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Mechanistic Insight into the Role of 2,3-Bisphosphoglycerate and Phosphorylated Amino Acids in Nonenzymatic Glycation and the Formation of Hemoglobin A1c

by

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Committee Approval

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Abstract

Nonenzymatic glycation (NEG) is a multi-step process that leads to the covalent modification of protein caused by glucose, and is related to the pathology of diabetes mellitus. A common clinical measurement to monitor glycemic control is glycated hemoglobin (HbA1c). It has been reported that inorganic phosphate and organic phosphate 2,3-bisphosphoglycerate (BPG) can enhance the rate of NEG and HbA1c formation, but the mechanistic actions have not been defined. BPG is known to bind only in the protein pocket responsible for HbA1c formation. ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy was used to assess if and how well BPG could interact with glucose in aqueous solution. In addition, computational modeling was used to identify possible mechanistic roles of BPG and phosphorylated amino acid residues on NEG formation in HbA1c. Overall, there are several mechanisms, both direct and indirect, for BPG to increase formation of HbA1c, but for phosphorylated amino acids there are no mechanisms to enhance NEG and the formation of HbA1c.

Chapter 1: Unifying Introduction

Type II diabetes mellitus is a chronic pathological condition that affects the body's metabolism of glucose. Glucose is a key source of energy for all bodily functions, especially the brain, because glucose is the predominant source of energy. The brain is considered the main consumer of glucose in the human body (1), but glucose is also an important energy substrate for contracting skeletal muscle, activating heart muscle, and maintaining homeostasis of blood glucose by the liver (2). With type II diabetes mellitus the body either resists the metabolic effects of insulin, or doesn't produce enough insulin to maintain normal blood glucose levels (3). Insulin increases the rate of glucose transport across select cell membranes, increases the rate of glycolysis in muscle, stimulates glycogen synthesis, and inhibits gluconeogenesis in the liver (4). In type II diabetes insulin's effect on glucose transport across cell membranes is compromised, leading to persistent hyperglycemia. Glycemia is defined as the presence of glucose in the blood, where normal fasting glycemia ranges from 3.9 to 5.6 mmol/L and hyperglycemia is any consistent range above 7 mmol/L (5).

Type II diabetes has been reported to affect over 347 million people globally (6) and 29.1 million people nationally in the United States (7). Extensive research has been conducted to gain a molecular understanding of the initial processes that give rise to diabetic complications, such as neuropathy, retinopathy, nephropathy and cardiovascular disease (8). In each case, prolonged interactions between glucose and protein molecules, and the resulting changes in protein structure and function may

define the progression to these complications.

Nonenzymatic glycation (NEG) describes an array of chemical processes that give rise to covalently modified proteins without the use of enzymes. The reagents involved in NEG consist of proteins interacting with monosaccharides such as glucose, fructose and ribose. Reagent is defined as a molecule(s) needed for a chemical process or reaction to take place. Even though there are multiple monosaccharides that can act as a reagent in NEG (9), glucose is proposed to be the primary reagent due to glucose having a high concentration, around 5 mmol/L in the serum and about 75% of that (~4 mmol/L) in the erythrocyte (10). This concentration is significant when compared to that of fructose in the serum which is around 0.01 mmol/L (11). However, glucose is not a single molecule and exists as one of five interconverting isomers (2 glucopyranoses, 2 glucofuranoses, and 1 ring-opened) in aqueous solution (Fig. 1). The predominant glucose isomers are the 6 membered rings: α- and β- glucopyranoses. The reactive form necessary for NEG is the ring-opened aldehydic isomer (12,13). Protein glycation involving glucose occurs through nonenzymatic binding (both non-covalent and covalent) resulting in chemistry between the glucose isomer and the protein it is interacting with. When compared to many of the enzymatic processes in the body, NEG is a very slow process due to the presence of multiple, reversible steps involving multiple reagents and both non-covalent and covalent chemistry.

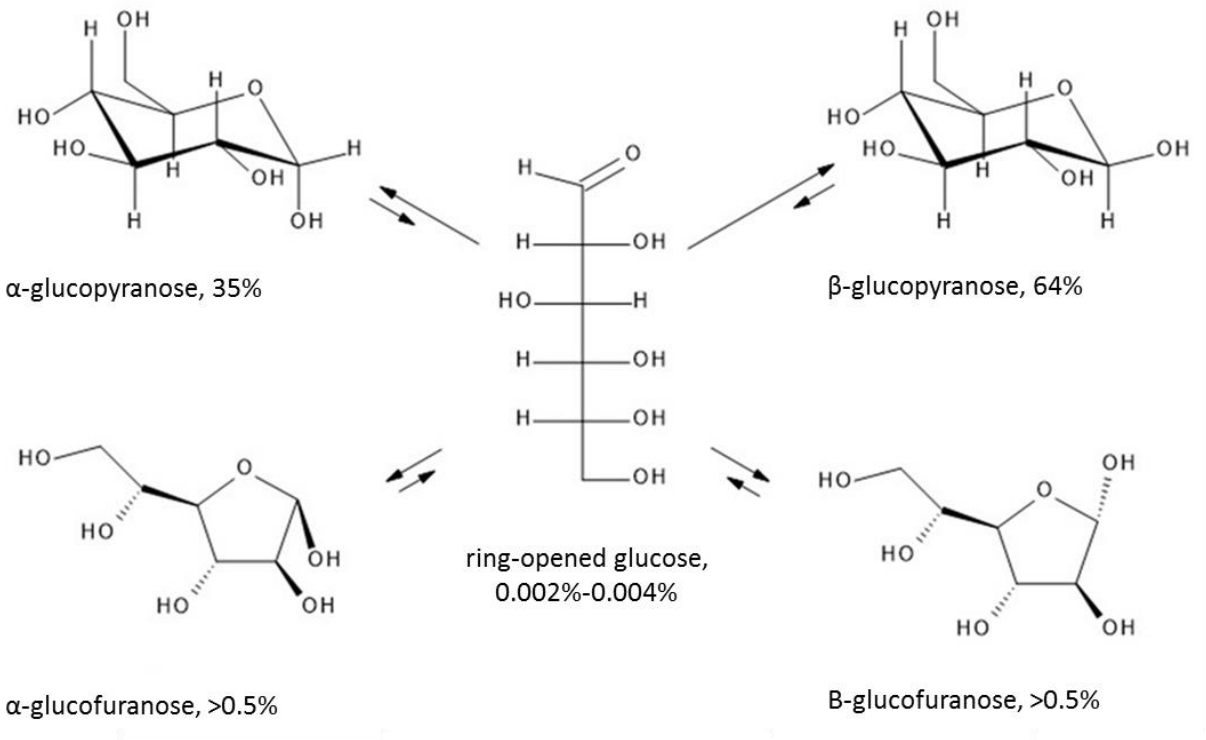
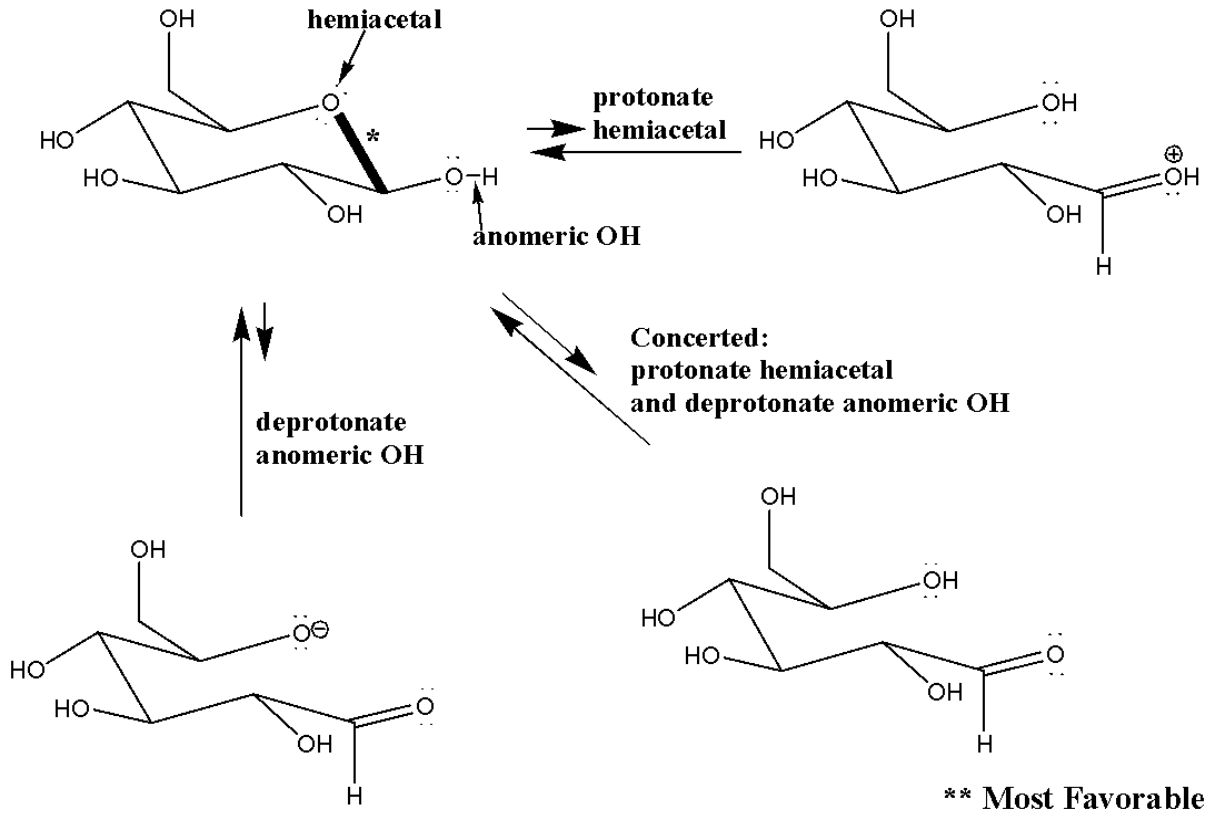


Figure 1. The five interchangeable isomers of glucose with respect to their equilibrium concentrations in aqueous solution as denoted by arrow lengths and percentages. This shows that glucose will almost always be found in one of its glucopyranose forms, since they are the predominant isomers. (11-13). The predominance of the β-glucopyranose isomer is further illustrated but the relative length of reversible arrows between the reactive ring-opened isomer and the corresponding ring-closed isomer.

How does the non-reactive ring-closed glucopyranose isomer ring-open to the reactive aldehydic isomer? In order to ring-open the glucopyranose isomer, the hemiacetal oxygen/anomeric carbon bond must break, which can be achieved in 3 ways (Fig. 2). First, the hemiacetal oxygen can be protonated, requiring an acid (good proton donor, either from an acidic amino acid residue or from another acidic reagent that binds) and would be favorable at low pH. This ring-open species is likely to be short lived and the equilibrium strongly favors rapid ring closure. Second, the anomeric OH can be deprotonated on carbon one. This requires a base (good proton acceptor, either from a basic amino acid residue or from another basic reagent that binds) and would be favorable at high pH. This ring-open species is also likely to be short lived and the equilibrium again strongly favors ring closure. The third option is to deprotonate the anomeric OH and protonate the hemiacetal oxygen in events that overlap in time (a concerted process). This requires either two reagents, an acid and a base (Fig. 3A), or one reagent that can act as an acid and a base (amphoteric) (Fig. 3B). This option is the most favorable of the three, because the first two options would produce ring-opened forms that are charged species which are not as likely to occur, and the third produces a neutral ring-open form and is therefore more thermodynamically stable and less likely to ring-close.



*** Bond broken to ring open**

Figure 2. Three possible mechanisms to ring-open a glucopyranose isomer by: protonating the hemiacetal oxygen, deprotonating the anomeric OH, or by doing both is a concerted fashion. Each method ring-opens the glucopyranose isomer by breaking the bond between the hemiacetal oxygen and the anomeric carbon, but the ring-opened species is different in each case.

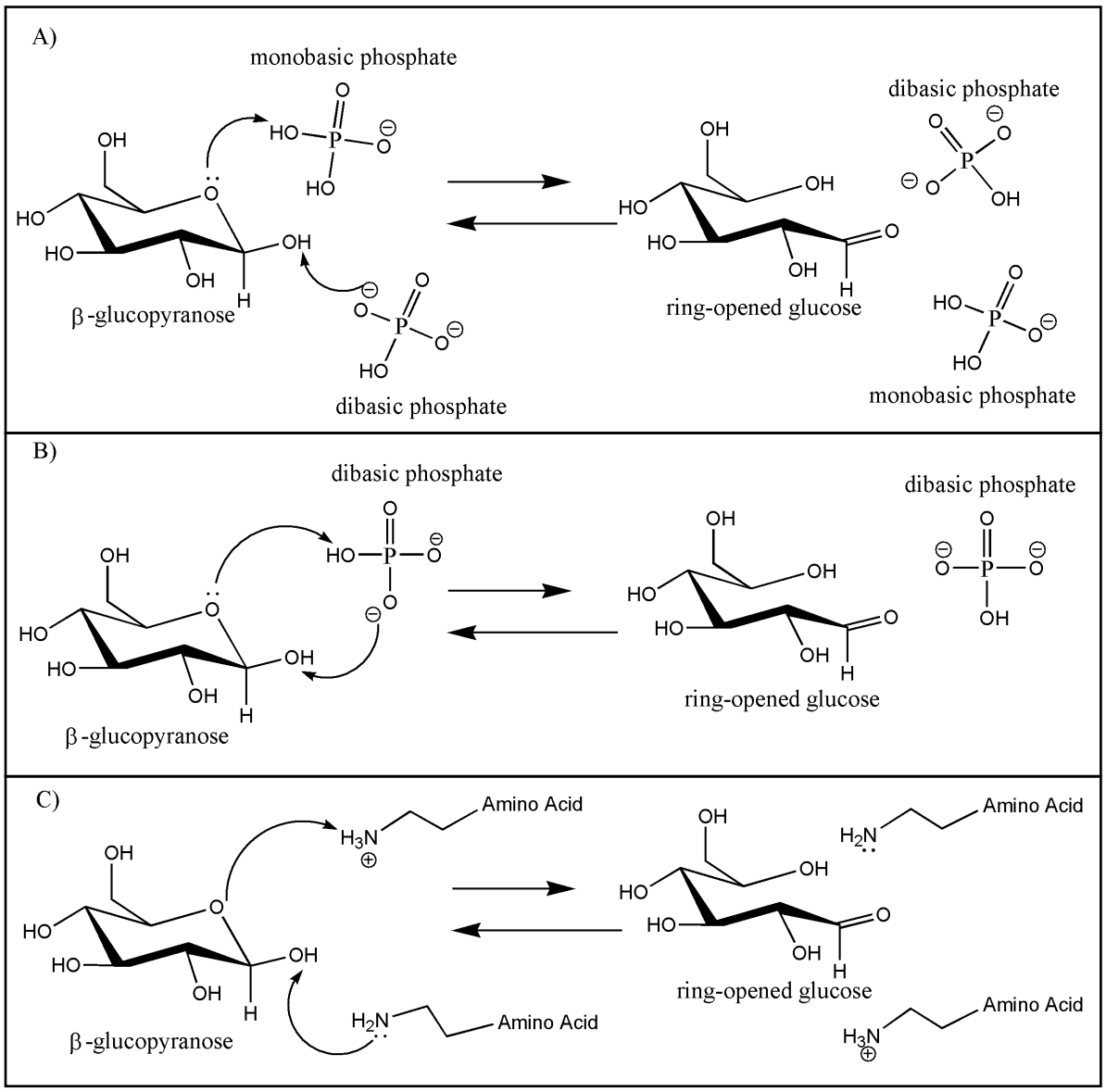


Figure 3. Possible mechanisms to ring-open a glucopyranose molecule in the most favorable way. **A)** A mechanism (simultaneous or sequential) in which two different inorganic phosphate molecules act to ring-open a glucopyranose isomer in solution. One monobasic inorganic phosphate acting as the acid and a dibasic inorganic phosphate acting as the base. **B)** A bridging mechanism in which a glucopyranose isomer is ring-opened using an inorganic dibasic phosphate which simultaneously acts as both an acid (by donating a proton to the glucopyranose) and a base (by abstracting a proton from the glucopyranose). Inorganic phosphate is shown, but it is not the only reagent capable of ring-opening a glucopyranose molecule; other physiological anions may also play this role. **C)** A mechanism in which a glucopyranose isomer is ring-opened using only amino acid residues (e.g., valines, lysines, and histidines) - serving as either an acid or base - that are contained within a protein pocket.

What can serve as the potential proton donors and acceptors that can facilitate ring-opening of glucose? Proton donors (nitrogen atom in ammonium ion form: $R\text{-}NH_3^+$) can include the following amino acid residues: lysine, histidine, and N-terminal amino acid residues. The following amino acid residues act as a proton acceptors: (nitrogen atom in amine form: $R\text{-}NH_2$) lysine, histidine, and N-terminal amino acid residues. Non-amino acid reagents serving as potential proton donors and or proton acceptors include physiological species such as water (14), bicarbonate (15), and phosphate (16), among others. All of these non-amino acid reagents are amphoteric in nature, but have varying strengths of effectiveness. For instance, water can act as either a weak acid or a weak base and, in turn, will not facilitate this chemistry very efficiently. Bicarbonate is a stronger acid and base than water, but can still only act as a moderate acid/base. Finally, phosphate - both inorganic and organic - can act as a strong acid/base, and can facilitate this chemistry in a much more efficient manner than the other two reagents. This information had led to the previous focus on phosphate's potential role in NEG (12,15-19).

For a reagent such as inorganic phosphate (Pi) to play a role in NEG, there must be concomitant binding with a glucopyranose isomer to the protein. Concomitant binding is the non-covalent process of two or more molecules binding in the same location at the same time. Binding interactions are reversible and involve electrostatic forces, hydrogen bonds, hydrophobic interaction, and van der Waals forces. For effective chemistry to occur, the two reagents must not only bind concomitantly but also obtain a proper geometry and achieve favorable energy. In this case, achieving

favorable energy requires an exothermic value (-ΔH), with the more negative value being more exothermic.

It is also worth noting that there are other ring-open reagents (electrophiles) found in equilibrium in aqueous solution that can bind to proteins (9). Sugars such as galactose and ribose have ring structures that are significantly more unstable than glucose, thus these monosaccharides are ten times more likely than glucose to be found ring-open (9). Furthermore, permanently electrophilic reagents (aldehydes and ketones) exist physiologically, and they can also serve as effective electrophiles in NEG.

Proteins serve as the final reagent in the NEG process. Proteins are amino acid polymers that are intracellular or extracellular molecules. Each protein contains an N(amino)-terminal amino acid residue, and a C(carboxy)-terminal amino acid group. The term protein is a very broad term that can encompass thousands of different species that are found within the human body. The glycation of proteins is a common, time-dependent process that is directly related to the pathology of diabetes (20). Given adequate time and reaction conditions, glucose can potentially interact with all proteins resulting in NEG. This interaction begins with the non-covalent binding of a ring closed glucose molecule in a pocket of the protein (17, 21). Facilitation must occur to ring-open the glucose to its reactive electrophilic form, which will then be attacked by a nucleophilic residue in the protein, resulting in the covalent interaction between the glucose and the protein (Fig. 4). The initial reaction of NEG is not inherently problematic for normal health. However, complications arise when the interaction becomes covalent, which then can lead to permanent changes in protein structure and function,

and possibly diabetic complications (20).

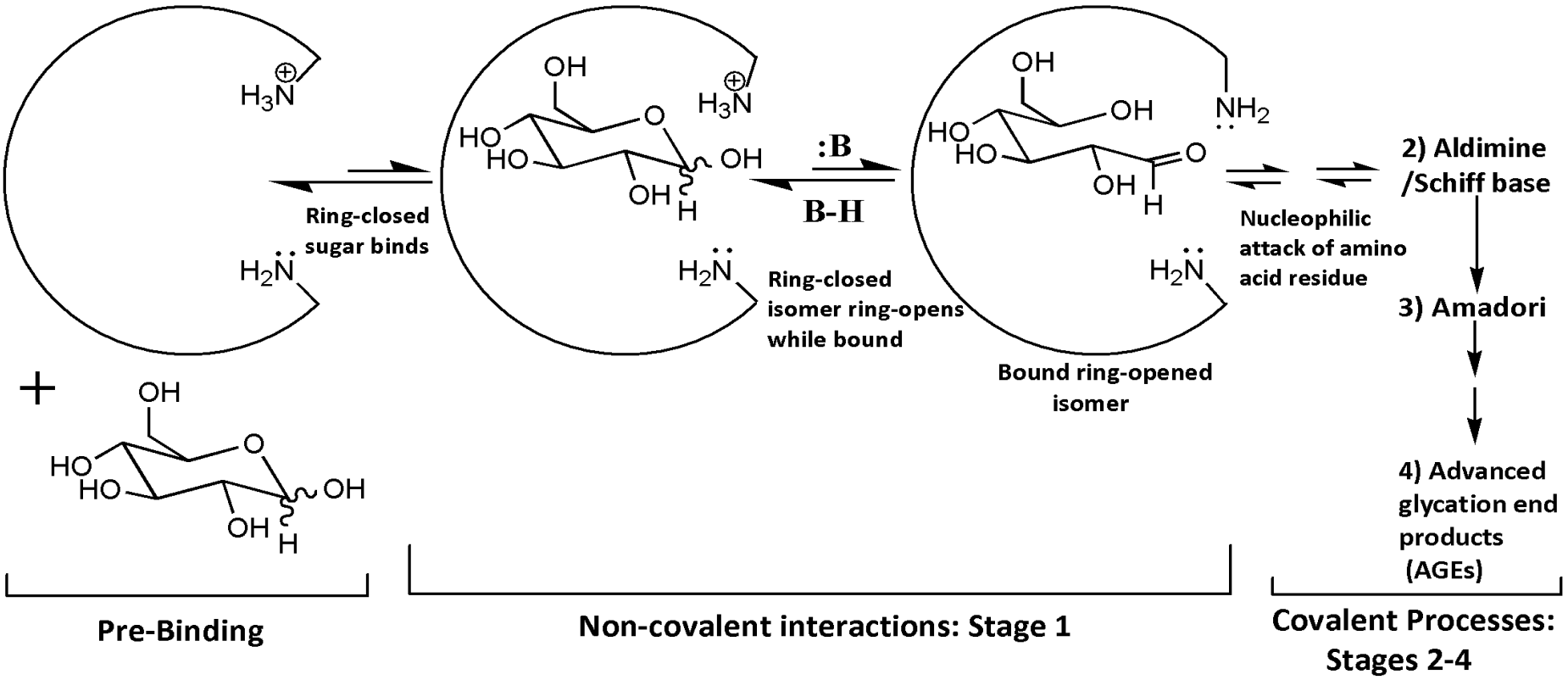


Figure 4. Proposed mechanistic scheme for glycation broken down into the pre-binding and the 4 consecutive stages. The pre-binding stage shows a protein pocket and a ring-closed β-glucopyranose molecule binding. Stage 1 shows the non-covalent interaction between the protein pocket and the ring-closed glucopyranose, which leads to the ring-opening of the glucopyranose molecule. Stages 2-4 show the covalent processes, from the nucleophilic attack of an amino-acid residue through the Schiff base and Amadori rearrangements ending with the formation of the Advanced glycation end products (AGEs). Note: the positive charge in the drawing represents an anion stabilizing group (H-bond donor, NH_3^+ acidic amino acid residue, etc.), and the **:B** represents a generic basic molecule needed for proton abstraction (H-bond acceptor, inorganic/organic phosphate, NH_2 amino acid residue.

Before any NEG chemistry can occur between glucose and a target protein, a ring-closed glucopyranose must bind non-covalently to a protein pocket. The chemistry between a bound glucose and the protein is a nonenzymatic process and involves multiple steps leading ultimately to advanced glycation end products (AGEs) (Fig. 4). Glucose must ring-open to its reactive aldehydic isomer while bound to the protein, thus serving as the necessary electrophile. For the glucose isomer to ring-open, it must be facilitated by another molecule. This facilitation is possible with the use of a basic species (such as an inorganic or organic phosphate species). Another requirement for effective chemistry to occur between sugar and protein is the presence of a nucleophile. Given the appropriate distance, geometry, and energy, the nucleophile will attack a given electrophile. The N-terminal amino acid residue and lysine residues in the protein can exist either as an ammonium ion (NH_3^+) or the reactive amine (NH_2). This is determined in part by the pKa of the R-groups of amino acids or the N-terminus. In some cases, the nucleophilic site exists as the ammonium ion and must be deprotonated for it to act as a nucleophile. Similar, to the facilitation of glucose ring-opening, the facilitation of the nucleophile must be done by another basic molecule. This molecule can be a nearby inorganic or organic phosphate, such as 2,3-bisphosphoglycerate (BPG), which is the organic phosphate in question (Fig. 5). This basic reagent must obtain a geometry within a 5 Å distance to the potential nucleophilic residue in order to act as a base and deprotonate the residue converting it to its reactive amine form. If the electrophilic and nucleophilic sites obtain a proper geometry, then reversible chemistry can occur, and an aldimine (or Schiff base) is formed (Fig. 4). From

the Schiff base, the reaction can continue forward in an irreversible manner to form an Amadori intermediate, which in turn can react further to form AGE (17), or reverse.

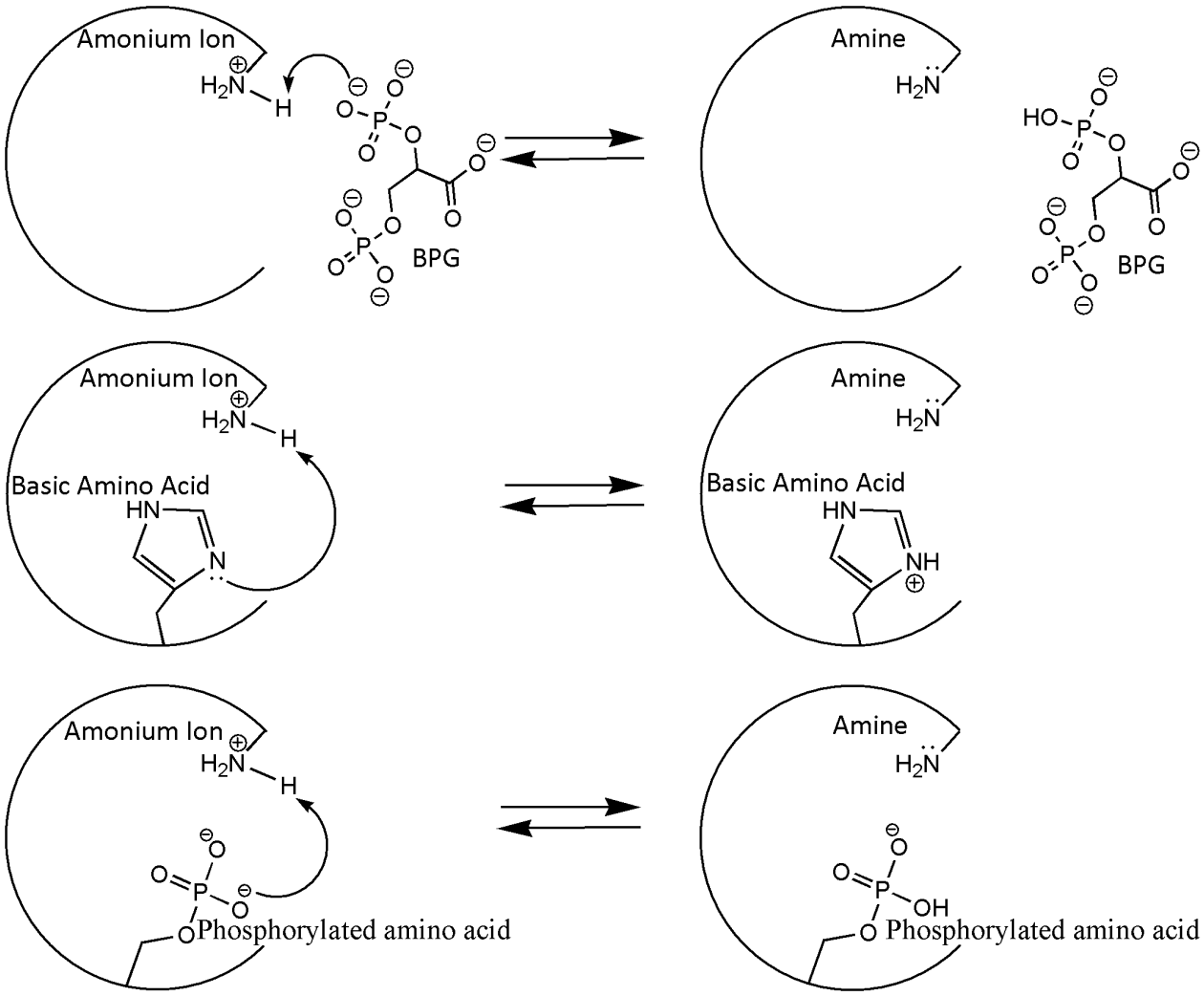


Figure 5. The above mechanisms show multiple ways to facilitate the nucleophile (amine from an ammonium ion) in a protein pocket. **A)** The nucleophile is being facilitated by an organic phosphate (tetrabasic 2,3-BPG in this case). **B)** The nucleophile being facilitated by a different amino acid residue (Histidine in this case). **C)** The nucleophile being facilitated by a phosphorylated amino acid (serine in this case). The examples used are not inclusive of all reagents in the above mechanisms.

Glycated proteins are found all individual in different concentrations, and are mostly associated with long-lived proteins such as collagens, lens crystallins, and nerve proteins (22, 23). Glycated proteins have a modified structure that may lead to a change in function, potentially causing biological damage *in vivo* (8,22-20). This change in function can also lead to disease in extreme cases, such as glycation in the lens crystallin of the eye can lead to retinopathy and ultimately blindness (8, 23, 20). However, glycation of some proteins cause little-to-no structural and/or functional changes, and consequently does not lead to disease states. One of the most common examples of these glycated proteins is human hemoglobin A (HbA). Glycated proteins do not typically form in excess in the body and in most cases, are non-problematic due to the body's ability to break down these proteins. Glycated proteins can be broken down in multiple ways, whether it be through the ubiquitin-proteasome pathway, lysosome proteolysis (24), or deglycated by an enzyme called fructosamine-3-kinase (25). Complications start to occur once an individual forms glycated proteins faster than their immune system and lysosomes can break them down. This causes a buildup of glycated proteins in the body, which can then (depending on the protein) may cause deleterious effects in the body (23, 26).

The most commonly measured glycated protein is HbA. HbA is a critical intracellular protein that is found within the erythrocyte of most healthy adults in high concentrations (~5 mM) (27, 28). This protein is responsible for transferring oxygen molecules throughout the body. Each hemoglobin protein is made up of a tetrameric structure that consists of two α subunits and two β subunits all linked together in the

center of the molecule (Fig. 6). When HbA is glycated it does not lead to pathogenic complication (29), but it is a good model protein since it is highly abundant, long-lived, and easy to measure. Glycation of HbA can be measured using a wide variety of methods, with one of the most common methods being high-performance liquid chromatography (HPLC) (30). All of the HbA measurement methods measure the percentage of Hemoglobin A1c (HbA1c). HbA1c is commonly used because erythrocytes have an average lifespan of 120 days, this means that the HbA1c value reflects the average glucose concentration over an 8-12 week period (31). Human red blood cell hemolysate contains several nonenzymatically modified minor hemoglobin components, the most abundant being HbA1c. This hemoglobin component, whose percentage (normally about 3-5% of total hemolysate) is directly related to the blood glucose concentration, and therefore provides useful information about patient's glycemic status (32-34). This percentage of HbA1c can be increased 2-fold or more in patients with diabetes mellitus (29,32), but any measurement greater than 6.5% is considered pathologic (35). HbA1c is the specific result of the nonenzymatic reaction of glucose with the α-amino groups of the valine residues at the N-terminus of β-chains (32).

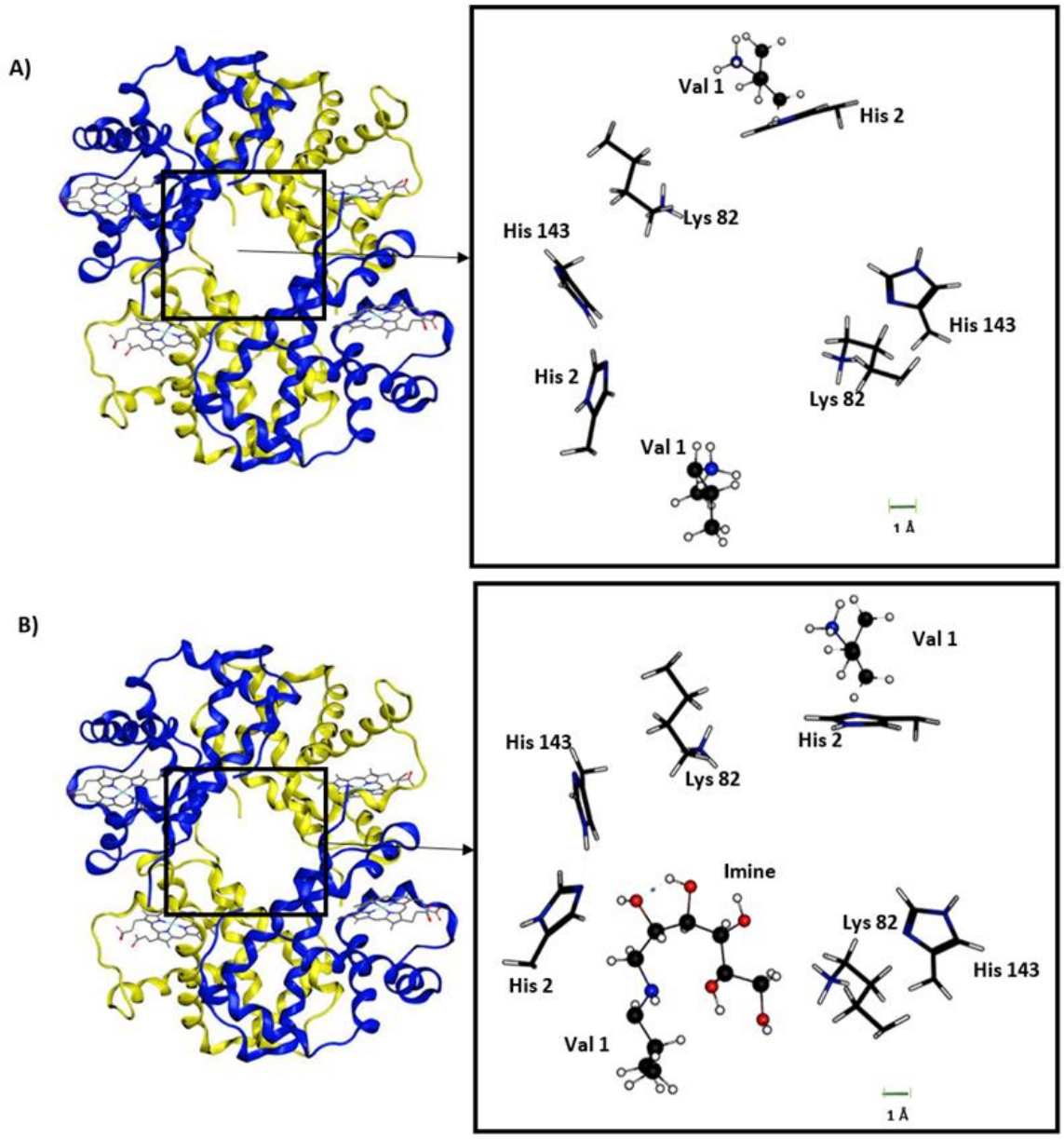


Figure 6. The entire deoxygenated hemoglobin A tetramer. The yellow ribbons indicate the α subunits, and the blue ribbons indicate the β subunits. **A)** Shows the whole HbA protein with the Val1/Lys82 (A1c) pocket magnified. **B)** Shows the whole HbA protein with the Val1/Lys82 (A1c) pocket magnified and with the Val1 glycated showing the Schiff Base or imine.

Hemoglobin is the most commonly measured glycated protein, but it is not the only measured glycated protein in clinical medicine. Albumin is used as a model for extracellular proteins and is glycated through NEG more rapidly, and to greater extent than hemoglobin, due to albumin's lysine groups reacting better than hemoglobin's N-terminal valine on the β-chains (36). Albumin has a higher turnover rate than hemoglobin, thus the measurement of glycated albumin reflects the mean glucose over a period of 2-3 weeks (19). Albumin is glycated predominantly on ε-groups of lysine residues and this may be enhanced by neighboring aspartic and glutamic acid side chains (36, 37).

NEG will initially form chemically reversible Schiff base and Amadori products in proportion to glucose concentration (38). Several factors have been reported to influence the rate of glycation of HbA: pH, glucose concentration, and catalysis by inorganic phosphate and 2,3-BPG (15, 16, 39). There are four stages to the NEG process as well as an initial pre-binding stage (Scheme 1). These stages are: 1) reversible, non-covalent binding of a ring-closed glucopyranose isomer to select pockets of the protein. The isomer then reversibly ring-opens to a transient ring-opened isomer while bound, 2) reaction between the bound transient ring-opened isomer and nucleophilic amine residues (terminal α-amino valines and internal ε-amino lysines) to generate a covalently bound Schiff base, 3) rearrangement of the Schiff base to an Amadori intermediate (amino-1-deoxyfructose) and 4) the non-reversible formation of AGE (Scheme 1, (17)). Once the Amadori or the AGEs are formed, the process becomes irreversible, unless acted upon by certain enzymes such as fructosamine-3-kinase which

has been studied as an enzymatic method to deglycate an intracellular and serum proteins (25). Without the assistance of enzymes, these proteins will remain glycated permanently. Some proteins when glycated have their secondary structure altered in such a way that they cannot function normally (8, 20, 22). This altered function can lead to very serious deleterious effects, such as blindness and nephropathy (8, 20-22). Due to these deleterious effects, the main focus has been looking at the stage 1 to 2 transition in the glycation process.

Stage 1 of NEG is the non-covalent interaction where a ring-closed isomer of a glucose molecule binds and ring-opens to provide a needed electrophile. The transition between Stage 1 and Stage 2 occurs when a nucleophilic amino acid residue (e.g., N-terminal valine on the β-chains of HbA, or lysine residue) attacks the ring-opened glucose to form a Schiff base (Stage 2). According to a study performed by Gil *et al*. (15), the rate determining step must come before the formation of the Amadori intermediate. This leads to the rate-determining step in this process being at stage1, 2 or the intermediate step between the two phases.

Stage 1 in NEG initially requires the interaction of a ring-closed glucose molecule with the pocket of a protein. The glucose molecule must then be able to ring-open, which can only be done through the assistance of either a concomitantly bound base or a nearby basic amino acid residue, as well as a nearby by acidic proton that may be located on a concomitantly bound acid or a nearby amino acid residue, such as valine1 or any lysine residue. By ring-opening, the sugar molecule provides the needed electrophile to progress in NEG. Since ring-opening the sugar is essential for this process

to continue forward, one strategy to reduce NEG might be to mitigate against ring-opening of the sugar molecule. Consequently, the NEG could be further slowed down even more, thus reducing deleterious effects that may arise.

Much is known about the role of inorganic phosphate (Pi) in NEG (11,15-19,40). The interests of this research are in organic phosphates (Po) and their potential role(s) in the NEG process. According to Gil *et al*., (15), Pi and Po(s) play important roles in the glycation of HbA. However, the roles of Pi and Po may be different from one another, where the role of Po is the abstraction of the proton in the Amadori rearrangement, and Pi plays a role to induce hemoglobin to catalyze its own glycation (15). Organic phosphates are esters of Pi, examples of Po include BPG, and phosphorylated amino acids present on intracellular proteins. We are interested in BPG because it is produced via a natural enzymatic pathway (glycolysis) and is only found inside of erythrocytes of mammals (including humans). BPG is an example of a Po with two phosphate groups attached, each one having its own pKa value (Fig. 7) (41). This difference in pKa values is thought to cause a difference in reactivity between the 2 phosphate groups, where the phosphate group in the third position acts as a better catalyst in NEG (40). BPG is an important regulator of intracellular hemoglobin function. More specifically, BPG modulates hemoglobin oxygen affinity, and therefore directly affects both blood oxygen transport and delivery (42). Within mammalian red cells, BPG is present in high concentrations that are approximately equimolar with the concentration of the hemoglobin tetramer (around 5 mM) (27). From a clinical perspective, BPG concentrations are increased in subjects with high rates of glycation (37).

The binding of BPG to HbA has been characterized using X-ray crystallography (28,43). 2,3-BPG is known to bind to the central cavity with Val1, Lys82, His2, and His143 of the β-chains of hemoglobin (43). BPG also binds HbA much better than Pi *in vitro* (43). Unlike Pi, very little attention has been devoted to defining potential mechanism(s) of action of BPG on increasing the glycation of HbA. It has been theorized that BPG may reduce the positive charge on the amino terminus, thus creating a more effect nucleophile at Val 1 (34). A BPG bound to HbA might also cause a conformational shift improving steric factors (34) creating better geometries for nucleophile – electrophile interactions, and the binding of a glucopyranose isomer.

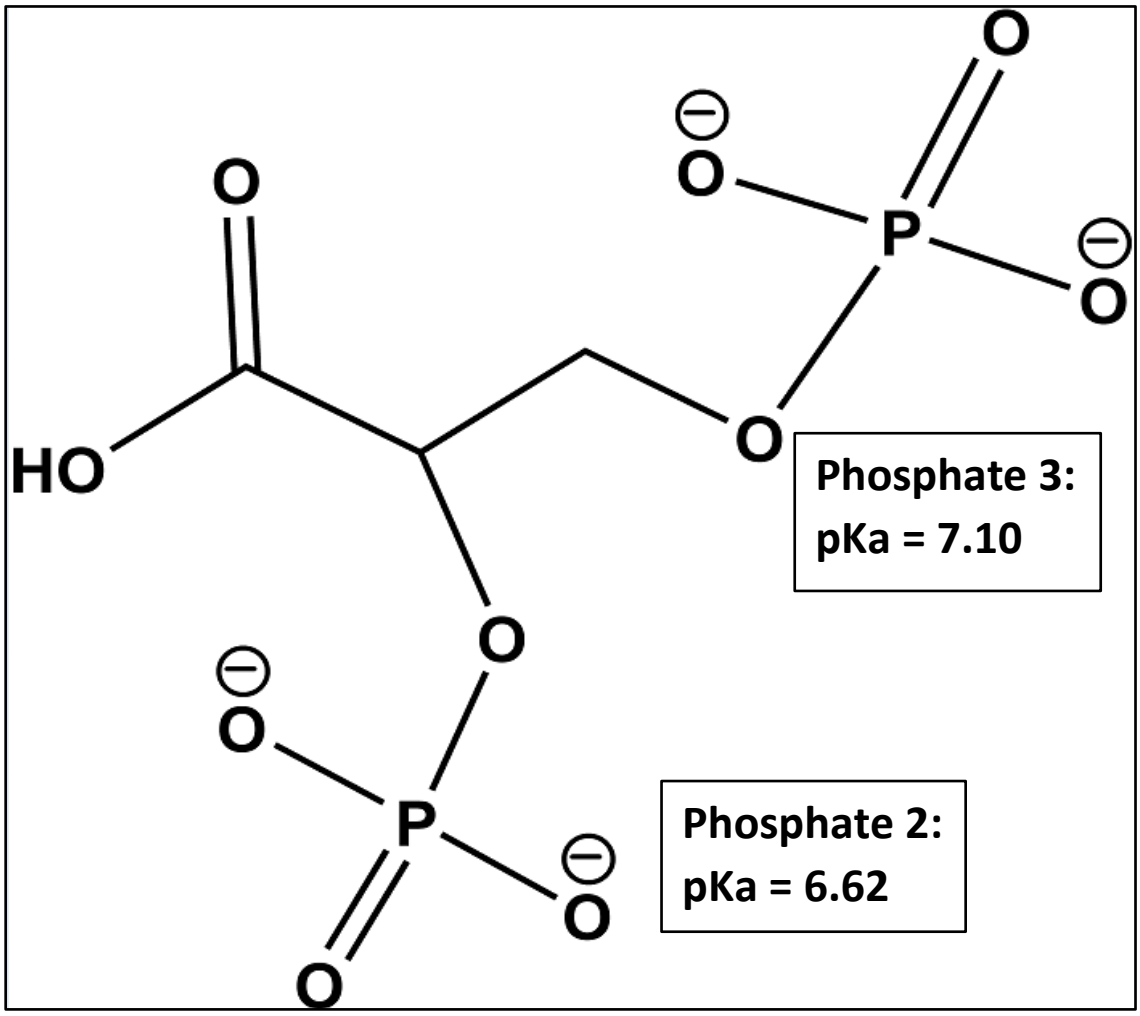


Figure 7: Shows the structure of tetrabasic 2,3-Bisphosphoglycerate, and the corresponding pKa's to each of the phosphates on the molecule (38).

We are also interested in covalently phosphorylated amino acids residues, because it would potentially eliminate the need for concomitant binding of an outside base in a protein pocket to facilitate formation of the electrophile or nucleophile. Phosphorylation is a process through which enzymes called kinases covalently bind inorganic phosphate molecules to a molecule or protein, which is essential for the regulation of cellular processes like metabolism, proliferation, differentiation, and apoptosis (44). Serine, threonine, and tyrosine amino acid residues are potential targets for phosphorylation events due to their hydroxyl groups (44). Although not explored previously, their importance in NEG is that they might potentially be able to perform chemistry reactions in a sequential manner similar to Pi or Po species. To the best of our knowledge, nothing is known about the role of potential phosphorylated amino acid residues in the role of NEG of proteins.

The research described within this thesis is divided into 4 chapters. Chapter 1 is this unifying introduction. Chapter 2 describes an investigation into the mechanistic possibilities for BPG to affect NEG in the formation of HbA1c, with a small added section on phosphorylated amino acids included. In this chapter, a detailed introduction as well as results and discussion are also featured. Chapter 2 is entitled *Possible Mechanistic Roles of 2,3-Bisphosphoglycerate (BPG), and phosphorylated amino acids in the formation of Hemoglobin A1c (HbA1c).* Chapters 3 and 4 contain the unifying methods and conclusions, respectively. The primary questions addressed in Chapter 2 are: a) Can BPG interact with a glucopyranose isomer to ring-open it, b) What mechanistic roles can BPG play in the formation of Hemoglobin A1c (HbA1c), and c) Could phosphorylated

amino acids increase NEG of proteins, specifically hemoglobin in the A1c pocket?

The methods utilized in Chapter 2 include computational modeling using Molecular Operating Environment (MOE) and ^{1}HNuclear Magnetic Resonance Spectroscopy (NMR).

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Chapter 2. Possible Mechanistic Roles of 2,3-Bisphosphoglycerate (BPG), and Phosphorylated Amino Acids in the Formation of Hemoglobin A1c (HbA1c).

2.1 Introduction:

NEG describes a common post-translational process by which D-glucose interacts slowly with both intracellular and extracellular proteins, which results in glucose ring-opening and eventual binding covalently to the protein (1). The most common protein studied in NEG is human hemoglobin A_0 (HbA), which is glycated at several sites both *in vivo* and *in vitro* (2). The most favored of these sites is the amino group of the N-terminal valine of the β-chains (3,4). To date, four distinct species of hemoglobin modified at this site have been identified (4). The most abundant of these species is Hemoglobin A1c (HbA1c) which is formed by the addition of glucose to the α-amino (N-terminal) group of the β-chains (3-5). HbA1c has been reported to range between 3-7% of total HbA in healthy individuals (6-12) and is found increased up to 10-15% in diabetic patients (7,8,11,12).

As mentioned earlier, HbA1c results from NEG of human HbA, and this chemical process occurs as a result of a site-specific reaction between glucose and the valine 1 residues on the β-chains of the protein. This reaction is best described by the following four sequential stages (Fig. 1, Clark et al., 2013): 1) reversible non-covalent binding of a ring-closed glucopyranose molecule to the β-valine 1 pocket on the protein, and ring-opening of the glucopyranose molecule to the electrophilic glucose form, 2) reaction

between the electrophilic glucose and one of the β-valine 1 residues to generate a covalently bound Schiff base/aldimine, 3) rearrangement of the Schiff base to an Amadori intermediate and, 4) The non-reversible formation of advanced glycation end products (AGEs) (1). The initial covalent product formed is a labile Schiff base derivative of the protein which slowly isomerizes to the more stable ketoamine via Amadori rearrangement (13). Even though this is a slow process there are several factors that can accelerate NEG. The most notable of these factors is the presence of different phosphate species (both inorganic and organic) (1,14). Other factors include additional basic species such as bicarbonate (1), arsenate (14), and even water (15).

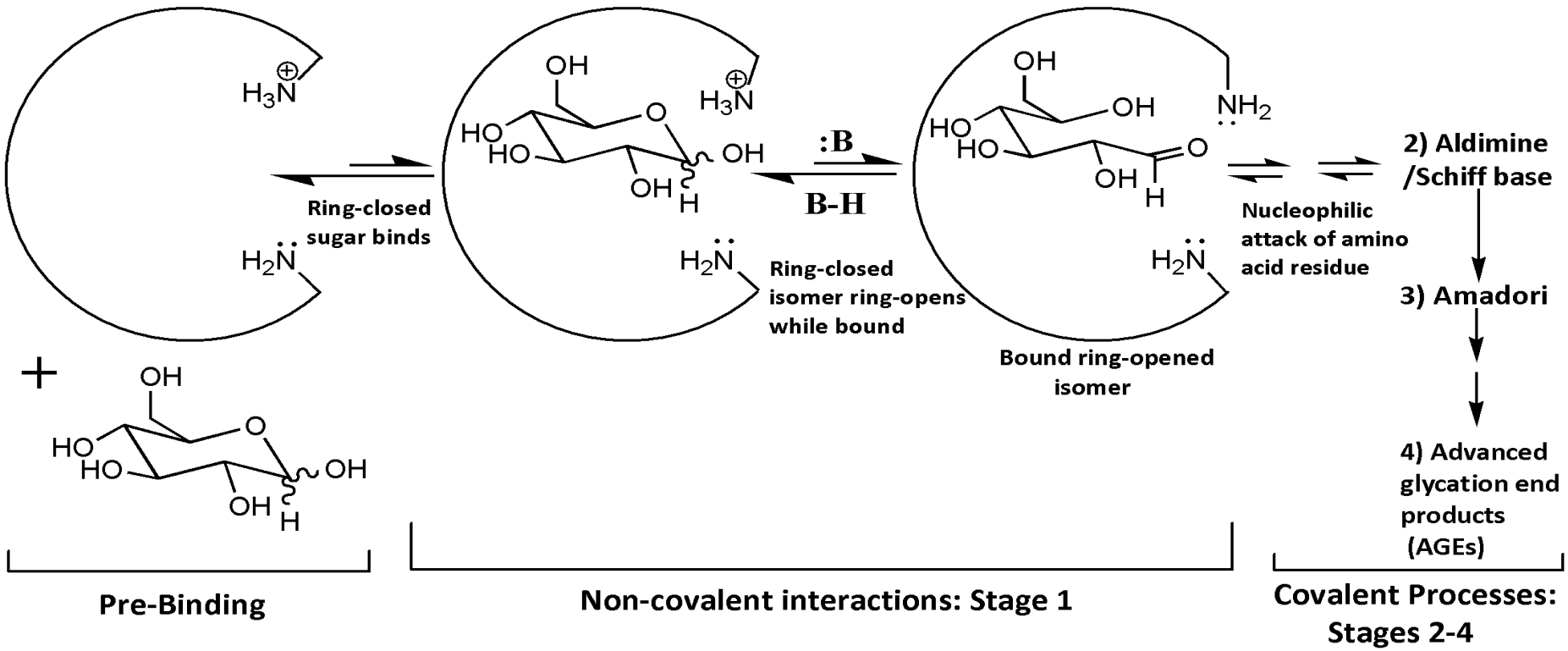

Figure 1. Proposed mechanistic scheme for glycation broken down into the pre-binding and the 4 consecutive stages. The pre-binding stage shows a protein pockets and a ring-closed glucopyranose molecule binding. Stage 1 shows the non-covalent interaction between the protein pocket and the ring-closed glucopyranose which leads to the ring-opening of the glucopyranose molecule. Stages 2-4 show the covalent processes, from the nucleophilic attack of an amino-acid residue through the Schiff base and Amadori rearrangements ending with the formation of the Advanced glycation end products (AGEs). Note: the positive charge in the drawing represents an anion stabilizing group (H-bond donor, NH^{3+} acidic amino acid residue, etc.), and the **:B** represents a generic basic molecule needed for proton abstraction (H-bond acceptor, inorganic/organic phosphate, NH_2 amino acid residue, etc.).

Of the factors mentioned, phosphate species are the strongest bases. These species (effector reagents) are known to affect NEG leading to a phenomenon called the phosphate effect (16). This effect is explained by the possible roles that these phosphate species play in NEG. It is believed that one of the roles of inorganic phosphate (Pi) is to induce HbA in such a way that HbA catalyzes its own glycation (17, See below). Phosphate is also known to enhance NEG directly, as well as increases the proportion of the ring-opened, reactive form of reducing sugars, such as glucose, promoting the enolization of the sugars. Pi also serves as an acid-base catalyst of numerous reaction under physiological conditions, such as the Amadori rearrangement (14). There has been considerable focus on Pi, but little is known about organic phosphates effects on the NEG process.

Organic phosphates (Po) are esters of inorganic phosphates, that may contain one or more phosphate groups. Similarly, to Pi, Po are thought to play an important role in the NEG of HbA (17). Although there is not a lot known about organic phosphates role in nonenzymatic glycation, it has been suggested that its role is different from Pi, where the role of Po is the abstraction of the proton in the Amadori rearrangement, and Pi plays a role to induce hemoglobin to catalyze its own glycation (17) (Scheme 1). Unlike Pi, which is a single molecule, Po are a category of many molecules that contain one or more phosphate groups, examples of which are BPG, and phosphorylated amino acids. Of the examples mentioned, BPG is the Po molecule that is the focus of this research.

BPG contains 2 phosphate groups on a 3-carbon chain. This molecule acts as an allosteric modulator of oxygen release in human erythrocytes (18). When BPG binds to

HbA it reduces the affinity for oxygen in HbA, which favors the transport of oxygen in the body (19). This allosteric effect happens by BPG binding to HbA in the central cleft between the two β-chains (6, 18-20), and has been reported to interact with multiple residues including: valine 1, histidines 2 and 143, and lysines 82 and 144 (18-20).

HbA and BPG are the principle organic constituents inside the health adult human erythrocyte (20). Both molecules have an *in vivo* concentration of 5 mM (10,21), but the function of BPG was unknown until the discovery that it lowers oxygen affinity of hemoglobin and thus facilitates the unloading of oxygen in active tissues (20). It has also been shown that the amount of BPG within the erythrocyte directly correlates with the serum concentration of Pi and the time average deoxyhemoglobin concentration (22). In addition, the concentration of BPG is elevated in individuals who have high levels of glycated hemoglobin (23).

We are also focused on phosphorylated amino acids due to their ability to potentially eliminate the need for a concomitant binding of an outside base in protein pocket to facilitate formation of the electrophile or nucleophile. This occurs through a post-translational modification called phosphorylation, which covalently attaches a pi group to an amino acid residue within a protein. Commonly serine, threonine, and tyrosine amino acid residues are phosphorylated due to their hydroxyl groups (24). Very little is known about phosphorylated amino acids role/s in NEG.

The focus of this chapter is: a) to determine if BPG can facilitate ring-open a glucopyranose, b) to theoretically determine the mechanism/s of BPG on NEG in the

formation of HbA1c, and c) to establish whether phosphorylated amino acids increase NEG of proteins, specifically hemoglobin in the A1c pocket? The methods to address these points included computational analysis and ^{1}HNuclear Magnetic Resonance Spectroscopy (NMR)

2.2 Results:

Question #1: Can 2,3-bisphosphoglycerate (BPG) ring-open a glucopyranose molecule?

To answer this question, pure α-glucopyranose was placed in an aqueous (D_2O) media at a pH in the physiological range and its interconversion (a reversible process) with the β-glucopyranose anomer at room temperature (~20°C) was followed as a function of time by ^{1}HNMR. The extent of α-glucopyranose- to- β-glucopyranose 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}HNMR signal of the anomeric C-H of the α-glucopyranose (which is diminishing with time) to equal the integration of the ^{1}H NMR signal of the anomeric C-H of the β –glucopyranose (which is increasing with time), equating to a 50:50 mixture of α and β anomers (Fig. 2). Using the inflection times for the reference (only α-glucopyranose converting to β-glucopyranose) (270 min) and for dibasic inorganic phosphate (Pi) ($\geq$9 min), we are able to compare to the inflection time for tetrabasic BPG (46 min) (Table 1).

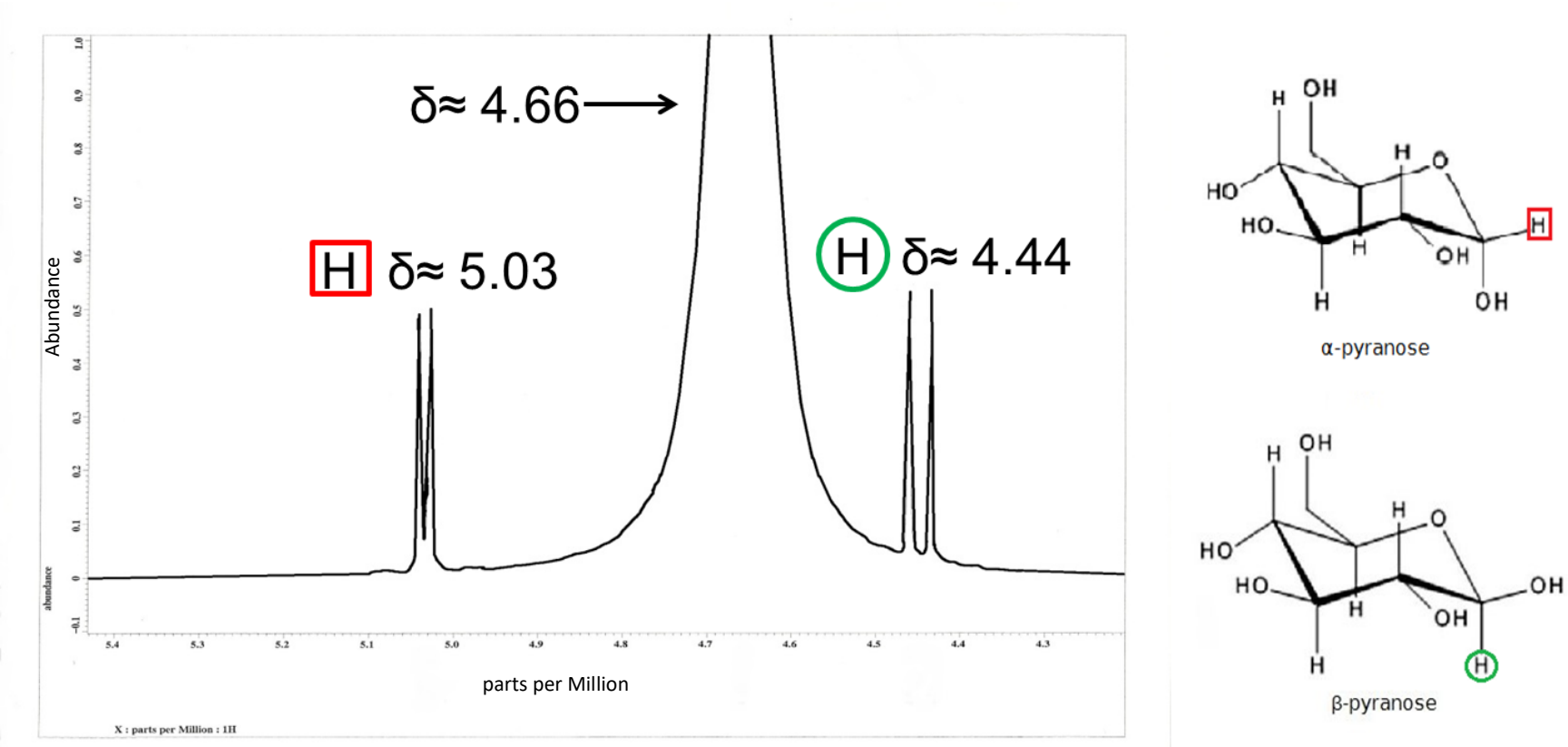

Figure 2. [1]HNMR spectra of the equilibrium formed between α- and β-glucopyranose, showing the doublets formed by the indicated protons (left). The inflection time is defined as the time when the area under the doublets are equal to one another when going from α- to β-glucopyranose.

Table 1: Time-dependent ^{1}H NMR data for α- to β-glucopyranose interconversion

Glucopyranose	Binding Molecule	Inflection Time (min)	Relative Rate Enhancement
α	-	270	Reference
α	**Pi**	9	30
α	**2,3-BPG**	**46**	**6**

*Charge state of BPG is tetrabasic in these experiments

With these inflection studies, we demonstrated that BPG, similar to Pi, can react with a glucopyranose molecule to ring-open it in an aqueous environment. However, tetrabasic BPG does not ring-open glucopyranose as well as dibasic Pi does in an aqueous environment.

Question #2: What are the Mechanisms of 2,3-bisphosphoglycerate (BPG) in the formation of Hemoglobin A1c (HbA1c)?

To be able to form HbA1c you need both a reactive electrophile and nucleophile. The electrophile is formed by the ring-opening of a glucopyranose isomer to its reactive electrophilic form. The nucleophile can be the N-terminal valine 1 residue found on either of the β-chains of HbA, which in turn attacks the electrophilic form of glucose. Roles on facilitating both the nucleophile and the electrophile were assessed to answer this question.

Our first approach was to look for chemistry mechanisms involving inorganic phosphate (Pi). As a result, we have determined 7 possible mechanisms for Pi (16). Of these mechanisms only 3 were theoretically possible for BPG (Fig. 3). These mechanisms have been denoted as 1) **the naked mechanism** (Fig. 3 panel A) which consists of BPG having a proximity effect which brings the other amino acid residues closer to a bound glucopyranose molecule to ring-open it, 2) **the bi-molecular bridge mechanism** (Fig. 3 panel B), which consists of BPG acting as a base to abstract the proton on the anomeric OH with an additional amino acid residue to act as an acid to donate a proton to the hemiacetal oxygen which in turns ring-opens the glucopyranose, and 3) **the bridge**

mechanism (Fig. 3 panel C) which consists of BPG abstracting a proton from a nearby valine 1 amino acid residue facilitating the necessary nucleophile, and in turn acts solely on a glucopyranose molecule as an acid (donates a proton to the hemiacetal oxygen) and a base (abstract a proton from the anomeric OH) to ring-open it. All 3 of these mechanisms are specific for the formation of the electrophile necessary in NEG.

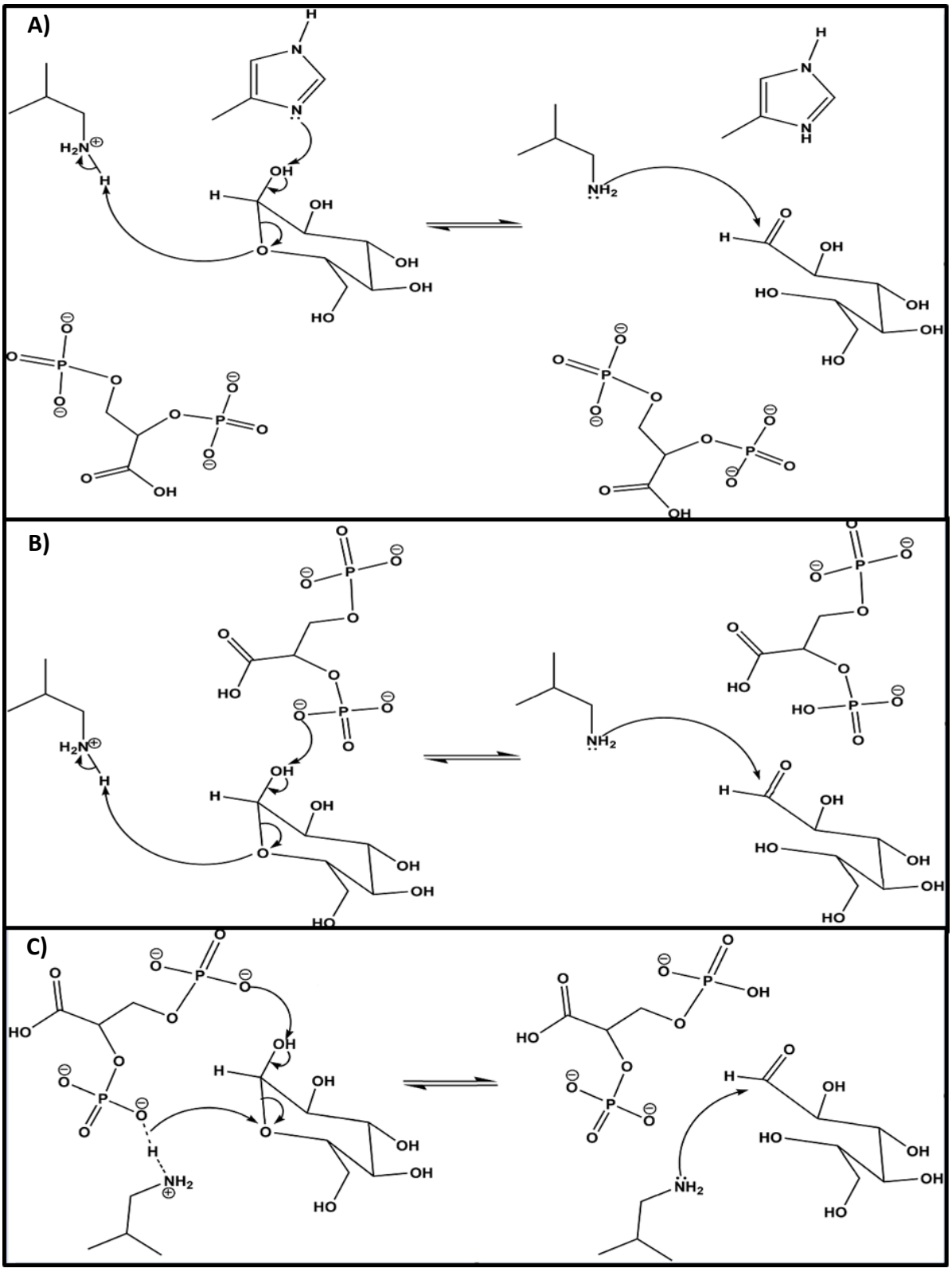

Figure 3: Proposed mechanisms for BPG in the ring-opening of a glucopyranose molecule to form the reactive electrophile. **A)** denoted as the **naked mechanism**, shows BPG acting in an allosteric method and not directly interacting with the amino acid residues or the

glucopyranose, **B)** denoted as the **bi-molecular bridge mechanism**, shows BPG directly interacting with the glucopyranose to help ring-open-open it, and **C)** denoted as the **bridge mechanism**, shows BPG interacting with both the glucopyranose and the amino acid residues to be able to ring-open the glucose pyranose without the direct influence of an amino-acid residue on the glucopyranose.

We followed up on this premise of the 3 mechanisms using computational modeling. This allowed us to create a protein pocket environment with a glucopyranose in the Val1/Lys82 pocket and hand place a BPG molecule in a favorable position and optimize the energy of the system. This energy optimization minimizes the energy in the system and geometrically altered the system into the most favorable position. From utilizing this method with our 5 Å threshold we were able to model each of the 3 mechanisms in question (Fig. 4).

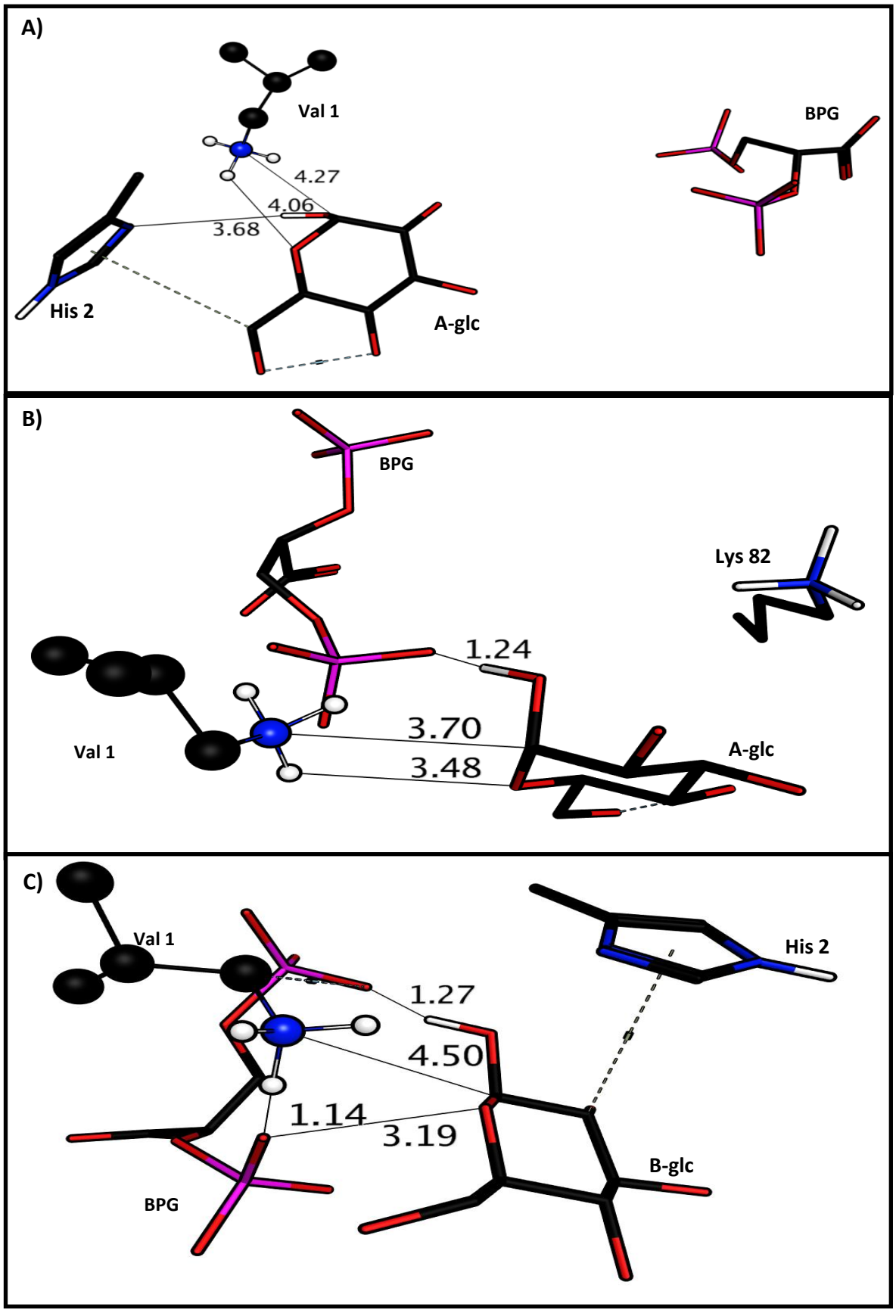

Figure 4: Proposed mechanisms for tetrabasic BPG in the ring-opening of a glucopyranose

molecule as shown by computational modeling. Color designation for atoms are as follows; Carbon atoms are black, oxygen atoms are red, nitrogen atoms are blue, phosphate atoms are pink, and hydrogen atoms are white. **A)** shows the **naked mechanism** in which BPG provides a needed allosteric effect in order to bring the nucleophile valine 1 and the base Histidine 2 within our 5Å range to be able to ring-open the glucopyranose molecule, **B)** shows the **bi-molecular bridge mechanism** in which BPG will act as a base to deprotonate the anomeric OH while the ammonium ion on valine 1 will act as an acid to donate its proton to the hemi-acetal oxygen, while Val 1 (now an amine) also become nucleophilic enough to attack the electrophilic site in the newly ring-opened glucopyranose, and **C)** shows the **bridge mechanism** wherein BPG acts as a base to deprotonate one of the nearby amino acid residues, valine 1 in this case, which facilitates the formation of the necessary nucleophile and then acts simultaneously with each phosphate group acting, one as an acid and one as a base, to deprotonate the anomeric OH, and donate a proton to the hemiacetal oxygen thus ring-opening the glucopyranose. In all 3 proposed mechanisms valine 1 will act as the nucleophile.

Question #3: Can phosphorylated amino acids increase nonenzymatic glycation of Hemoglobin A1c (HbA1c)?

To answer this question, we used a list of known phosphorylation sites in HbA (Table 2) which was provided by Phosphosite.org (25). From here we phosphorylated each residue using MOE and measured nearby nucleophilic amino acid residues (valine 1, or any lysine), which demonstrated no phosphorylation sites within 20Å from either of the valine 1's on the β-chains, which are responsible for the formation of HbA1c (Table 2).

Table 2: A list of the residues that are known to be phosphorylated in both the α and β-chains of human Hemoglobin A, as well as any lysine residues within 20 Å of the phosphorylated residues. Measured between one of the oxygen atoms on the phosphate group and the proton located on the nitrogen atom on Val 1/Lys residues.

Human HBA phosphorylation sites β-chains	Residues of interest near phosphorylation sites on the β-chains	Human HBA phosphorylation sites α-chains	Residues of interest near phosphorylation sites on the α-chains
Thr 5	Lys 8	Ser 4	-
Ser 10	Lys 132	Thr 9	-
Thr 13	Lys 8	Tyr 25	Lys 120 beta
Tyr 36	-	Ser 36	Lys 132 beta
Thr 39	Lys 59	Thr 40	Lys 132 beta
Ser 45	Lys 59	Thr 42	Lys 95 beta
Ser 50	Lys 59	Tyr 43	Lys 95 beta
Thr 51	Lys 61	Ser 50	Lys 120 beta
Thr 88	Lys 144	Ser 53	Lys 120 beta
Thr 124	Lys 144	Thr 109	Lys 120 beta
Tyr 131	Lys 17	Ser 139	-
Tyr 146	Lys 95	Tyr 141	-

With this information, it appears that phosphorylated amino acid residues on HbA cannot directly affect the formation of HbA1c, although these residues could directly affect glycation sites other than the HbA1c site. This lead to us consider a possible allosteric effect to the Val1/Lys82 pocket that may impact in the formation of HbA1c by either facilitating or inhibiting it. An allosteric effect is defined as the binding of a ligand to one site of a protein, which affects or alters a different site of that same protein. To address this possibility, each potential phosphorylation site was phosphorylated one by one and distance measurements were taken between the N-terminal nitrogen atoms of the two valine 1 ’s and between the two ε nitrogen atoms of the lysine 82’s on the β-chains, before and after the optimization of the system. The initial distances between the nitrogens of both valine 1’s on the β-chains was 18.95 Å, and the distances between the nitrogens of both lysine 82’s on the β-chains was (11.15-11.22 Å), depending on the oxygenation state (Table 3,4). The results show an average change between all of the residues to be $\geq$ 0.1 Å, with a few noteworthy changes seen as large as 0.57 Å.

Table 3: The average change in the distance (Å) between the nitrogens of both lysine 82's and both valine 1's in the β-chains of Human hemoglobin, from phosphorylation of dibasic Pi to specific residues in both the α- or β-chains, under fully oxygenated and deoxygenated conditions. Noteworthy changes are also shown as the specific residue on the specific chain with the distances listed as Lys82 first and Val1 second.

Protein oxygenation state/ α- or β-chain phosphorylation with Dibasic Pi	Average Change in Lys82-Lys82 (Å)	Average Change in Val1-Val1 (Å)	Noteworthy Changes* (Lys82-Lys82, Val1-Val1) (Å)
Oxygenated/ α-chain Residues	0.09	0.08	**ser 36**: α2(-0.17, 0.32) **thr 40**: α1(0.19, 0.31) **thr 40**: α2(0.05, 0.26)
Oxygenated/ β-chain Residues	0.09	0.09	**tyr 131**: β1(0.13, 0.57) **tyr 146**: β2(0.27, 0.23)
Deoxygenated/ α-chain Residues	0.05	0.06	**thr 40**: α2(0.33, 0.33)
Deoxygenated/ β-chain Residues	0.05	0.06	**thr 13**: β1(0.08, -0.27) **thr 124**: β1(0.11, 0.23)

* negative values indicate where the residues measured decreased in distance, and positive values indicate where the residues measured increased in distance

Table 4: The average change in the distance (Å) between the nitrogens of both lysine 82's and both valine 1's in the β-chains of Human hemoglobin, from phosphorylation of monobasic Pi to specific residues in both the α- or β-chains, under oxygenated and deoxygenated conditions. Noteworthy changes are also shown as the specific residue on the specific chain with the distances listed as Lys82 first and Val1 second.

Protein oxygenation state/ α or β chain phosphorylation with Monobasic Pi	Average Change in Lys82-Lys82 (Å)	Average Change in Val1-Val1 (Å)	Noteworthy Changes* (Lys82-Lys82, Val1-Val1) (Å)
Oxygenated/ α-chain Residues	0.1	0.05	**thr 42**: α1(-0.22, 0.09)
Oxygenated / β-chain Residues	0.1	0.07	**thr 88**: β1(-0.22, 0) **tyr 131**: β1(0.29, 0.43) **tyr 14**6: β2(-0.24, -0.1)
Deoxygenated/ α-chain Residues	0.04	0.04	**thr 40**: α2(0.22, 0.11)
Deoxygenated/ β-chain Residues	0.06	0.07	**thr 13**: β1(0, -0.27)

* negative values indicate where the residues measured decreased in distance, and positive values indicate where the residues measured increased in distance

2.3 Discussion:

The objectives of this study were to a) determine whether or not 2,3-bisphosphoglycerate (BPG) would interact with and ring-open a glucopyranose isomer, b) determine potential mechanistic role/s of BPG to enhance nonenzymatic glycation in the formation of HbA1c, and c) determine whether or not phosphorylation of amino acid residues in Human hemoglobin could affect nonenzymatic glycation in the formation of HbA1c.

Inflection studies using 1HNuclear Magnetic Resonance (NMR):

Compared with the inflection times in aqueous (D_2O) media for the reference value (no anionic effect to the ring-opening of α-glucopyranose) (270 min) and with dibasic inorganic phosphate (Pi) ($\geq$9 minutes), the inflection time for tetrabasic BPG (46 min) can be considered effective in creating the reactive electrophile in aqueous solution, although not as effective as Pi. This may be due to the geometric constraints limiting effective collisions between glucose and BPG, or BPG interacting with itself or the surrounding water in aqueous solution, thus prolonging its affect.

The objective of this experiment was to determine whether or not BPG could interact with and ring-open a glucopyranose isomer thus creating the necessary electrophile needed for NEG to occur. The results of this experiment in aqueous solution confirm that this is the case, however the comparison of BPG to Pi may not be physiologically representative because NEG occurs in a protein pocket that would contain very little to no water.

Using Computational Modeling to determine the Mechanistic Roles of 2,3-Bisphosphoglycerate in the Formation of HbA1c:

This objective stemmed from the observation that there is the considerable evidence that Pi accelerates NEG (1, 14, 17, 26-29), but very little is known about possible effects of organic phosphates (Po, specifically BPG) on HbA1c. BPG was chosen as our model molecule due to its high intracellular concentration and physiological ability to act as an allosteric modulator of oxygen release from HbA in erythrocytes (18). This allosteric effect occurs by BPG binding to HbA in the central cleft between the two β-chains (6, 18-20), and BPG interacts with multiple residues including; valine 1, histidines 2 and 143, and lysines 82 and 144 (18-20) - which is the same site where HbA1c formation occurs. This leads us to the question of whether BPG affects the formation of HbA1c? Using computational modeling, roles on facilitating both the nucleophile and the electrophile were assessed. According to Gil et al., (17) Pi and Po both play an important role in the glycation of hemoglobin. However, the roles of Pi, and Po are different from one another. In Gil's study, it is claimed that the role of Po is to abstract the proton in the Amadori rearrangement (Fig. 1, Stage 2-3) (17). According to Smith et al., (8) the mechanism of BPG is unknown, but it may act in decreasing the positive charge on the amino terminus, which would create the necessary nucleophile to react with the electrophilic ring-opened glucose (Fig. 1 Stage 1), or cause a conformational shift of the protein pocket that results in improved steric factors (Fig. 4 Panel A) (8).

Based on these studies and the current mechanistic studies done on Pi (16), we

posit several mechanistic possibilities of BPG in the formation of HbA1c (Fig. 3). These mechanisms are as follows: a) **the naked mechanism**, where BPG's role is to provide a proximity effect that improves steric factors for the formation of the electrophile to occur by ring-opening a glucopyranose isomer, and form the nucleophilic N-terminal valine 1 to be within our 5 Å cutoff of the electrophile (8) (Fig. 3 Panel A), b) **the bi-molecular bridge**, where one of the phosphate groups on BPG acts as a base to deprotonate the anomeric OH on a glucopyranose molecule in combination with a nearby acidic amino acid that donates a proton to the hemiacetal oxygen which ring-opens the glucopyranose to its reactive electrophilic form, near the nucleophilic N-terminal valine residue (Fig. 3 panel B), and c) **the bridge mechanism**, where both phosphates on BPG act on a glucopyranose molecule, one acts as a base to deprotonate the anomeric OH, and the other acts as an acid (requires prior proton abstraction from nearby amino acid residue) and donates a proton to the hemiacetal oxygen which ring-opens the glucopyranose to its reactive electrophilic form, near the nucleophilic N-terminal valine residue (Fig. 3 panel C). With these mechanisms identified, BPG most certainly is able to affect NEG in the formation of HbA1c. These mechanisms with comparison to the Pi mechanisms (16) show that Po and Pi can show similar effects although not entirely the same, due to the lack of acidic protons on Po that can be found on Pi. Pi, both mono- and dibasic forms, contain at least 1 acidic proton, where in Po due to the organic group on the molecule only the monobasic form will contain an acidic proton. This can be seen in figures 3c and 4c where BPG needs to deprotonate the ammonium ion in the valine residue prior to being able to act as an acidic residue. Since

BPG is only found within the erythrocyte in mammals, this molecule may be partly responsible for the glycation gap that has been identified between HbA1c and fructosamine values. Glycation gap is defined as the difference between the measured level of HbA1c and the level that would be predicted from its regression on the fructosamine level (30). Since BPG is known to bind within the A1c pocket of mammalian hemoglobin, and can affect the glycation of hemoglobin, then it is probably one of many factors that contribute to the glycation gap. The glycation gap is the difference between measured A1c and the value predicted by regression on fructosamine (31). With A1c being the only measurement included in the glycation gap that contains BPG as a variable, and with increased concentrations of BPG correlating to increased A1c levels (23), it can be assumed that BPG is a unique factor in the glycation gap.

All of the computational modeling was done using a BPG molecule that is tetrabasic (each phosphate group is dibasic). This form of BPG is the most common indicated by the individual pKa's of the phosphate groups as shown by Hobish and Powers (32). This form of BPG also limited the mechanistic possibilities for creation of an electrophile, due to its lack of an acidic proton on its phosphate groups, which can only be found in its dibasic or tribasic form (one or both phosphates are monobasic). When looking at Pi there is an acidic proton available in its mono- and dibasic forms, this allows it to be more versatile in its mechanistic possibilities, where BPG will need to accept a proton prior for it to act in any acidic mechanisms. When it comes to Pi and Po there are mechanistic similarities and differences. The largest difference between Pi and

Po is the number of acidic protons, in that Po will have one less proton if in the same protonation state as Pi. This is due to the ether group that makes an organic phosphate. Due to this difference in protons Po is not able to perform as many mechanisms as Pi is. As explained earlier Pi can perform 7 mechanisms (Pi paper) where Po can only perform 3 of the 7 mechanisms.

There are a few limitations to computational modeling that should be mentioned. First, by hand-placing molecules into favorable positions and then optimizing the system, it makes it difficult to duplicate results. Also, the data that we have reported is only geometric possibilities, and these possibilities may or may not be energetically favorable. However, based upon the energies given in the dockings between the glucopyranose isomers and the HbA, the docking or binding events are likely favorable ($\leq$ -2.5 kcal/mol). Another limitation depends on which crystal structure you use for the docking experiments. It was apparent that different crystal structures from the Protein Data Base RCSB (1BZ0, 1B86, and 1GZX) vary in conformation and specific coordinates and results vary according to which crystal that is used (Table 5).

Table 5: Shows the measurements between 3 different PDB files of Human Hemoglobin. The measurements are between the same nitrogen atom on both Val1's, Lys82's, His2's and His 143's on each β-chain of each crystal. The crystals were modified to be fully oxygenated/deoxygenated, and with repeated measurement taken with the presence of BPG in the protein.

PDB	Oxygenation State	BPG presence	Val 1-Val 1(Å)	Lys 82-Lys82(Å)	His 2-His 2 (Å)	His 143-His143 (Å)	reference
1BZ0	full oxygenated	no	20.3	8.7	21.9	15.4	**20**
1BZ0	full oxygenated	yes	20.3	7.3	21.4	15.2	**20**
1BZ0	full deoxygenated	no	20.8	9.5	21.6	16.9	**20**
1BZ0	full deoxygenated	yes	20.5	7.1	21.8	16.3	**20**
1B86	full oxygenated	no	17.0	7.9	13.2	12.1	**21**
1B86	full oxygenated	yes	14.6	5.2	11.7	11.4	**21**
1B86	full deoxygenated	no	15.5	6.0	11.8	13.3	**21**
1B86	full deoxygenated	yes	14.7	5.2	11.8	11.3	**21**
1GZX	full oxygenated	no	19.0	11.2	13.4	16.5	**33**
1GZX	full oxygenated	yes	17.1	8.8	13.4	15.0	**33**
1GZX	full deoxygenated	no	19.0	11.2	13.4	15.4	**33**
1GZX	full deoxygenated	yes	18.3	9.6	15.1	14.6	**33**

In summary, the variability in distance between the specific molecular landmarks presented in Table 5 depends on oxygenation states of the protein, as well as in the presence or absence of BPG. The variability between the valine 1s of the different crystals is as large as 5.9 Å for deoxygenated protein with BPG present and as small as 1.3 Å for oxygenated protein without BPG. The variability between the lysine 82s of the different crystals is as large as 5.2 Å for deoxygenated protein without BPG, and as little as 0.8 Å between oxygenated protein without BPG. The variability between the histidine 2s of the different crystals is as large as 10.0 Å between deoxygenated proteins with BPG bound, and as low as 0.2 Å between oxygenated protein without BPG. The variability between the histidine 143s of the different crystals is as large as 5.0 Å for the deoxygenated protein with BPG, and as little as 0.2 Å for oxygenated protein with BPG. What do these data tell you and how does this affect your study objectives?

Using Computational Modeling to determine if phosphorylation of amino acid residues in Human Hemoglobin enhance nonenzymatic glycation in the formation of HbA1c:

It was mentioned earlier that there is little known on organic phosphates role in NEG, and phosphorylated amino acids are no exception. Phosphorylation is a common post-translational modification of proteins caused and we identified the amino acid residues that are phosphorylated in HbA (Table 2). Next we used computational modeling to determine that these phosphorylated amino residues do not directly affect NEG in the formation of HbA1c. This was determined by manually phosphorylating each residue (one at a time) and looking in a 20 Å range for any lysine's and/or valine 1 (Table

2).

In as much as our computational analysis predicts that phosphorylated amino acid residues do not directly affect the formation of HbA1c, we explored whether there may be any allosteric effects from the phosphorylation of these residues on the Val/Lys82 pocket that could impact reaction distances. We measured the distances between the two valine 1's and the two lysine 82's, before and after each phosphorylation event to see if there was a conformational change in the pocket (Table 3, 4). The results of this showed very small changes in the protein pocket ($\geq$0.1 Å), but a few results were notable with changes seen as large as 0.57 Å. Based on these results it seems likely that the phosphorylation of amino acid residues would have a minimal effect on the protein pocket responsible for the formation of HbA1c, which in turn would not necessarily effect NEG there. In other proteins or other pockets of HbA, phosphorylation may enhance NEG, either directly or indirectly, but it does not seem to be the case in the pocket of question.

Clinical Relevance

BPG is known to be able to enhance NEG (4, 8, 23, 27), and its mechanisms were expounded upon in this study. The clinical relevance of BPGs effect in this process may be significant due to its ability to enhance this process. BPG has been seen in higher concentrations in individuals with higher rates of glycation, compared to individuals with lower rates of glycation (23), which confirms that BPG levels are a factor in the NEG of human hemoglobin (8). Since HbA1c is one of the most common measurements used

to help diagnose and monitor diabetes (34), a BPG measurement should also be included to determine if it is glucose, BPG, or both that are causing increased HbA1c levels.

BPG is not the only factor known to alter A1c measurements, there are multiple drugs and conditions that can alter A1c levels (Table 6) (35). These factors can be very common in the population and should be considered when measuring A1c values.

Table 6: Some factors that are known to alter A1c measurements.

Condition	Effect on A1c	comments
Chronic alcohol consumption	False Increase	Formation of acetaldehyde-HbA1 compound
Splenomegaly	False Decrease	Decreased erythrocyte lifespan
Vitamin E ingestion	False Decrease	Reduced glycation
Vitamin C ingestion	False Increase or Decrease	Depends on assay used, increased with electrophoresis and decreased with chromatography
Pregnancy	False Decrease	Decreased erythrocyte lifespan through 2nd trimester

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Chapter 3. Materials and Methods

Non-covalent binding of organic Phosphates and glucose to Human Hemoglobin.

Computational substrates-All non-covalent binding data was obtained via computational methodologies. The structures utilized in the docking computations were as follows: a) the crystal structure for the fully oxygenated Human Hemoglobin A (HbA) (1GZX) (1) was obtained from the RCSB Protein Data Bank (2), b) the α - (α-glc) and β-glucopyranose (β-glc) isomers were obtained from Heterocompound Information Centre, Uppsala, (HIC-UP) (3), and c) 2,3-bisphosphoglycerate (BPG) was built with the Spartan Student Edition (4). The decision to use the α- and β-glucopyranose isomers arose from their being the dominant forms, out of the 5 possible glucose isomers (Figure 1), found in aqueous solution. The α- and β-glucopyranose isomers represent ~99% of the available isomers at equilibrium Clark et al. (5).

The docking of various molecules was done to the HbA protein, more so the molecules were docked to both the clinically relevant glycation site, specifically the Val1/Lys82 pocket for HbA1c, and to the entire protein. The docking was accomplished with Molecular Operating Environment (MOE, ver. 2014.9, Chemical Computing Group Inc., Montreal, Canada). All MOE docking computations were done using default settings with the exceptions of: temperature being changed to 310 K, using an induced fit setting, and energy minimizing immediately before any docking is done. Within MOE several parameters were altered for these computational experiments: The protein (HbA) used was altered in two ways, the first being, a) the N-terminal Valine1 on each β-

chain was modified such that the charge state on Valine1 was either an R-NH_2, or R-NH_3^+ and the second being, b) the oxygenation state of the protein, which was modified to be either completely deoxygenated (having no oxygen on any of the heme-groups), or completely oxygenated (having oxygen on all four heme-groups). For all docking studies a cutoff distance of 5Å was established for effective geometry between reacting species within protein pockets. This is a more conservative geometric parameter predicated by the parameter set forth by Clark et al. (5), and a cutoff exothermicity threshold of -2.5 kcal/mol energy was established for effective reaction within protein pockets. This cutoff is to ensure that the effective reaction within the protein pocket is more favorable than any interaction with a potential water molecule that may be nearby.

Assessment of geometric possibilities-When the generated poses were assessed visually we were looking for possible facilitation of an electrophile or a nucleophile. The facilitation of an electrophile is determined by a maximum distance of 5 Å from either, a) the nitrogen atom from a lysine/valine1 amino acid (currently an R-NH_2), or b) from one of the negatively charged oxygens on the organic phosphate in question (BPG) to the hydrogen atom on the oxygen that is directly attached to the anomeric carbon on a glucopyranose molecule. This interaction can ring-open the glucopyranose molecule and generates the electrophile.

The facilitation of the nucleophile is determined by a maximum 5Å distance from either, a) a nitrogen atom found on a nearby Histidine residue (currently an R-NH_2), or b) from one of the negatively charged oxygens on the organic phosphate in question (BPG) to one of the protons on a R-NH_3^+ lysine/valine1 residue. This interaction

generates the nucleophile by deprotonating the R-NH_3^+ residue. Once both of these parameters are satisfied then a measurement was taken from the nitrogen atom on the deprotonated lysine/valine1 residue to the anomeric carbon of the glucopyranose molecule. If all of the distances measure were within the 5Å parameter set, then the specific pose in question had a favorable geometry and was consider to be a possible mechanism.

2,3-Bisphosphoglycerate binding-While it is well known that BPG binds non-covalently in the Val1/Lys82 pocket and affects oxygen affinity of HbA (6,7), it is not known if BPG plays a role in nonenzymatic glycation. A series of computational experiments were completed with BPG and HbA. The BPG ligand used was tetrabasic (each phosphate group being dibasic). With the parameters mentioned above, the non-covalent binding experiments were completed in which α-glc, and β-glc isomers were placed randomly within the Val1/Lys82 pocket. Next, the BPG ligand was hand placed within the same Val1/Lys82 pocket as the α-glc, and β-glc isomers, and the resulting poses were geometrically optimized and assessed visually using default settings. This was done to streamline the process in order to determine if specific roles of BPG in NEG were possible in a theoretical environment.

Phosphorylated amino acid residues- It is known that certain serine, threonine, and tyrosine residues can undergo phosphorylation via an enzyme kinase (8,9). From this information the question arose, can these covalently bound phosphates on HbA interact with nearby amino acid residues or a bound glucopyranose molecule in a way to facilitate NEG and the formation of Hemoglobin A1c (HbA1c)? A series of computational

experiments were completed with these phosphorylated residues (see residue list, Table 2 Ch. 2). Within MOE the protein was modified to be either completely deoxygenated, or completely oxygenated. Each residue in question was modified within MOE by manually building a phosphate group (mono or dibasic) onto the existing alcohol portion of the residue on each α- or β-chain that the particular residue resides on. This was done for one residue at a time and was energy minimized after each modification. Measurements were then taken of the Val1/Lys82 pocket between the two valine 1 residues, and the two lysine 82 residues before and after a particular residue was modified and energy minimized. The change in distance for each phosphorylated residue was then recorded to determine whether any allosteric effect are likely.

NMR inflection times-

Chemical reagents- All chemical reagents were obtained from Sigma-Aldrich.

Model reactions to assess the potential facilitation of glucopyranose ring-opening with selected organic phosphates- To test whether BPG (pentasodium salt)(Sigma-Aldrich product number D-5764), can facilitate glucose ring-opening, (10 mM,) of pure α-glucopyranose (Sigma-Aldrich product number 158968) was placed in aqueous (D_2O) media at a pH in the physiological range (~7.4) and its interconversion (a reversible process) with the β-glucopyranose isomer at room temperature (~20°C) was followed as a function of time by ^{1}HNMR (JEOL CPF-300). The extent of α-glucopyranose to β-glucopyranose 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}HNMR signal of the isomeric C-H of the α-glucopyranose to equal the integration of the ^{1}H NMR signal of the anomeric C-H of the β–glucopyranose equating to a 50:50 mixture of α-glucopyranose and β-glucopyranose isomers. The initial α-glucopyranose signal (measured after ca. 8 min in solution) is ca. 96% of the sum of the signal for the α-plus-β-glucopyranose and diminishes with time, while the initially negligible signal for the β-glucopyranose increases with time. Inflection time for α- to β-glucopyranose interconversion was then determined in the presence of equimolar BPG. The aqueous media in this reaction was at a physiological pH of 7.

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Chapter 4. Unifying Conclusions

Currently, there are over 347 million people globally affected by type II diabetes (1), with over 29 million people nationally affected (2), and this number is increasing. If left untreated, serious diabetic complications can arise, such as neuropathy, retinopathy, nephropathy and cardiovascular disease (3). Understanding the mechanism by which hyperglycemia (high blood glucose), as a result of diabetes, structurally modifies proteins is clinically important. Nonenzymatic glycation (NEG) describes an array of slow non-enzymatic chemical processes that give rise to covalently modified proteins, particularly with glucose. This process is clinically important because the measurement of glycated hemoglobin A1c (HbA1c) has been used to track diabetic conditions over a long period of 8-12 weeks (4). Since NEG is a natural process occuring during the lifetime of a given protein, it is normal to have an HbA1c value between 3-5% (5-7). HbA1c values in patients with diabetes mellitus are increased at least 2-fold (4,5,8,9) but any measurement greater than 6.5% is considered pathologic (10).

Understanding the mechanism by which glucose covalently binds to proteins is important because it reveals key reagents and processes that contribute to increasing the rate of glycation as well as potential targets for pharmaceutical intervention. NEG is comprised of four temporal stages. The first stage consists of the initial noncovalent binding of a ring-closed glucose isomer and perhaps an effector reagent, like inorganic phosphate, to a protein, followed by the ring-opening of glucose within a protein pocket, which creates a reactive electrophile. A nearby nucleophilic residue such as

valine 1 can then attack the electrophilic glucose (11). Stages two-four are the covalent stages, beginning with the formation of the Schiff base, then proceeding to the Amadori rearrangement and ending with the formation of advance glycation end-products (AGE) (12).

Until recently, most of the research focus has been on the covalent stages of NEG and very little has been presented about the initial, noncovalent stages. However, our lab has recently developed an increased understanding and application of the early noncovalent stages, highlighting the importance of inorganic phosphate (Pi) toward generating the ring-opened glucose electrophile while bound in a protein pocket (12). This work has expanded the understanding of the noncovalent stages of NEG and has shown that it is much more complex and diverse than previously thought, with multiple mechanisms, both including and excluding phosphate, leading to a covalently bound glucose (Fig. 1).

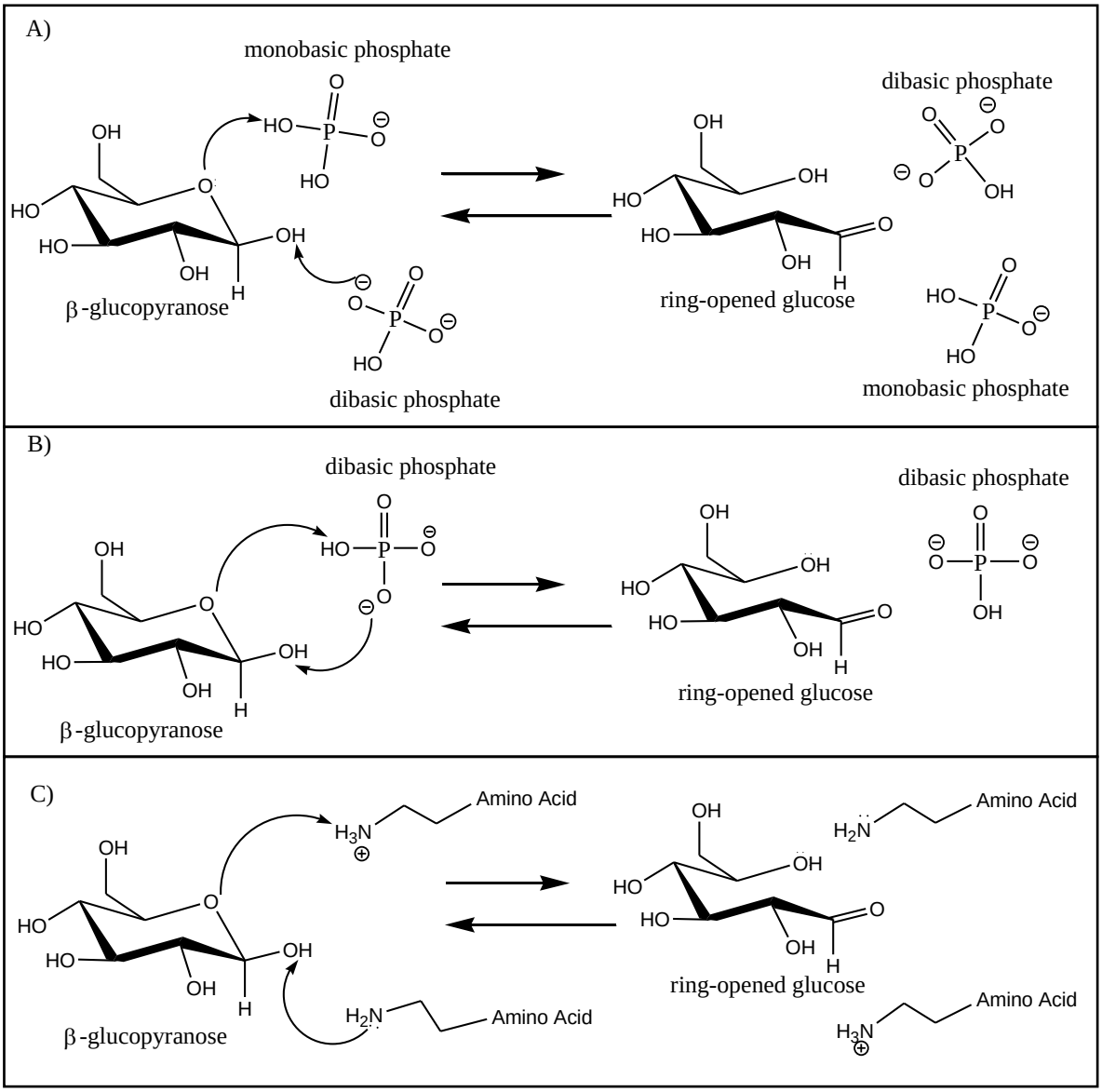

Figure 1. Possible mechanisms to ring-open a glucopyranose molecule in the most favorable way. **A)** A mechanism (simultaneous or sequential) in which two different inorganic phosphate molecules act to ring-open a glucopyranose isomer in solution. One monobasic inorganic phosphate acting as the acid and a dibasic inorganic phosphate acting as the base. **B)** A mechanism in which a glucopyranose isomer is ring-opened using an inorganic dibasic phosphate which simultaneously acts as both an acid (by donating a proton to the glucopyranose) and a base (by abstracting a proton from the glucopyranose). Inorganic phosphate is shown, but it is not the only reagent capable of ring-opening a glucopyranose molecule; other physiological anions may also play this role. **C)** A mechanism in which a glucopyranose isomer is ring-opened using only amino acid residues (valines, lysines, and histidines) that are contained within a protein pocket.

Despite considerable knowledge about Pi and its role in the NEG process (12-18), little is known about organic phosphates, specifically 2,3-bisphosphoglycerate (BPG) and phosphorylated amino acid residues. BPG is the main focus of this thesis because it is an important regulator of intracellular hemoglobin function and it occurs at high concentrations in mammalian erythrocytes. In human erythrocytes BPG is present in equimolar concentrations with the hemoglobin tetramer (around 5 mmol/l) (10), and is known to bind to the central cavity of the β-chains (A1c cavity) in HbA (19). It is also noteworthy that BPG binds to HbA better than Pi does (19).

An additional focus of this thesis is on the mechanistic potential of phosphorylated amino acids on NEG. In theory, phosphorylated amino acids could provide a local source of acid and eliminate the need for an outside base to concomitantly bind in a protein pocket with a glucose molecule. Phosphorylation is a process through which enzymes called kinases covalently bind inorganic phosphate molecules to a molecule or protein, which is essential for the regulation of cellular processes like metabolism, proliferation, differentiation, and apoptosis (20). This regularly occurs among serine, threonine, and tyrosine amino acid residues in proteins due to their hydroxyl groups (20). To the best of our knowledge, the importance of phosphorylated amino acid residues in NEG has not been investigated.

This information led to the questions which were posited in the Unifying Introduction of Chapter 1 that were answered in detail within Chapter 2. Specifically, in Chapter 2 entitled *Possible Mechanistic Roles of 2,3-Bisphosphoglycerate (BPG), and Phosphorylated Amino Acids in the Formation of Hemoglobin A1c (HbA1c),* three

questions were posed. Each of these three questions will be generally answered in the order in which they appear in Chapter 1.

Question 1: *Can 2,3-Bisphosphoglycerate (BPG) interact with a glucopyranose isomer to ring-open it?* Based on the results of the Nuclear Magnetic Resonance (NMR) inflection studies, it is clear that in aqueous (D_2O) media tetrabasic BPG is capable of accelerating the ring-opening of a glucopyranose isomer, and creating a necessary electrophile for NEG. Although BPG does not ring-open glucopyranose isomers as well as dibasic inorganic phosphate (Pi) does in aqueous media.

Question 2: *What mechanistic roles can 2,3-bisphoglycerate (BPG) play in the formation of Hemoglobin A1c (HbA1c)?* Based on a prior study performed on Pi (21), we were able to determine the potential roles that BPG may play in the formation of HbA1c. In total, there were 3 potential mechanisms available to BPG, these mechanisms are as follows; a) **the Naked Mechanism**, b) **the bi-molecular bridge mechanism**, and c) **the bridge mechanism**.

Of the 3 mechanisms listed only **the naked mechanism** requires that BPG plays an indirect role. For this mechanism to work BPG provides a necessary allosteric effect. This brings together the necessary components (a protonated valine 1 and neutral histidine in this case), to ring-open the glucopyranose isomer forming the necessary electrophile, and having valine 1 nearby to act as the nucleophile which is necessary for the mechanism to work. The other mechanism requires BPG to provide a more direct approach (Fig 2 panel A).

The next mechanism listed is **the bi-molecular bridge mechanism**. This method requires BPG to perform a single action in the process of ring-opening a glucopyranose isomer to form the necessary electrophile. The action required is that of a base abstracting the proton on the anomeric OH of the glucopyranose isomer. This mechanism requires the facilitation of a secondary molecule, either a positively charged amino acid, or a secondary acidic molecule. The role of this secondary molecule is to donate a proton to the hemiacetal oxygen is the glucopyranose isomer, thus completing the ring-opening of the glucopyranose isomer (Fig 2 panel B). This all must occur near valine 1 which acts as the necessary nucleophile.

The third and final mechanism listed is **the bridge mechanism**. Unlike the bi-molecular bridge mechanism, this one requires BPG to act in both roles that are required to ring-open a glucopyranose isomer forming the necessary electrophile. This means that one of the phosphate groups will act as a base to deprotonate the anomeric OH, while the other phosphate group acts as an acid to donate a proton to the hemiacetal oxygen. This all must occur near valine 1 which acts as the necessary nucleophile. This mechanism requires that the phosphate group, that acts as an acid, be in a monobasic protonation state. This means that the phosphate group must first interact with a positively charged amino acid to abstract the proton and become monobasic prior to interaction with the glucopyranose isomer (Fig 2 panel C).

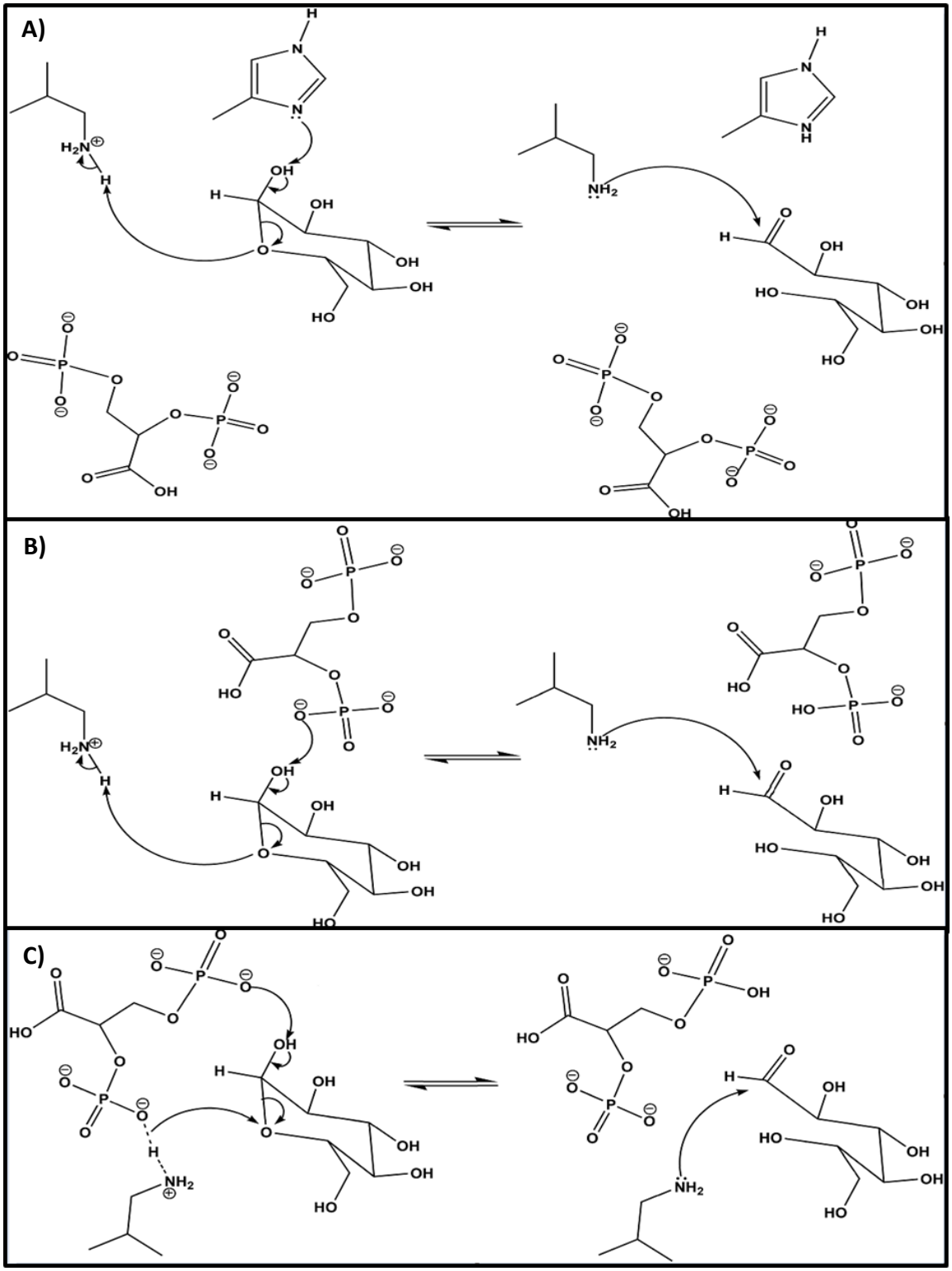

Figure 2: Proposed mechanisms for BPG in the ring-opening of a glucopyranose molecule. **A)** denoted as the **naked mechanism**, shows BPG acting in an allosteric method and not directly interacting with the amino acid residues or the glucopyarnose, **B)** denoted as the **bi-molecular bridge mechanism**, shows BPG directly interacting with the glucopyranose to help ring-open it,

and **C)** denoted as the **bridge mechanism**, shows BPG interacting with both the glucopyranose and the amino acid residues to be able to ring-open the glucose pyranose without the direct influence of an amino-acid residue on the glucopyranose.

Based on the results from the computational modeling, all 3 mechanisms were possible with favorable geometries - using a 5 Å threshold for distances. This threshold, which was used in a prior study (12) is very conservative when compared to another study (17). This provides confidence in the validity of these mechanisms in a geometric sense, but based on the method of computational modeling used, gives no information into the energetics of these mechanisms. Although based on the initial docking information used ($\leq$-2.5 kcal/mol), it is implied that the mechanisms would most likely occur with favorable energies. In conclusion, these mechanisms for BPG's affect in the formation of HbA1c are possible in a geometric sense, but nothing specific is known on the energetic of these mechanisms.

Question 3: *Could phosphorylated amino acids increase nonenzymatic glycation of proteins, specifically hemoglobin in the A1c pocket?* Based on the computational modeling performed, where each potentially phosphorylated amino was individually phosphorylated, it was concluded that none of the residues directly affect the formation of HbA1c. This was determined from a 20 Å threshold from the residues to surrounding areas. Then allosteric effects were considered as a possible indirect method to affect the formation of HbA1c, similar to that of the naked mechanism for BPG. Further computational modeling has shown that the phosphorylation of the potential amino acid residues has little to no effect on the specific pocket for the formation of HbA1c.

This suggests that the phosphorylation of amino acid will not play a direct role, and may only slightly play an indirect role, in the nonenzymatic glycation for the formation of HbA1c, although these residues could directly affect glycation sites other than the HbA1c site.

Future Research: The findings presented within this thesis rely heavily on computation modeling, which has its limitations. While this method allows us to see interactions inside of a protein pocket, it does not give us insight on the retention time of these molecules, nor does it give specific energetics to manipulated systems. Future research should include the use of Molecular Dynamics to better examine these mechanisms and gather information on the temporal components of NEG, as well as the energetics necessary to confidently assess the mechanisms that have been reported here.

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