% fructose and are used in breads, cereals, breakfast bars, lunch meats, yogurts, soft drinks, soups, and condiments. The HFCS used in soft drinks consists of 55% fructose and 42% glucose (*19*). Consumption of one 24 oz carbonated soft drink

sweetened with HFCS by healthy, non-diabetic subjects results in fructose increasing from 5.4 μM to nearly 300 μM after just 30 min and not returning to baseline values until approximately 4 h (*16*). Corresponding glucose values increased from 4.5 mM to 6.3 mM, peaking after just 30 min and returning to baseline after 90 min. It should be noted that neither D-galactose nor D-xylulose will be found in significant concentration in the erythrocyte of adult humans. Thus, physiological interaction with HbA is unlikely. That said, D-galactose is found in blood, particularly in infants (galactose is the sugar found in milk) and in adults from societies that regularly consume milk will also exhibit higher than normal levels in the blood (*20*). D-xylulose is not a physiologically relevant monosaccharide but is investigated here so as to provide information relevant to the question; do other reducing sugars besides D-glucose bind to HbA as a ring-closed species and then open while bound?

D-glucose undergoes reversible mutarotation in aqueous media whereby five different isomers interconvert (Figure 2a). 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 aldehyde (making glucose an aldose). The corresponding equilibrium distribution of the five glucose structures is: 35% α-pyranose, 64% β-pyranose, less than 1% for the sum of α- + β-furanoses (*21*), and just 0.002%-0.004% for the ring-opened isomer (*22*).

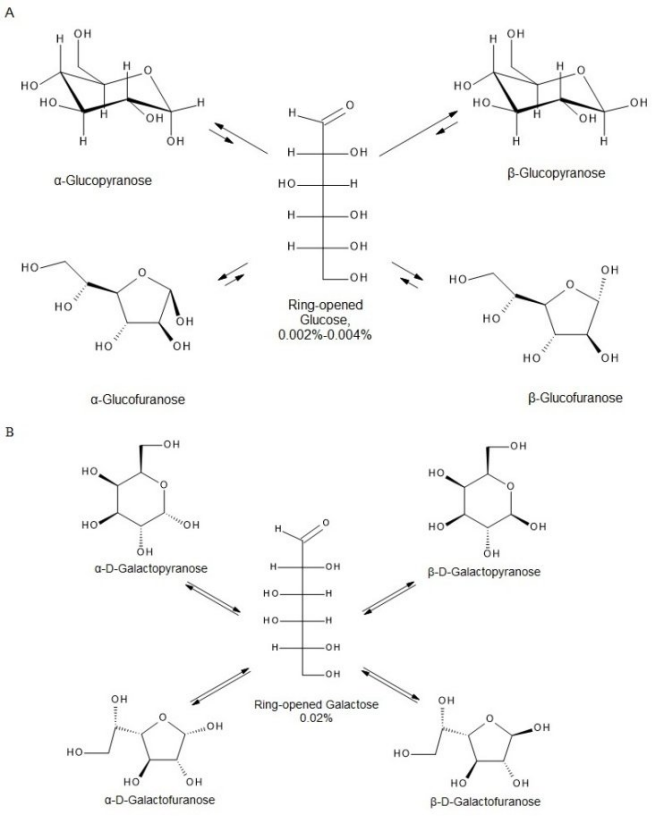

Figure 2. Isomer manifolds for the aqueous mutarotation of D-glucose (A) and D-galactose (B). The ring-opened isomer is an aldehyde in each manifold (D-glucose and D-galactose are thus aldoses).

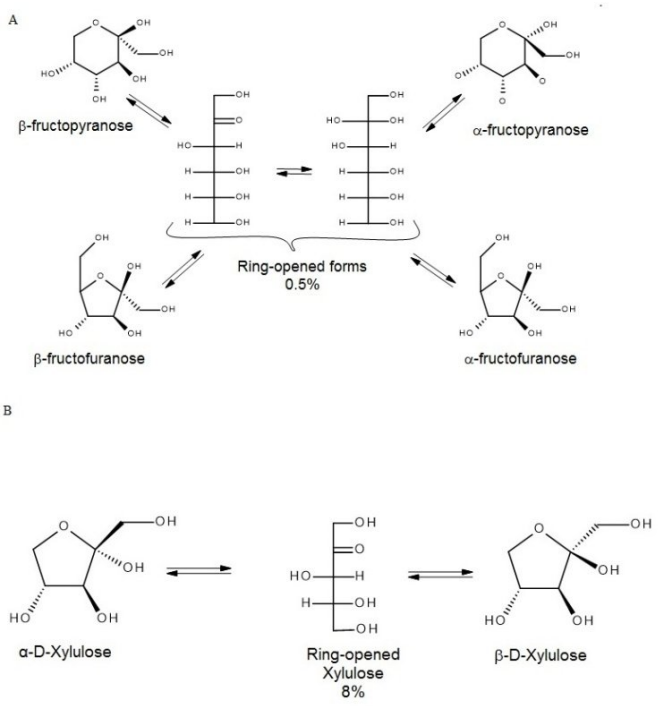

Figure 3. Isomer manifolds for the aqueous mutarotation of D-fructose (A) and D-xylulose (B). The ring-opened isomer is a ketone in each manifold.

The mutarotation of fructose involves five interconverting isomeric species (Figure 3a): β-fructopyranose (68% at equilibrium), α-fructopyranose (3%), β-fructofuranose (22%), α-fructofuranose (3%), and a ring-opened ketone isomer (0.5%; fructose is a ketose). At equilibrium in aqueous solution the ring-opened form of glucose is 0.002% (relative to all glucose isomers). As such, the ring-opened fructose isomer (a ketose) is ca. 250 times more abundant (compared to the cyclic fructose isomers) than is the ring-opened glucose isomer (compared to the cyclic glucose isomers). However, there is a significant contribution from a fructose ring-opened hydrate form (not an isomer) that arises from reaction

between water and the ring-opened fructose isomer (*23*). The hydrate does not possess an electrophilic carbon and cannot proceed to Schiff base/ketimine.

D-galactose has four isomeric ring-closed isomers (Figure 2b) with a singular ring-opened form, an aldose, through which the four isomers interconvert. At equilibrium in aqueous solution the ring-opened form of ring-opened galactose isomer is at 0.02% (*1*) which is 10 times more abundant (relative to all galactose isomers) than is ring-opened glucose relative to all glucose isomers. Finally, D-xylulose exists at equilibrium in two ring-closed anomeric forms with the ring-opened form (a ketose) found in an abundance of 8.0%. The ring-opened form of xylulose is ca. 4,000 times more abundant than that for glucose at equilibrium (*1*).

Docking Computations with HbA: Glucose, Fructose, Galactose, and Xylulose Isomer Specificity. Based on the computational binding of the five isomers of glucose to fully oxygenated human hemoglobin, no preferential binding of the ring-opened glucose anomer to the protein is predicted (Table I). If any binding selectivity is manifest, it would be in favor of ring-closed glucopyranose anomers. This prediction is consistent with the initial binding of ring-closed glucose isomers that ring open upon binding (*3*). In the computational assessment of the initial binding of the isomers of fructose, galactose, and xylulose to HbA, each monosaccharide is predicted to bind with low isomer specificity. No specificity towards binding the ring opened form of any of these monosaccharides exists (based upon binding

exothermicities). Thus, if any of these monosaccharides are to proceed to glycated protein then, like glucose, the initial binding will involve ring-closed species that bind and ring open while bound.

Docking Computations with HbA: Glucose, Fructose, Galactose, and Xylulose Amino Acid Residue Selectivity. The manifold of glucose isomers noncovalently bind to a β-chain of HbA in multiple protein cavities that can theoretically lead to the glycation of seven residues (Val1, Lys17, 59, 66, 82, 120, and 144; Table I). As such, low amino acid residue selectivity is predicted. Interestingly, the α- and β-glucopyranoses and the ring-opened glucose bind in the same cavities, consistent with the premise that the ring-closed α- and β-glucopyranoses binding and then ring open while bound. Is this the case for the other monosaccharides?

The manifold of fructose isomers also bind in multiple protein cavities that can theoretically lead to the glycation of five residues (Val1, Lys17, 66, 82, and 120; Table I). Like glucose isomer noncovalent binding, the fructose isomers also bind with low amino acid residue selectivity (sharing many residues in common with the glucose isomers). Further, the ring-closed fructose isomers and the ring-opened fructose isomer bind in common cavities with comparable energies (Table I). This is consistent with ring-closed fructose isomer binding followed by ring opening while bound. Finally, like the manifold of glucose isomers, the manifold of both the galactose and xylulose isomers noncovalently bind with low amino acid residue selectivity. Further, the ring-closed isomers and the ring-opened

isomers bind in common cavities (relative to one another) consistent again with ring-closed isomer binding followed by ring opening while bound.

Docking Computations with HSA*:* There is a great deal of recent interest in the clinical significance of glycated HSA (*24*), specifically in terms of binding effects with various drugs, fatty acids and other solutes. Moreover, glycated HSA is viewed as a complimentary tool to glycated HbA_{1c} to monitor long-term glycemia in diabetic patients. That said, of the over 300 papers on HSA glycation since 2006, to the best of our knowledge, only one paper has addressed the initial binding of glucose to HSA (*25*).

Similar to HbA, the extracellular protein HSA can undergo glycation by circulating glucose and serve as short-term (2-3 week) marker of glycemia (*26*). If initial binding of monosaccharides to HSA also involves a ring-closed isomer that ring opens while bound (which is general for monosaccharide binding to HbA), then it is possible that HSA glycation rate may be affected by initial binding events (as is the case for HbA glycation rate). As such, understanding the initial binding of monosaccharides to HSA in terms of both isomer specificity and amino acid residue selectivity is important.

Docking Computations with HSA: Glucose and Fructose Isomer Specificity. Overall, the binding of the five glucose structures to HSA is not specific for any one isomer based upon thermodynamic exothermicities (Table II). Thus, the initial specific binding of the ring-opened glucose to HSA in the presence of the ring-closed structures (α- and β-pyranose and α-

and β-furanose) is unlikely. This mimics what is observed in initial noncovalent Stage 1 glucose binding to HbA. Our computations indicate that selective binding of the ring-opened fructose isomer to HSA is also unlikely (Table III). While the initial binding of the ring-opened fructose competes thermodynamically with the binding of the β-pyranose isomer of fructose (Table III), at equilibrium (in water) there will be ca. 150 available β-pyranose isomers of fructose for every ring-opened fructose. Hence, selective binding of the ring-opened isomer is unlikely and, overall, the binding of the five fructose structures to HSA is not specific for any one structure (Table III).

Docking Computations with HSA: Glucose and Fructose Amino Acid Residue Selectivity. The manifold of glucose isomers interact with HSA such that binding in many protein cavities is predicted, leading to the potential glycation of 43 different HSA lysine residues (Table II). This remarkably low amino acid residue selectivity is as well characteristic of fructose binding to HSA. Fructose binding to HSA can lead to 39 potentially glycated lysine residues (each of these in common with amino acid residues potentially glycated by the glucose isomers; Table II-III). Thus, like both glucose and fructose binding to HbA, the initial binding of these monosaccharides to HSA is predicted to proceed with low amino acid residue selectivity.

3.4. Conclusion

In general, HSA has a more exothermic initial noncovalent binding interaction with monosaccharide isomers than does HbA (ca. 20% relative increase in binding exothermicity; Table I compared to Table II-III). Neither protein exhibits isomer specificity with any of the monosaccharides investigated. While both proteins bind these monosaccharides with low amino acid residue selectivity, HSA (the more reactive protein), binds in cavities that can lead to the potential glycation of approximately three times as many residues as does the β-chain of HbA (modest variation depending upon what monosaccharide is bound). Further, our computations predict that HSA can have suitable geometry within binding cavities to make theoretically possible the glycation of 73% of the glycatable sites on the protein. In contrast, our computations predict only 58% of the total sites on the β-chain of HbA are theoretically glycatable sites. HSA is predicted to bind monosaccharides to a greater extent than does HbA. With either protein, and with any of the monosaccharides investigated, the initial binding is likely to be a binding of a ring-closed isomer, consistent with the prediction that has been forwarded by Wang and coworkers (*25*).

The comparison of HSA and HbA initial noncovalent binding with the four monosaccharides in this study supports the generality of the initial noncovalent binding of monosaccharaides to proteins as being energetically favored, transient, reversible events proceeding with low isomer specificity and low amino acid residue selectivity primarily involving ring-closed species that must ring open while bound if they are to proceed in the glycation process. While

the general binding trends for both HbA and HSA are the same, based on the binding of the glucose and physiological adducts it is possible that the presence of the physiological anions could have a greater effect for the initial binding event of HSA than for HbA. While the adduct binding for HbA is close exothermically to the binding of glucose alone, the adduct binding to HSA is much higher than the binding of glucose to HSA alone. This alone could aide in expanding the explanation for the glycation gap. The relative concentrations of physiological anions in patients' intracellular and extracellular compartments may also affect the extent of a patient's glycation gap. Consequently a deeper understanding of the possible explanations for the glycation gap will provide physicians with a better platform from which to treat their diabetic patients.

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Chapter 4. Unifying Methods

All binding data were obtained via computational methodologies. The protein structures utilized were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (http://www.pdb.org/pdb/home/home.do) (*1*). The crystal structure of both the oxygenated and deoxygenated Human Hemoglobin A (HbA) tetramer (id code 3B75 and 2DN2 respectively) (*2-3*) and the crystal structure of the Human Serum Albumin (HSA) dimer (1A06) (*4*) were cleaned using Deep View-Swiss PDB Viewer (WINE; http://spdv.vital-it.ch/wine.html) (*5*). Cleaning involved the removal of extraneous anions and duplicate structures. The same program was used to isolate the β chain of the fully oxygenated HbA as well as divide the HSA dimers. The ligands used to computationally dock to the proteins were obtained from multiple sources. Select monosaccharides: α- (glc) and β-glucopyranose (bgc) anomers, α- (gla) and β-galactopyranose (gal) anomers, and the ring-opened xylulose (xul) and the physiological anions BPG (dg2) and bicarbonate (bct), were obtained from Hetero-compound Information Centre Uppsala, (three-letter compound designations)(HIC-UP , (http://xray.bmc.uu.se/hicup/) (*6*). All other monosaccharides and physiological anions were generated using The GlycoBioChem PRODRG2 Server (*7*); (http://davapc1.bioch.dundee.ac.uk/prodrg/index.html). 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/). The glucose isomer adducts as well as the fructose isomer adducts were generated using the program Spartan (*8*). HbA, HSA, and all monosaccharides as well as monosaccharide-anion adducts were non-hydrated species.

Computational binding was performed on only one β-chain of HbA for simplicity in view of spatial resolution limitations. Moreover, the clinically relevant glycation site, specifically Val1 for HbA_{1C}, is on the β-chain where this residue accounts for approximately 30% of total glycation sites (9-13). Although focusing on one β-chain will not account for inter-chain interactions, this simplified approach is consistent with the objective and scope of these investigations.

Once the β-chain was isolated, the resulting file (xxxx.pdb) was imported into the AUTODOCK program (*14*, 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 ligands and the target protein structure are pre-calculated in a three-dimensional grid surrounding the binding region (our grid calculation was set at a spacing of 1Å, with X, Y, and Z dimension points set at 108, 126, and 126 respectively for the β-chain of HbA (*15*) with the x, y, and z center at -24.839, 7.221, and 98.244 respectively (0.375Å spacing). The coordinates for the HSA were more difficult to establish given the large size of the HSA dimer. The alpha and beta chains were separated from one another; the alpha chain was sectioned into three separate coordinate areas as follows: 126

points in all dimensions, 1) 16.35, 26.186, 4.517 (X, Y, and Z center respectively) and 0.375Å spacing; 2) 44.07, 30.752, 25.375 (X, Y, and Z center respectively) and 0.497Å spacing; 3) 16.125, 32.245, 38.647(X , Y, and Z center respectively) and 0.408Å spacing. The beta chain was sectioned into three separate coordinate areas as follows: 126 points in all dimensions, 1) 46.393, -4.444, 38.894 (X , Y, and Z center respectively) and 0.392Å spacing; 2) 5.268, -16.702, 35.112 (X , Y, and Z center respectively) and 0.392Å spacing; 3) 32.392, -17.927, 8.207 (X , Y, and Z center respectively) and 0.525Å spacing. The default settings within AUTODOCK were utilized throughout all computations. Computations were also performed to assess whether both monosaccharide and physiological anion can reside in known glycation cavities at the same time. Specifically, the monosaccharide anomers/isomers and the physiological anions were computationally generated as a sugar/anion adduct and then the adduct was allowed to bind to the β-chain of HbA. Although this process does not allow for competitive binding, it does give valuable and comparative insights regarding how HbA and HSA accommodate binding of multiple molecules within the same region. Each docking computation was replicated in triplicate to assess reproducibility and all docking computations reported are within ± 5% relative measurement.

Each AUTODOCK analysis produces a specified number of energetically favorable (exothermic) conformations for the binding event which we set between 10 and 40 conformations depending upon protein size and duplication of

conformations. The readout describing each conformation 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, vs. 12.3, http://jmol.sourceforge.net/) and the UCSF Chimera package (*16*) (Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco supported by NIGMS P41-GM103311), which allows for selection and visualization of salient regions of the molecule, and enables an assessment of through-space atom-to-atom distances. Amino acid residue selectivity for binding to the β-chain of HbA and to HSA was assessed by determining how many nucleophilic amino acid residues have a non-covalently bound glucose with geometry suitable for a potential reaction to proceed to the formation of the aldimine/Schiff Base. That distance is 9Å, assuming a 3-5Å distortion enabling a ca. 3Å interaction (*17-18*).

All ^{1}H and ^{31}P data were collected on a JEOL-EM300 at room temperature (~20°C). All kinetic data utilized pure α-glucopyranose (Sigma-Aldrich) as a starting material dissolved in D_2O (Sigma-Aldrich). ^{1}H NMR spectra were generated as a function of time and analyzed for the percent α- and β-glucopyranose by integrating the doublets at 5.05ppm and 4.46ppm, respectively. The equilibration time is defined as the point at which the percent of α-glucopyranose approaches 35% and remains constant and β-glucopyranose nears 64% and remains constant.

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Chapter 5. Unifying Summary and Conclusions

This summary is designed to reassess those questions which were posited in the Unifying Introduction of Chapter 1 that were answered in detail within Chapters 2 and 3. Specifically, in Chapter 2 entitled *The initial noncovalent binding of glucose to human hemoglobin in nonenzymatic glycation,* four questions were posed. In Chapter 3 entitled *A comparison of the initial noncovalent binding of D-glucose and D-fructose with hemoglobin and albumin in nonenzymatic glycation,* three questions were forwarded. Each of these eight questions will be generally answered in the order in which they appear in Chapter 1.

Question 1: *Which of the five glucose isomers are initially non-covalently bound to HbA?* Based on the results of the AutoDock computations it is clear that each one of the five glucose isomers has the ability to bind to the β-chain of HbA with varying degrees of exothermicity such that no singular isomer will bind selectively in the presence of the other isomers. Most critically, the ring-opened isomer, which is thought to be the reactive species that proceeds in the glycation process, will not selectively bind.

Question 2: *How is the Schiff base generated from initially bound glucose isomer/s?* The predominant initial non-covalent binding of glucose to the β-chain of HbA will overwhelmingly involve the binding of the α-pyranose and β-pyranose isomers because there is no selective binding and these two isomers

constitute ca. 99% of the available glucose. Based on the solution equilibrium concentrations of glucose (35% α-pyranose, 64% β-pyranose, less than 1% for the sum of α- + β-furanoses (*1*), and just 0.002%-0.004% for the ring-opened isomer (*2*) there will be no selective binding of the ring-open isomer because it competes with the other four ring-closed isomers. Further, the ring-closed pyranose isomers bind with greater exothermicity to the protein than will the ring-open isomer. These ring-closed isomers, once bound to the protein, will then by definition have to ring open once bound so as to present the ring-open isomer bound to the protein for further reaction and thus progress in the glycation process. Thus, the initial bound species is ring-closed and then must ring open while bound generating a transient reactive ring-opened glucose isomer via a reversible process. Within the lifetime of the bound ring-opened isomer, a nucleophilic attack by a N-terminal Valine or an internal Lysine residue is necessary in order to generate a covalent bond in the formation of a bound Schiff Base.

Question 3*: Is the initial binding event limited to the participation of the sugar and the protein or are physiological anions that are known to be present in the body involved in the initial binding event as well?* Some of the most prevalent physiological anions found in the body include inorganic phosphate, bicarbonate, and BPG. Each of these anions is known to be present in the erythrocyte cytosol and is found in significant concentrations. As observed in our theoretical docking computations, inorganic phosphate, BPG, and bicarbonate each bind the β-chain of HbA with exothermicities comparable to or exceeding that of the glucose

isomers and can compete against and/or complement the binding of glucose to HbA. These anions bind in similar locations to the glucose isomers within HbA and can bind near known glycation sites in HbA. Further, the interpretation of our docking data leads to the conclusion that each of the physiological anions can theoretically undergo concomitant binding with glucose isomers with exothermicities similar to the sugars alone in known glycation regions. As such, each of the anions can participate mechanistically with bound sugars in HbA.

Question 4: *If physiological anions are involved, what role do they play in the mechanism?* Physiological anions are involved because they can bind together with the glucose isomers in the same protein regions of HbA at the same time. Regarding the role of the bound physiological anions, we must extrapolate from model studies involving NMR and bimolecular reactions in aqueous solution. Based upon the interpretation of the data obtained through NMR methodology we assert that dibasic phosphate and bicarbonate will interact with glucose in solution to form adducts. Further, in solution, both dibasic phosphate and bicarbonate will increase the rate at which α-glucopyranose converts to β-glucopyranose. Because the conversion between ring-closed isomers must pass through the ring-opened isomer of glucose, an enhanced rate of α-to β-glucopyranose conversion equates to a greater time average concentration of the ring-opened isomer of glucose. Extrapolating to the protein chemistry of interest, the only reactive form of bound glucose that can make progress in the glycation process is the bound ring opened isomer. Because this bound species is transient, the greater the time average

concentration of the bound ring-opened isomer, the greater the progression in glycation. Based upon computational results, the physiological anions investigated will bind in areas that are known to glycate as well as concomitantly bind with glucose in the same regions (addressed under Question 3). As such, the presence of a bound physiological anion in the same glycation region as the bound α-glucopyranose or β-glucopyranose enables the two bound species to interact. Since we know that these physiological anions will increase the time average concentration of solution ring-opened glucose isomer, we assert that the concomitantly bound anions will enhance the time average concentration of bound ring-opened glucose isomer in HbA. Therefore, the role of the physiological anions is to enhance the ring-opening of the bound ring-closed isomers and thus facilitate the glycation event.

For HbA interactions with glucose there is a) no isomer selectivity in terms of initial binding to the β-chain of HbA, b) the predominant initially-bound species are ring closed isomers (α-glucopyranose or β-glucopyranose in this case), c) the bound ring-closed isomer must ring open while bound in order to proceed in the glycation process, d) as the time average concentration of the bound ring-opened glucose isomer increases, progress in glycation increases, e) physiological anions within the erythrocyte can co-bind with glucose concomitantly and serve to enhance the time averaged concentration of the bound ring-opened glucose isomer and thus progress in glycation increases such that these anions can be referred to as facilitation agents. The remaining three questions (addressed and answered in

detail within Chapter 3) are designed to assess the generality of the answers to the first four questions. Specifically, are these points applicable only to glucose and only to HbA? Will other sugars and other proteins also be governed by similar principles and interactions? The featured additional protein evaluated to assess generality is human serum albumin (HSA) while additional reducing sugars are fructose, xylulose, and galactose.

Question 5: *Will other reducing sugars (fructose, xylulose, and galactose) exhibit the same binding characteristic as glucose?* The interpretation of the computational data of fructose, xylulose, and galactose binding with the β-chain of HbA leads to the conclusion that these reducing sugars initially bind HbA as ring-closed isomers. Like glucose binding to HbA, if progress is to be made in glycation, the bound ring-closed isomer must ring open while bound. This result indicates that each reducing sugar investigated will undergo binding characteristics in common and suggest that this may be general for all reducing sugars. That is, reducing sugars will bind HbA as ring-closed isomers that must ring open while bound to proceed in glycation. Further, it is reasonable to assert that Schiff base formation involving these non-glucose reducing sugars proceeds via a similar mechanism as glucose Schiff base formation.

Question 6: *Which of the five glucose isomers are initially non-covalently bound to HSA and how does this compare to the binding event for HbA?* The results for the binding of the glucose isomers to HSA are nearly identical to the binding for the β-chain of HbA. Granted the exothermicity of the binding of the

glucose isomers to HSA is more stable than the binding to HbA, the binding trends are nearly identical to HbA in that the pyranose isomers have a more stable binding exothermicity than the ring-open isomer. Thus, the glucose isomers that will initially bind HSA will primarily consist of ring-closed α-glucopyranose or β-glucopyranose. Like glucose binding to HbA, if progress is to be made in glycation the bound ring-closed glucose must ring open while bound.

Question 7: *Is Schiff base formation in HSA the same or different than Schiff base formation in HbA?* Based on binding exothermicities obtained via AutoDock, the ring-closed pyranose isomers have a more stable exothermicity than the ring-open isomer. This indicates that the initial binding of glucose to HSA will be similar mechanistically to the initial binding of glucose to HbA. This initial binding will proceed with the binding of the ring-closed isomers which will subsequently ring-open once bound and thus present the ring-open isomer for nucleophilic attack in order to generate the Schiff Base. Docking computations were also performed to assess the possible affect physiological anions phosphate and bicarbonate could have on the binding of glucose to HSA. It was found that the same trend was present for the binding of HSA as was present for the binding of HbA. The physiological anions will concomitantly bind to areas on HSA that are known to glycate with a more stable exothermicity than the binding of glucose alone. This indicates that the possible role of the physiological anions in the binding of glucose to HSA will also be to facilitate the ring-opening

of glucose as well as possibly aid in the initial binding of the ring-closed glucose to the protein and protein glycation.

Question 8: *What are the clinical implications associated with the answers to the preceding questions?* A clinically observed phenomenon is the difference between intracellular glycated HbA_{1C} and extracellular glycated proteins known as fructosamine (primarily HSA). Both of these measurements are used to predict the levels of average glucose concentration. The difference between these measurements is known as the "glycation gap." Based upon the findings reported upon in this thesis, the biological variation of intracellular glycation of HbA versus extracellular glycation of serum proteins like HSA may not be a simple reflection of the prevailing explanations for these observed differences. Specifically inter-individual differences in the glucose gradient across the erythrocyte membrane and differences in protein life span (*3-4*). This research has established that the glycation rate for both HbA and HSA can be affected by the presence of physiological anions. The relative concentrations of physiological anions in patients' intracellular and extracellular compartments may also affect the extent of a patient's glycation gap. The glycation gap has been shown clinically to correlate directly to the pathology of nephropathy (*4*). Consequently a deeper understanding of the possible explanations for the glycation gap will provide physicians with a better platform from which to monitor and treat their diabetic patients which includes the use of certain medications that could be affected by the presence of differing concentrations of physiological anions.

Future Research: The findings presented within this thesis have directed the continuation of this research such that the next investigation will focus on the incubation of both HbA and HSA with differing concentrations of reducing sugars, glucose and fructose, so as to measure the time-dependent degree of glycation in these proteins. This will serve as a standard for comparison to the time-dependent degree of glycation that arises from protein/sugar incubations in the presence of varying concentrations of physiological anions. These results will then be assessed to determine if anion-facilitated degree of glycation observed in the incubation studies correlates to the predicted degree of anion facilitation that our NMR and computational investigations reported upon here predicts.

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