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Mechanistic Insight into Taurine's Role in Reducing Nonenzymatic Glycation

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To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Marie Elizabeth Finkbeiner find it satisfactory and recommend that it be accepted.

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Abstract

Nonenzymatic glycation (NEG) is a multi-step process that involves the covalent modification of protein by glucose, and is related to the pathology of diabetes mellitus. Taurine (2-aminoethanesulfonic acid) has been shown to act as a glycation-reducing agent. ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy was used to determine that taurine reacts with the glucose derivative glyoxal in aqueous solution to form both mono-imines and di-imines, suggesting that taurine can remove reactive carbonyls before they bind to protein. Computational modeling was used to test for taurine effects in the initial, noncovalent stages of the NEG process. Taurine can to bind to known glycation sites of human hemoglobin (HbA) and human serum albumin (HSA) alongside glucose and other effector reagents to interfere with glucose ring-opening. Taurine was also able to inhibit the nucleophilic attack inside protein pockets. Overall, there are multiple mechanisms in which taurine can reduces NEG in the early noncovalent stage.

Chapter 1. Unifying Introduction

According to the American Diabetes Association, diabetes mellitus is a diverse metabolic disease that is characterized as “hyperglycemia resulting from the bodies inability to use blood glucose for energy” (Drive, 2017). There are two subcategories of diabetes mellitus, type 1 and type 2, which describe the physiological processes that lead to hyperglycemia. Type 1, which accounts for 5-10% of diabetic patients, is characterized by the bodies failure to produce insulin, which prevents blood glucose from entering select cells to be used for energy. This form of diabetes mellitus is an autoimmune disease and occurs when the immune system attacks insulin producing beta cells of the pancreas. The exact reason why the immune system targets these cells is unknown. The rate of beta cell destruction is quite variable between individuals, often rapid in infants and children, while slower in adults (American Diabetes Association, 2017) The incidence and prevalence of type 1 diabetes is increasing in the United States, but is far less frequent than type 2 diabetes (Dabelea et al., 2014). Type 2 diabetes mellitus accounts for 90-95% of all diabetics and is unique from type 1 in that the body is able to produce insulin, but cells become resistant to the insulin that is produced. The body tries to compensate for this, and keep blood glucose levels in the appropriate range, by producing more insulin. However, the body cannot keep up with the need for insulin so the individual experiences hyperglycemia (Drive, 2017). Insulin resistance may improve with weight reduction and/or

pharmaceutical intervention, but hyperglycemia seldom returns to normal (American Diabetes Association, 2017).

Diabetes mellitus, especially type 2, is very prevalent in the American population as well as around the world. The American Diabetes Association reported that in 2012, 9.3% of the American population, or 29.1 million individuals suffered from diabetes mellitus. Each year they estimate that an additional 1.4 million Americans are newly diagnosed with diabetes. Additionally, in 2012 the US spent $245 billion dollars on people diagnosed with diabetes (Drive, 2017).

When glucose is not being used for cellular energy production, it can covalently attach to intracellular and extracellular proteins, modifying their structure through a process known as nonenzymatic glycation (NEG). NEG is a multistep process that consists of the initial noncovalent binding of glucose to a protein, followed by the irreversible covalent attachment of glucose to the protein, leading to an array of structurally modified proteins, known as advanced glycation end-products (AGE) (Fig. 1).

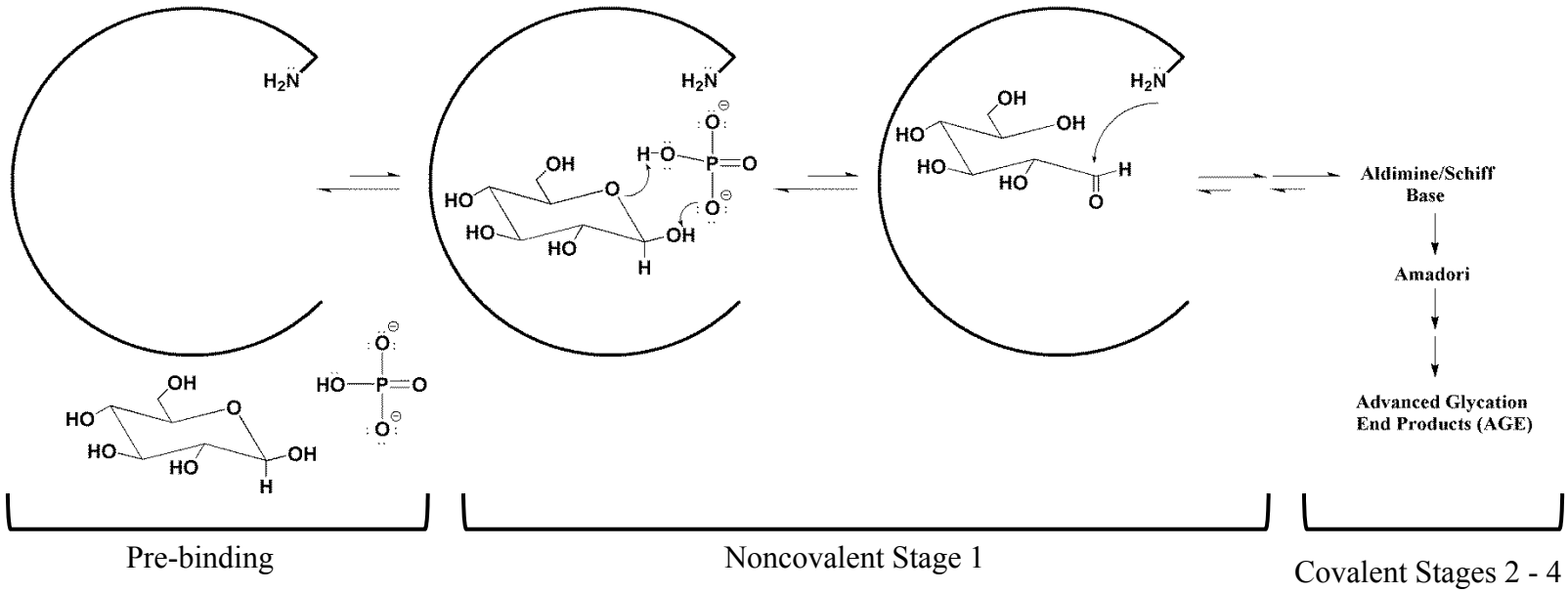


Figure 1. A mechanistic scheme of nonenzymatic glycation. Pre-binding occurs before a glucose molecule reaches a protein pocket, like the Val1/Lys82 pocket of HbA. Glucose noncovalently binds in a protein and can be ring-opened by a concomitantly bound effector regent, inorganic phosphate (Pi). A nucleophilic amino acid residue, Val1, attacks a ring-opened glucose to form the Schiff base. This product rearranges to form the Amadori product and eventually leads to the production of a host of advanced glycation end products (AGE). Double arrows indicate the mechanistic steps that are reversible, while single arrows represent mechanistic steps that are irreversible (Clark et al., 2013).

In order to investigate how NEG can be reduced from a mechanistic perspective, it is necessary to have an understanding of the bond making/bond breaking mechanistic scheme of the NEG process. NEG describes the process whereby glucose covalently bonds to a protein at select amino acid residues. However, glucose exists in five isomers that interconvert with each other through mutarotation. These structures include α- and β-glucopyranose, α- and β-glucofuranose and a ring opened structure, which contains an electrophilic carbonyl at the anomeric carbon (Fig. 2).

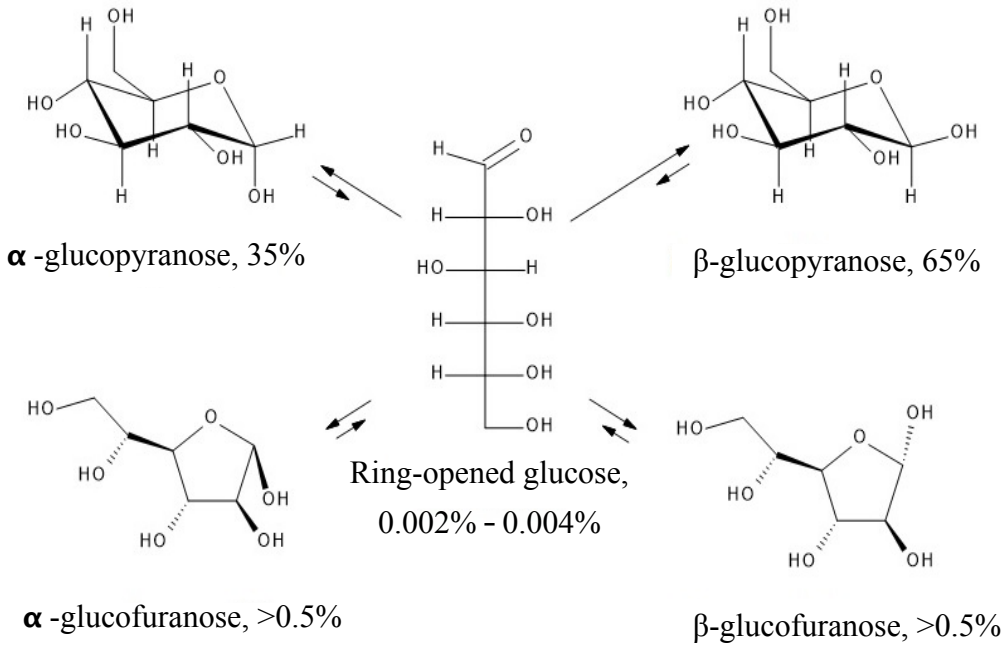


Figure 2. The five isomers of glucose in aqueous solution with respective solution equilibrium concentration. The reversible arrows indicate the relative rate of glucose ring-opening.

The α- and β-glucopyranoses exist in the highest concentrations (35% and 64% respectively) at equilibrium, whereas α- and β-glucofuranoses and the ring-opened isomer exist in much lower concentrations (0.5%, 0.5% and 0.002% respectively) (Clark et al., 2013). Although all 5 isomers are capable of binding in a protein pocket on the β-chain of human hemoglobin (HbA), the α- and β-glucopyranoses bind with better exothermicities than do the furanoses or ring-opened isomer (Clark et al., 2013). The ring-opened isomer of glucose must be present in a protein pocket in order to proceed with NEG. Because the relative concentration of the ring-opened isomer is so low and its binding energies are less favorable than the glucopyranose forms, it has been shown that a ring-closed isomer of glucose (α- or β-glucopyranose) initially binds to a protein and subsequently undergoes ring-opening within a protein pocket (Clark et al., 2013).

The NEG process exists in four stages as depicted in Fig. 1. Any pre-binding events occur prior to Stage 1, the noncovalent stage. In this stage, α- or β-glucopyranose enters the protein pocket and undergoes ring-opening with the assistance of both an acid and base to generate the electrophilic carbonyl. The roles of the acid and base may be fulfilled by intrinsic amino acid residues on the proteins itself as well as by effector reagents, like inorganic phosphate (Pi) that concomitantly bind in the protein pocket with glucose. The ring-opened glucose electrophile is then attacked by a nucleophilic amino acid residue, such as an N-terminal valine to form the covalently bound Schiff base in Stage 2. In Stage 3,

the Schiff base rearranges to form the Amadori product, which goes on to form AGE in Stage 4 (Clark et al., 2013).

The covalent modification of proteins by a glucose molecule can lead to the altered function of protein (Philippe and Bourdon 2011) and may ultimately contribute to diabetic pathology and complications (Brownlee et al., 1984). Covalent modification of protein may take many forms, thus why there are a host of AGE that are produced from NEG through a series of different mechanisms. Examples of covalently modified proteins include the irreversible formation of cross-links between proteins and AGE that can ultimately cause damage to blood vessels, kidneys, and eyes. A process like this is observed in the lens of the eye with the protein crystallin. Because this lens protein turns over very slowly in the body, it is a target for glycation as a normal process of ageing as well as a result of high blood glucose levels in diabetic patients. Compared to healthy individuals, those with diabetes experience a twofold increase in the percent of glycated crystallin. However, because this lens protein does not have as many reactive lysine residues as human serum albumin (HSA) or HbA, and the concentration of glucose in the lens is a fraction of that in the blood plasma in healthy individuals, the ratio of glycated crystallin to unmodified crystallin is smaller than the ratio of glycated to unmodified serum proteins. (Garlick et al., 1984).

The proteins that are involved in NEG are found throughout the body, including both intracellular proteins such as HbA in erythrocytes, as well as serum or extracellular proteins, such as HSA (Brede et al., 2016). Clinically, the

diabetic condition and associated hyperglycemia over extended periods can be monitored by measuring the levels of glycated protein within the body. The most common measurement of glycated proteins *in vivo* is the HbA1c measurement (Brownlee, 2005). HbA1c defines a covalently modified HbA by glucose at Val1 on one or both of the β-chains (Bookchin and Gallop, 1968; Bunn et al., 1975).

Although glycated HbA does not have a direct pathological implication in diabetes, it is a useful clinical measurement for a number of reasons. First, it can be used as a marker for the glycation levels of many other proteins in the body. Second, it is easier to isolate and measure than certain tissue proteins and has a relatively long lifetime (~120 days) (Brownlee et al., 1984). This measurement is useful over a single blood glucose test because blood glucose levels are subject to frequent change and can be misleading, whereas the HbA1c is an indirect measurement of average glycemia and tracks the diabetic condition over a longer period of time (~2-3 months). However, HbA1c measurements must be interpreted carefully in the presence of certain comorbidities. For example, during anemia HbA1c values will be lowered independently of glucose concentrations in the blood because lifespan of erythrocytes and HbA are shorter than normal (Ford et al., 2011).

HSA, the second most common serum protein, can also be used clinically to measure the amount of glycated protein and assess the average concentration of blood glucose. Fructosamine, a glycated amine (often glycated HSA) can be measured from serum (Johnson et al., 1983). This measurement describes a

shorter period of glycation of proteins (~2-3 weeks) than does HbA1c (~2-3 months), so it can be useful to track more rapid changes in blood glucose and glycated protein levels (Brede et al., 2016). Normal HbA1c levels are between 5% - 6%, whereas diabetics typically have levels greater than 6.5% (Brede et al., 2016). Normal fructosamine levels are 200 μmol/L, whereas a diabetic level would at or above 288 μM/L (Cohen et al., 2003).

Interestingly, some species of birds do not undergo significant amounts of NEG. For example, chickens do not experience the same degree of NEG of serum albumin or hemoglobin as humans do (Zuck et. al., 2017). Beuchat and Chong, (1998) found that chickens, duck and turkey experience only 0.5% - 1.0% glycated hemoglobin, even though these species can have plasma glucose levels that are 1.5 – 2 times higher than mammals with similar body sizes (Braun and Sweazea, 2008). Even hummingbirds, which have fasting blood glucose levels of 300 mg/dL (over 3 times the normal human value) experience on average 3.7% - 4.6% glycated hemoglobin, which is less than the percentage used a marker for diabetes in humans (>6.5%). An interest correlate is that birds, like chickens, have high levels of taurine in their red blood cells (~ 5.5 mmol/L) (Porter and Martin, 1992), which may contribute to the low levels of glycated hemoglobin that chickens experience.

Taurine (2-aminoethanesulfonic acid, Fig. 3) is a low molecular weight organic chemical that is found in high concentrations within cells (~5-50 mmol/L), in humans and in lesser concentration in the serum (~100 μmol/L)

(Lourenco and Camilo, 2002). Although many authors refer to taurine as a nonessential amino acid, it does not exist in the same basic structure as an amino acid.

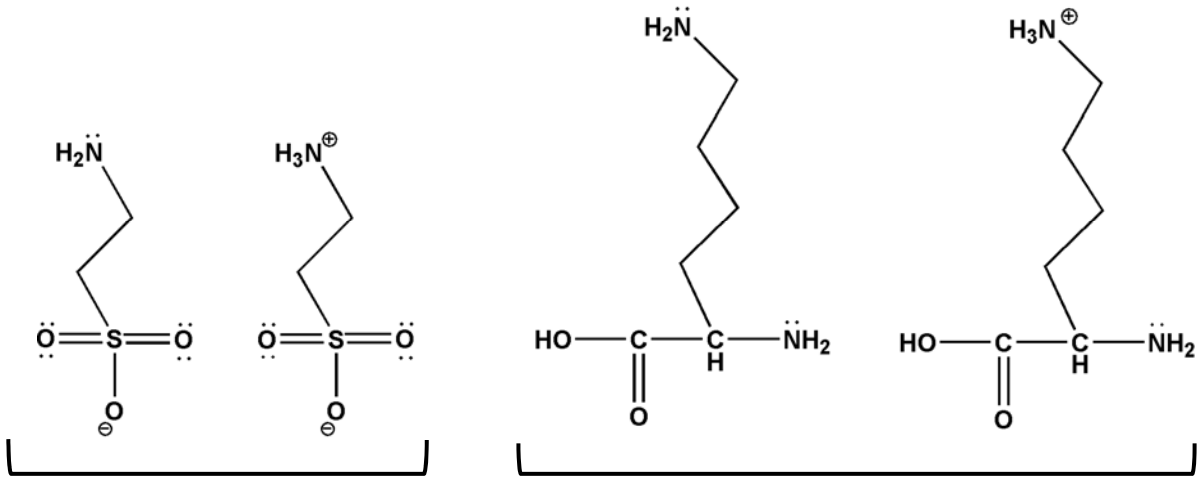


Figure 3. Comparison between the structural forms of taurine and a representative amino acid, lysine. Taurine is not technically an amino acid because it does not contain the common backbone that all amino acids share. However, both taurine and lysine share the common feature of the amine/ammonium ion group.

In humans, taurine is obtained through both dietary intake and biosynthesis from metabolic conversion of cysteine in the liver (Learn et al., 1990). Once taurine is produced *in vivo*, it is exported from the liver into the plasma to be transported around the body (Stipanuk, 2003). The concentration of taurine varies throughout the body, with cardiac muscle cells and neurons of the central nervous system having some of the highest concentrations (mM range) (Lourenco and Camilo, 2002). Although taurine is produced in the liver, it is able be taken up by a variety of cells via the TauT transporter (Napoli et al., 2016).

As previously mentioned, it is possible to synthesize taurine *in vivo* from cysteine (Learn et al., 1990), however, much of the taurine that we have available in our bodies come from our diet. Meats such as fish, beef and lamb contain concentrations of taurine between 3.4 μmoles/g and 12.4 μmoles/g (Pasantes-Morales et al., 1989). Taurine is also a common additive to energy drinks, although the purpose for this addition is not well understood. For example, a 250 ml can of Redbull contains 1000 mg of taurine (Hohnmann et al., 2014).

The potential applications and functions of taurine throughout the body are vast. One of the most important and widely recognized roles of taurine is as an antioxidant. In diseases like type 2 diabetes mellitus, hyperglycemia often results in the overproduction of reactive oxygen species (ROS). These ROS lead to oxidative damage to cells and contribute to the pathology of diabetes (Hosseini and Abdollahi, 2013). Taurine has been observed to reduce oxidative stress caused by hyperglycemia. When ROS are produced, taurine is able to bind to

oxygen free radicals, to eliminate the oxidative threat. This prevents an excess of oxidants from being transported into cells, like neurons and protects cells from mitochondrial damage. Additionally, taurine has been observed to increase the activity of antioxidant enzymes superoxide dimutase (SOD), catalase and glutathione peroxidase (GSH-Px). In some cases, such as in the presence of high levels of iron, these enzymes have reduced activity. However, supplementation with taurine returns these enzymes to their normal activity (Zhang et al., 2014).

In addition to its roles as an antioxidant, taurine has also been shown to be an effective osmoregulatory agent. By affecting ion pump activity, taurine regulates cellular concentration of Na^+, K^+ and Ca^{2+}. However, in some diseases, such as Type 2 diabetes mellitus, the activity of Na^+/K^+ ATPase and Ca^{2+} ATPase pumps is decreased. This decreased activity causes a buildup of Ca^{2+} in cells which can be damaging. However, taurine is able to increase ion pump activity by reducing lipid peroxidation and protein glycation, which is the cause of the pump's decrease in activity. Additionally, taurine is able to modify the opening and closing of the Ca^{2+} pump, and Ca^{2+} delivery to the pump so as to prevent Ca^{2+} overload in cells (Nandhini and Anuradha, 2003).

Taurine is also thought to be involved in limiting the extent of glycated proteins in the process of NEG. Studies from Nandhini et al. (2004) have shown that in the presence of taurine, glycated hemoglobin levels are reduced *in vivo* in fructose fed rats, as well as *in vitro* (Nandhini et al., 2004). Although there is a reduction in glycated hemoglobin in the presence of taurine, the reduction is

modest (~10% reduction of control values, or slightly less than 1% absolute reduction of actual glycated hemoglobin values). Studies by the same group have also shown taurine to reduce the level of glycated bovine serum albumin (BSA) over a 30-day incubation period (Nandhini and Anuradha, 2003).

Although there is evidence that taurine plays a role in limiting the extent of NEG, little mechanistic detail has been provided. Ogasawara et al., (1993) have given some insight into potential mechanistic action of taurine, by showing that taurine can react with glucose similarly to other amino acids, specifically glycine and alanine. However, reactions performed by this group were done over a 34-day incubation period at 50°C, therefore the results are not physiologically relevant. They also show that when melondialdehyde (MDA) is allowed to react with low-density lipoproteins (LDLs) in the presence of taurine, the amount of LDLs modified by MDA is reduced, suggesting that the amine group on taurine competes with the amino acids of the protein to bind with MDA (Ogasawara et al., 1993).

Some have suggested that taurine limits the amount of NEG of proteins by reacting with carbonyl groups intracellularly in aqueous media prior to protein interactions, generating a taurine-glucose adduct and preventing the formation of AGE (Nandhini et al., 2004). The taurine-glucose adduct is thought to have antioxidative properties toward lipid peroxidation (Ogaswara et al., 1993; Nandhini et al., 2004,), although mechanistically these postulates have not been investigated.

We seek to determine at what stage(s) of NEG does taurine play a role in reducing glycation or if the taurine effect is prior to any of the formal NEG stages (that is, pre-binding). Looking prior to the first stage, we postulate that there is an opportunity for taurine to tie up certain carbonyl species before these species can enter a protein to covalently bond. The ring-opened form of glucose represents a reactive carbonyl species (RCS). Reactive carbonyl species are those which contain one or more electrophilic carbonyl groups. Fig. 2 shows that glucose, in the ring-opened form contains a carbonyl group, thus giving it the characterization as an RCS. RCS may exist transiently, like glucose, which quickly ring-closes on itself to form one of the other five isomers, or permanently, like glyoxal which contains two carbonyl groups yet does not have the ability to form a ring on itself. Because taurine contains a nucleophilic amine group, it has the potential to covalently bond with a ring-opened glucose or glyoxal and eliminate the reactive electrophilic centers before the electrophile can enter a protein pocket. This reaction of taurine and carbonyls has been proposed in previous literature and is referred to as "scavenging carbonyls" (Li et al., 2010). We ask: "does taurine scavenge carbonyls and bind up RCS before they have a chance to glycate proteins (Fig. 4)?"

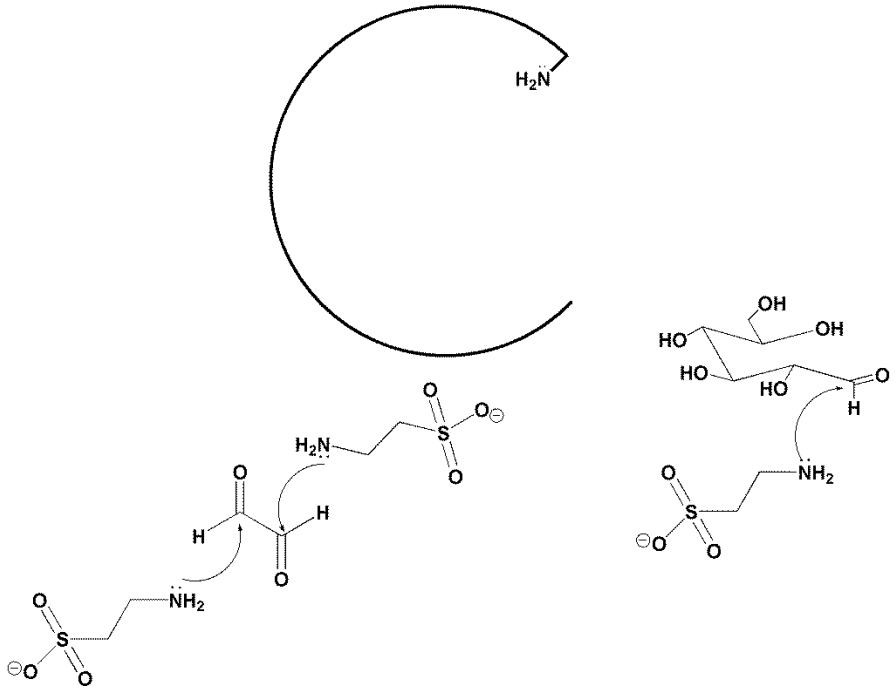


Figure 4. Taurine interacting with RCS (glyoxal (left) and a ring-opened glucose (right)) before RCS enter the protein pocket.

Additionally, in order for glucose to covalently bond to protein and proceed in NEG, it must go through ring-opening. Given that the ring-opened structure of glucose exists transiently and in very low concentrations relative to the other isomers, and that the α- and β-glucopyranoses bind with more favorable exothermicities than does the ring opened form, it has been concluded by Clark et al. (2013) that glucose enters the protein pocket in a ring-closed form (predominantly α- and β-glucopyranoses), and must ring-open while inside the pocket (Clark et al., 2013). This leads to the question, "does taurine inhibit glucose from ring-opening while bound to proteins, thus limiting the forward progression of NEG (Fig. 5)?"

Clark et al. (2013) also reported that a base is necessary to ring-open glucose once it is in the protein pocket. Possible bases include intrinsic amino acid residues, water, or inorganic phosphate (Pi) (Fig. 5). Pi is shown to be the best base in order to ring open a glucose. Tracking the conversion of α-glucopyranose to β-glucopyranose in the presence of Pi has shown that Pi increases the rate of glucose ring opening by a factor of 30, known as the Pi effect (Clark et al., 2013). This presents the question, "does taurine limit NEG by geometrically inhibiting the Pi effect within a protein pocket (Fig. 6)?"

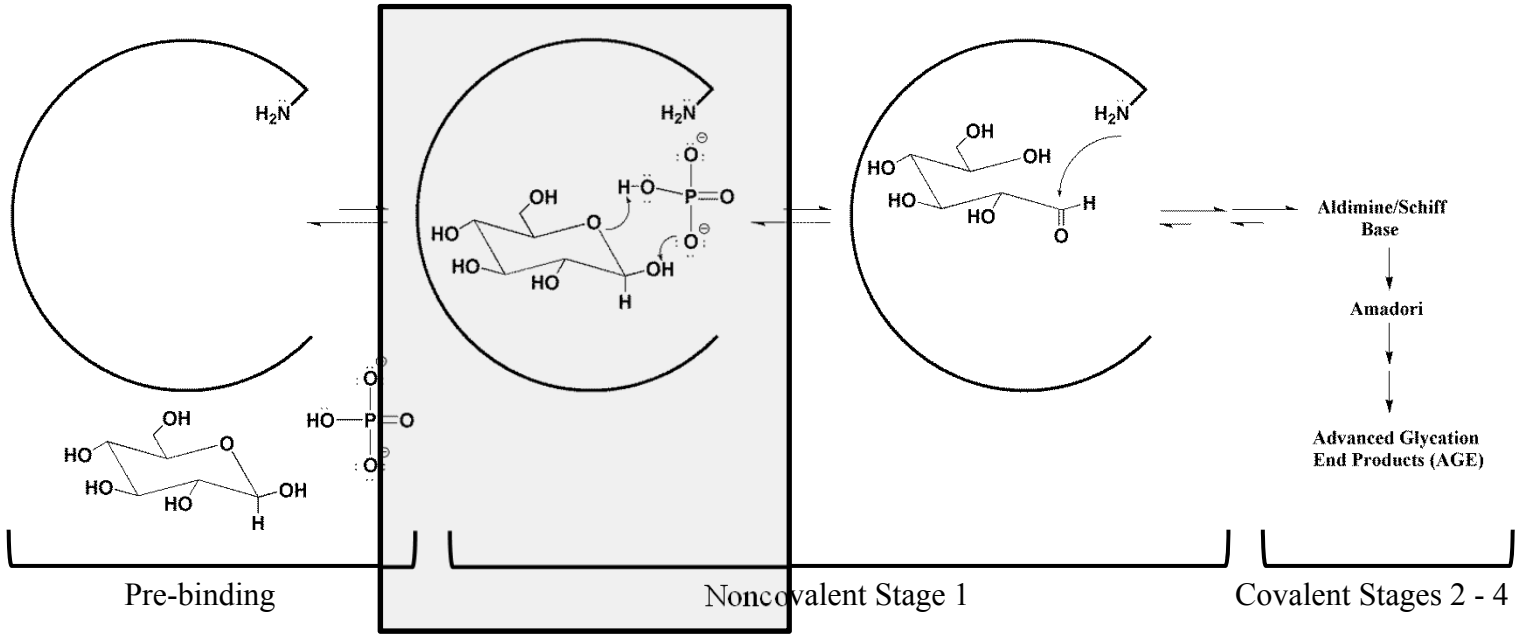


Figure 5. Scheme for nonenzymatic glycation highlighting the noncovalent step of dibasic phosphate (Pi) acting as both an acid and base to ring open glucose within the protein pocket (Pi effect).

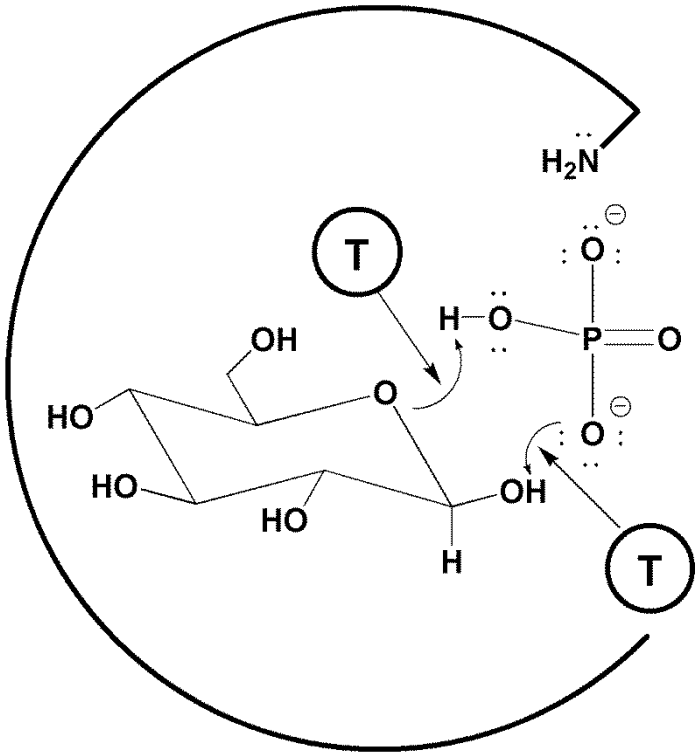


Figure 6. Taurine (represented as "T" enclosed in a circle) may interfere with the Pi effect by inhibiting either the base (deprotonating the hemiacetal OH) or acid (protonating the anomeric O^-) step.

In order to proceed to Stage 2 of NEG, a nucleophile is required to bond to the electrophilic carbonyl generated by ring-opening glucose within the protein pocket. The most common nucleophile on human hemoglobin is the Val1 residue on the β-chain of the protein, although other sites have been shown to also act as nucleophiles, such as select lysine residues (Delpierre et al., 2004; Clark et al., 2013). Given the structure of taurine (pKa ~ 9.06) as well as the results from Ogasawara et al., (1993) on their study with MDA and LDLs, we pose the question: "does taurine reduce the amount of NEG by competing with the amino acid nucleophiles to react with the electrophilic, ring-opened glucose (Fig. 7)?"

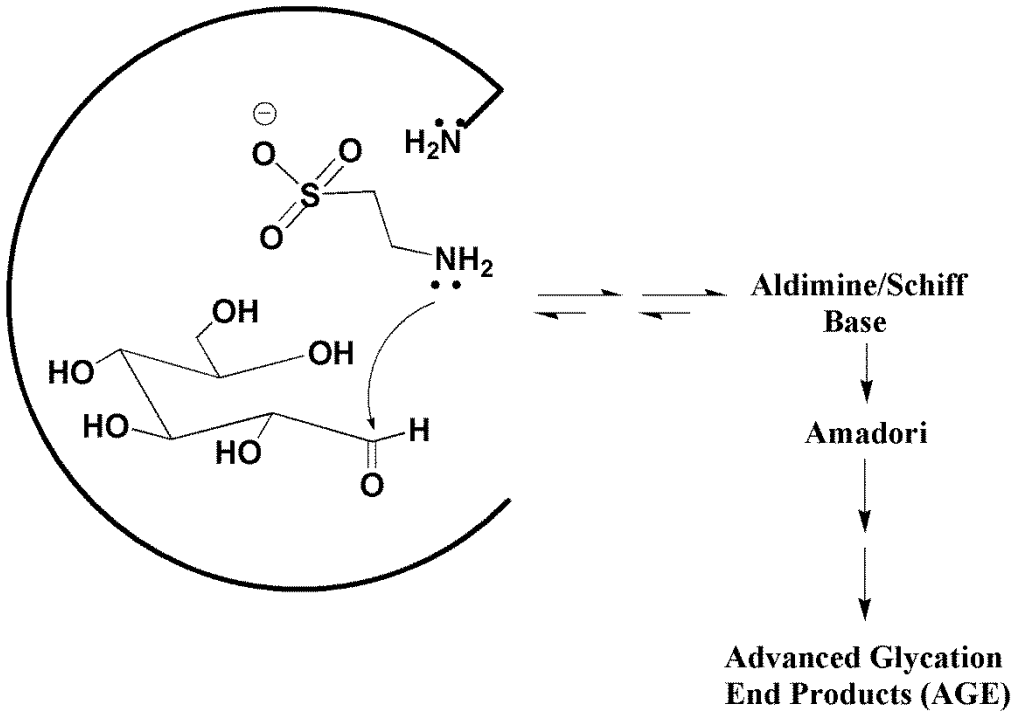


Figure 7. Taurine and a ring-opened glucose bound in a protein pocket, with taurine acting as the nucleophile rather than the amine group on a protein amino acid residue.

The primary goal of this thesis is to determine the role that taurine plays in the NEG process, specifically answering whether taurine scavenges carbonyls and binds glucose prior to glucose entering the protein pocket; whether taurine inhibits glucose ring opening once it is in the protein pocket, specifically by inhibiting the Pi effect; or whether taurine competes with amino acid residues to bind glucose in the protein pocket? Resolving these questions is of value because taurine might be applicable in the battle against human diabetes mellitus. Further, our goal is to extend previous work on taurine to other reagents that are used clinically as a treatment for diabetes, metformin and aminoguanidine, which share common structural features with taurine (i.e. amine group) In particular, our emphasis is on the comparison between the three species in their mechanistic action.

The methods that are utilized to address the research questions will be computational modeling using Molecular Operating Environment (MOE) and nuclear magnetic resonance (NMR) investigations of model reactions.

References

1. American Diabetes Association. (2017) Classification and Diagnosis of Diabetes, Diabetes Care 40 (Suppl. 1), S11-S24.
2. Beuchat, C. A., Chong, C. R. (1998) Hyperglycemia in hummingbirds and its consequences for hemoglobin glycation. *Comp Biochem Physiol A Mol Integr Physiol* 120, 409–416.
3. Bookchin, R. M., Gallop, P. M. (1968) Structure of hemoglobin A1c: nature of the N-terminal beta chain blocking group. *Biochem Biophys Res Comm* 32, 86-93.
4. Braun, E. J., Sweaxea, K. L. (2008) Glucose regulation in birds. *Comp Biochem Physiol Biochem Mol Biol* 151, 1–9.
5. Brede, C., Hop, B., Jørgensen, K., Skadberg, Ø. (2016) Measurement of glycated albumin in serum and plasma by LC-MS/MS. *Scand J Clin Lab Invest* 76, 195–201.
6. Brownlee, M., Vlassara, H., Cerami, A. (1984) Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med.* 101, 527-537
7. Brownlee, M. (2005) The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54, 1615-1625.
8. Bunn, H. F., Haney, D. N., Gabbay, K. H., Gallop, P. M. (1975) Further identification of the nature and linkage of the carbohydrate in hemoglobin A1c. *Biochem Biophys Res Comm* 67, 103-109.

9. Dabelea, D., Mayer-Davis, E. J., Saydah, S., Imperatore, G., Linder, B., Divers, J., Bell, R., Badaru, A., Talton, J. W., Crume, T., Liese, A. D., Merchant, A. T., Lawrence, J. M., Reynolds, K., Doan, L., Liu, L. L., Hamman, R. F. (2014) Prevalence of type 1 and type 2 diabetes among children and adolescents from 2001 to 2009. *JAMA* 311, 1778–1786.
10. Drive, A. D. A. 2451 C., Arlington, S. 900, V. 22202. (n.d.). Statistics About Diabetes. Retrieved April 11, 2017, from http://www.diabetes.org/diabetes-basics/statistics/.
11. Clark, S. L., Santin, A. E., Bryant, P. A., Holman, R. W., Rodnick, K. J. (2013). The initial noncovalent binding of glucose to human hemoglobin in nonenzymatic glycation, *Glycobiol* 23, 1250-1259.
12. Cohen R. M., Holmes Y. R., Chenier T. C., Joiner C. H. (2003). Discordance between HbA1c and fructosamine: evidence for a glycosylation gap and its relation to diabetic nephropathy. *Diabetes Care*. 26 (1): 163–167. doi:10.2337/diacare.26.1.163.
13. Delpierrre, G., Vertommen, D., Communi, D., Rider, M. H., and Schaftingen, E. V. (2004). Identification of fructosamine residues deglycated by fructosamine-3-kinase in human hemoglobin. *J Biol Chem* 279, 27613–27620.
14. Ford, E. S., Cowie, C. C., Li, C., Handelsman, Y., Bloomgarden, Z. T. (2011). Iron-deficiency anemia, non-iron-deficiency anemia and HbA1c among adults in the US. *Diabetes* 3, 67–73.

15. Garlick, R. L., Mazer, J. S., Chylack, L. T., Tung, W. H., and Bunn, H. F. (1984). Nonenzymatic glycation of human lens crystallin. Effect of aging and diabetes mellitus. *J Clin Invest* 74, 1742–1749.
16. Hohnmann, M., Felbinger, C., Christoph, N. (2014) Quantification of taurine in energy drinks using 1HNMR. *J Pharm Biomed Anal* 93, 156-160.
17. Hosseini, A., Abdollahi, M. (2013) Diabetic neuropathy and oxidative stress: therapeutic perspectives. *Oxid Med Cell Longev* 10.1155/2013/168039.
18. Johnson, R. N., Metcalf, P. A., Baker, J. R. (1983) Fructosamine: a new approach to the estimation of serum glycosylprotein. An index of diabetic control. *Clin Chim Acta* 127, 87–95.
19. Learn, D. B., Fried, V. A., Thoman, E. L. (1990) Taurine and hypotaurine content of human leukocytes. *J Leuk Bio* 48, 174-182.
20. Li, G., Tang, T., Peng, M. (2010) Direct reaction of taurine with malondialdehyde: evidence for taurine as a scavenger of reactive carbonyl species. *Redox Rep* 15, 268-274.
21. Lourenco, R. Camilo, M. E. (2002) Taurine: a conditionally essential amino acid in human? An overview of health and disease. *Nutr Hosp* 17, 262-270.
22. Nandhini, T. A., Anuradha, C. V. (2003) Inhibition of lipid peroxidation, protein glycation and elevation of membrane ion pump activity by taurine in RBC exposed to high glucose. *Clin Chim Ac* 336, 129-135.
23. Nandhini, T. A., Thirunavukkarasu, V., Anuradha, C. V. (2004) Stimulation of glucose utilization and the inhibition of protein glycation and AGE products by taurine. *Acta Physiol Scand* 181, 297-303.

24. Napoli, Z., Seghieri, G., Bianchi, L. (2016) Taurine transporter gene expression in mononuclear blood cells of type I diabetic patients. *J Diab Res*. 10.1155/2016/7313162.

25. Ogasawara, M., Nakamura, T., Koyama, I (1993) Reactivity of taurine with aldehydes and its physiological role. *Chem Pharm Bull* 41, 2172-2175.

26. Pasantes-Morales, H., Quesada, O., Alcocer, L., and Olea, R. S. (1989) Taurine content in foods. *Nutr Rep Int* 28, 413-421.

27. Porter, D. W., Martin, W. G. (1992) Taurine flux in chicken erythrocytes. *Comp Biochem Physiol Comp Physiol* 102, 117–122.

28. Rondeau, P., Bourdon, E. (2011) The glycation of albumin: structural and functional impacts. *Biochimie* 93, 645–658.

29. Stipanuk, M. H. (2004) Role of the liver in regulation of body cysteine and taurine levels: a brief review. *Neurochem Res* 29, 105-110.

30. Terrill, J. R., Pinniger, G. J., Graves, J. A. (2016) Increasing taurine intake and taurine synthesis improves skeletal muscle function in the mdx mouse model for duchenne muscular dystrophy. *J Physiol* 592, 3095-3110.

31. Wang, Y., Yu, H., Shi, X., Luo, Z., Lin, D., Huang, M. (2013) Stuctural mechanism of ring-opening reaction of glucose by human serum albumin. *J Biol Chem* 288, 15980-15987.

32. Zhang, Z., Liu, D., Yi, B. (2014) Taurine supplementation reduces oxidative stress and protects the liver in an iron-overload murine model. *Mol Med Rep* 10, 2255-2262.

33. Zuck, J., Borges, C. R., Braun, E. J., Sweazea, K. L. (2017) Chicken albumin exhibits natural resistance to glycation. *Comp Biochem Physiol Biochem Mol Biol* 203, 108–114.

Chapter 2. Investigation into Taurine's Mechanism to Reduce Nonenzymatic Glycation.

2.1 Introduction

Diabetes mellitus is a metabolic disease that affects a large number of Americans. As of 2012, 29.1 million Americans were diagnosed with type 2 diabetes mellitus, while 128,000 of these individuals reside in Idaho (American Diabetes Association, 2012). Although the exact cause of type 2 diabetes is not known, genetic and dietary factors are implicated (Brutsaert, 2017). Type 2 diabetes mellitus is characterized by long-term hyperglycemia that initially results in insulin resistance, and ultimately progresses to insulin fatigue. In the early, or pre-diabetic stages, high blood glucose levels stimulate the pancreas to produce more and more insulin (insulin resistance), but eventually the demand for insulin due to chronically high levels of blood glucose in the blood is too great, and the pancreas cannot keep up (insulin fatigue) (Gardner et al., 2011; American Diabetes Association, 2012;).

Although type 2 diabetes can be treated with a combination of medications to lower blood glucose levels and lifestyle changes (i.e., eating healthier and increasing exercise), further complications can arise. Vascular, renal and peripheral nerve damage can result from prolonged and untreated type 2 diabetes (Melmed et al., 2011; American Diabetes Association, 2012). Additionally,

hyperglycemia can result in an increased level of covalently modified (including glycated) proteins throughout the body (Smith et al., 1982). Glycated proteins are proteins that are structurally modified by a covalently attached glucose which leads to a host of advanced glycation end products (AGE), like cross-linked proteins (Ganea et al., 1994). However, glucose is not the only molecule that can covalently modify proteins. Reactive carbonyl species (RCS) are species that contain an electrophilic carbonyl either transiently, like ring-opened glucose, or permanently, like glyoxal. RCS like glyoxal are able to bind to and covalently modify proteins, in a process that is similar to NEG called nonenzymatic covalent protein modification (NECPM).

When proteins are covalently modified, their structure is altered in such a way that protein function may also be altered, which is the ultimate problem with glycated proteins (Sen et al., 2004). One common example of a glycated protein is HbA1c, the glycated form of human hemoglobin (HbA) at one or both of the N-terminal valine residues on the β-chains of the HbA tetramer. HbA1c is a clinical marker for diabetes (both type 1 and type 2). A reasonable goal for adults is <7% although some health care providers may suggest a more stringent HbA1c goal such as 6.5% (Diabetes Care, 2017). The normal range of HbA1c is between 5% and 6% (Brede et al., 2016)).

The small organic molecule taurine has been implicated as an antiglycation agent due to the finding that when incubated *in vitro* with glucose and HbA, taurine will reduce the amount of glycated HbA formed (Nandhini et

al., 2004). Furthermore, plasma taurine is found in high concentration in non-humans, like chickens (Porter et al., 1992). Interestingly, these species have low glycated plasma protein levels despite having high blood glucose concentrations (Zuck et al., 2017).

In humans, the concentration of taurine varies throughout the body. In plasma, the concentration of taurine is 29-49 μmol/L, yet in the vastus lateralis muscle is ranges from 50 – 62 mmol *(kg dry weight)-^{1} (~60-80 mmol/L, Cuisinier et al., 2002). Taurine is well-characterized as an antioxidant and osmoregulator, having diverse functions throughout the body (Huxtable, 1992). However, it is unclear the mechanism by which taurine reduces protein glycation.

The focus of this chapter is to assess the mechanistic possibilities of taurine to inhibit the early, noncovalent stages of NEG. We seek to understand the bond-making/bond-breaking mechanism by which taurine may limit a glucose or glucose derivative from covalently attaching to protein. However, it is possible that taurine may reduce NEG through a mechanism that does not involve bond-making or bond-breaking. We hypothesize that taurine may serve multiple mechanistic roles to limit NEG, starting by irreversibly scavenging glucose derivatives (glyoxal), as well as concomitantly binding to protein pockets at glycation hotspots and interfering with the protonation state of key players in the NEG process (i.e. inorganic phosphate, lysine residues, and histidine residues).

2.2 Methods

General Methods:

Nuclear Magnetic Resonance (NMR) **-** NMR data was collected on a JOEL-EM300 at room temperature (~20°C). The NMR data obtained consisted of both ^{1}HNMR as well as ^{31}PNMR. All reactions were performed in D_2O obtained from Sigma-Aldrich (lot# MKBV2445V). Taurine was obtained from Acros Organics (lot# A0242256); glyoxal (40% w/w aq. soln.) was obtained from Alfa Aesar (lot# 10171751); dibasic phosphate was obtained from Fisher Scientific (lot# 081308), and monobasic phosphate was obtained from Fisher Scientific (lot# 974537); pure α-glucopyranose was obtained from Sigma-Aldrich (lot# MKBH5500V). Standards for all chemical were conducted using ^{1}HNMR, expect dibasic phosphate, and monobasic phosphate, which were conducted using ^{31}PNMR. Default settings were used, with the exception of number of scans run, which varied with experiment and reagents.

Computational Modeling – Non-covalent binding data was obtained in the program Molecular Operating Environment (MOE, ver. 2015.1001, Chemical Computing Group Inc., Montreal, Canada). The proteins used in the docking procedure were obtained from Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) and included: 1) A single β-chain of human hemoglobin (HbA) was isolated from the fully oxygenated HbA crystal tetramer, 1B86 (obtained from the RCSB PDB (http:// www.pdb.org/pdb/home/home.do)) using Deep View-Swiss PDB Viewer (WINE,

http://spdv.vital-it.ch/wine.html), and 2) Human Serum Albumin (rHSA, 1AO6, Wang et al., 2013). The substrates that were used in the docking procedure included: 1) the α-glucopyranose and β-glucopyranose isomers were obtained from Heterocompound Information Centre, Uppsala, (HIC-UP, http:// xray.bmc.uu.se/hicup/), 2) metformin, taurine and aminoguanidine multiple protonation forms were built in MOE, and 3) HPO_4^{2-} and $H_2PO_4^-$ were built with The PyMOL Molecular Graphics System (ver. 1.5.0.4 Schrödinger, LLC, http://www.pymol.org/).

All dockings were done under default parameters with the exception of the temperature being set to a physiological value (310 K), induced fit setting being used, and energy minimization before each docking. For all dockings a distance of 5Å was used as a cutoff for effective geometry in protein pockets, and an energy of -2.5 kcal/mol.

Specific Methods:

Methods for Question #1: Can taurine covalently bond with glucose in aqueous solution to effectively reduce glucose concentration and thereby reduce the extent of NEG?

Taurine and pure α-glucopyranose were reacted in D_2O and followed with ^{1}HNMR to observe if there was evidence of a taurine-glucose adduct formation.

The reaction was followed over time, with 36 NMR scans taken every 20 min for 150 min.

Methods for Question #2: Can taurine covalently bond to glyoxal in aqueous solution to effectively reduce glyoxal concentration and thereby reduce the extent of NECPM?

Taurine and permanent dicarbonyl glyoxal were reacted in D_2O at their native pH (pH ~ 6), and were followed by ^{1}HNMR for 21 days, with scans taken every 7 days. Taurine, glyoxal, and potassium dibasic phosphate were followed by ^{1}HNMR and ^{31}PNMR at their native pH (~ 7.5). These reactions were done to observe if taurine and glyoxal formed permanent adducts in solution.

Methods for Question #3: Can taurine inhibit glucose ring-opening in solution, thus RCS formation?

Inflection time of α-glucopyranose in the presence of taurine was obtained by ^{1}HNMR, measuring the percent of α- and β-glucopyranose generated as a function of time. The inflection time is defined as the time it takes for the concentration of both α- and β-glucopyranose to be approximately 50%. This is determined by measuring the integration of the doublets at 5.05 ppm (α-glucopyranose) and 4.46 ppm (β-glucopyranose).

Methods for Question #4: Does taurine theoretically compete with glucose binding in protein pockets, thus limiting NEG?

The HbA PDB file was altered in MOE so that Val1 and all histidine residues were deprotonated to amines ($R\text{-}NH_2$). Conversely, all lysine residues were protonated to ammonium ions ($R\text{-}NH_3^+$) in both HbA and HSA. The docking done to HbA was tethered to the Val1 and all lysine residues, due to these residues being the most commonly glycated sites on HbA (Delpierre et al., 2004). Independently, α-glucopyranose, β-glucopyranose, and taurine ($R\text{-}NH_2$, $R\text{-}NH_3^+$) were docked with 50 poses produced. For each pose, we examined the substrates geometry and calculated an average exothermicity (ΔH).

In order to assess if taurine competes with glucose binding in a protein pocket, sequential docking was done with taurine ($R\text{-}NH_2$ or $R\text{-}NH_3^+$) and either α-glucopyranose or β-glucopyranose. The sequential docking was done by first docking one substrate, then saving a single pose and docking the next substrate to that pose. All 8 docking combinations were done. We observed if the second substrate would dock given the presence of the first substrate in the Val1/Lys82 protein pocket of HbA and the Lys195/Lys199 protein pocket of HSA. The exothermicities (ΔH) were assessed for the docking of the second substrate.

Methods for Questions #5 & #6: Can taurine theoretically prevent glucose ring-opening in a protein pocket and thereby reduce NEG? Can taurine theoretically prevent a protein nucleophile from attacking a ring-opened glucose in a protein pocket?

Computational modeling was used to determine if the presence of taurine ($R\text{-}NH_2$ or $R\text{-}NH_3^+$) in the Val1/Lys82 pocket of HbA or Lys195/Lys199 pocket of HSA can prevent the acid or base from ring-opening a bound glucose or nucleophilic attack on a ring-opened glucose. Using MOE, we compared three potential mechanisms of ring-opening. The baseline mechanisms we used were the naked mechanism, bimolecular mechanism and bridge mechanism. Starting from each of the three baseline mechanisms (each using both α-glucopyranose and β-glucopyranose, and the bimolecular and bridge using both monobasic and dibasic Pi), we hand placed a taurine ($R\text{-}NH_2$ or $R\text{-}NH_3^+$) into the pocket and energy minimized. We then assessed the baseline mechanism again with taurine in place to see if taurine obtains the proper geometry to interfere with either the acid or base, by measuring the distance between reactive centers (i.e. nitrogen of amine groups on protein residues or taurine, and the anomeric carbon of the glucopyranoses). We also assessed the baseline mechanisms to determine if taurine had the correct proximity and orientation to glucose to transfer a proton to the amino acid nucleophile, thus inhibiting the nucleophile. We measured the distance between the reactive centers on glucose, the protein and taurine, using a cutoff for reaction distance of 6Å.

2.3 Result and Discussion

Pre-binding Questions:

Question #1: Can taurine covalently bond with glucose in aqueous solution to effectively reduce glucose concentration and thereby reduce the extent of NEG?

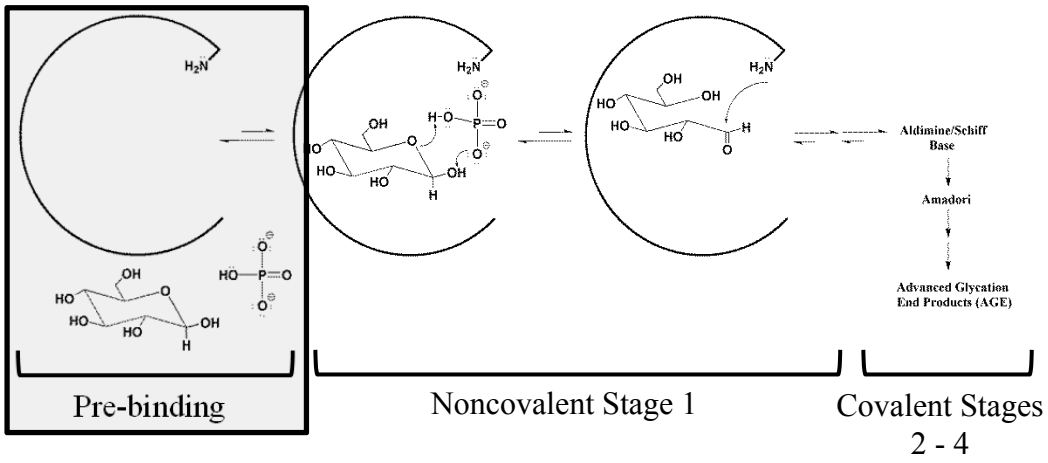


Figure 1. Nonenzymatic glycation (NEG) scheme with the temporal region of interest in question one highlighted in grey.

When we followed the reaction between taurine and pure α-glucopyranose with ^{1}HNMR we did not find evidence of an adduct forming between taurine and glucose after 150 min. These results indicate that taurine does not bond with glucose in solution to reduce the effective concentration of glucose. Therefore, taurine's role in reducing NEG is likely not explained in the pre-binding temporal region due to interactions with glucose.

Question #2: Can taurine covalently bond to glyoxal in aqueous solution to effectively reduce glyoxal concentration and thereby reduce the extent of NECPM?

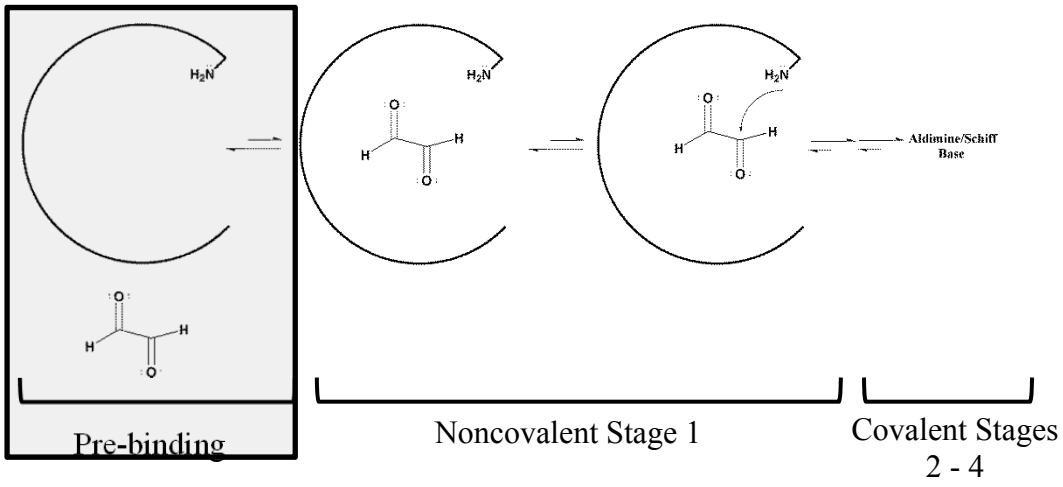


Figure 2. Nonenzymatic covalent protein modification (NECPM) scheme for glyoxal with the temporal region of interest in question two highlighted in grey.

When following a 1:1 reaction of taurine and glyoxal by ^{1}HNMR, we found that taurine and glyoxal interact to form a 1:1 mono-imine (Fig. 3, panel A) and a 2:1 di-imine (Fig. 3, panel B). However, the reaction to the di-imine is slow as it takes approximately 14 days to see it appear on the NMR, even though there is evidence of the mono-imine at the first time point (approximately 30 min). Additionally, when this reaction is followed for 21 days, we see there is an equilibrium that forms between the mono- and di-imine, which would suggest that the reaction between taurine and glyoxal is reversible, albeit slowly reversible (Fig. 4).

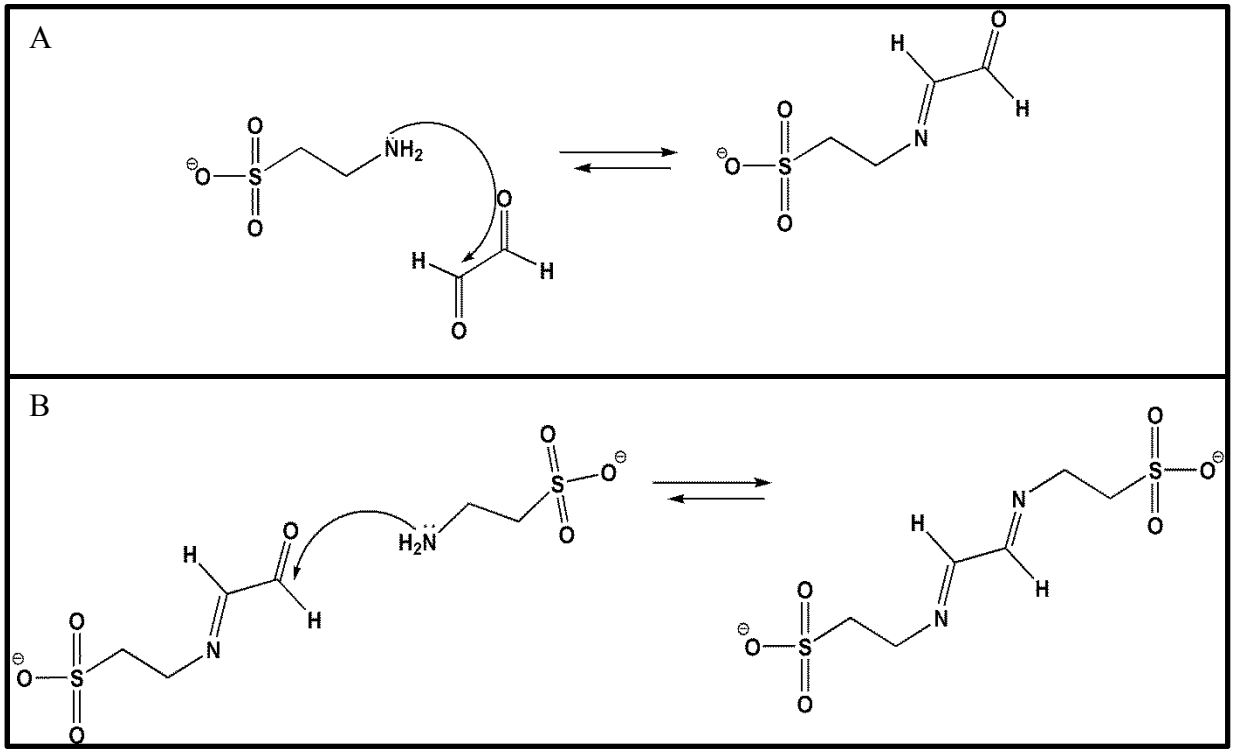


Figure 3. Proposed interaction between taurine and glyoxal in aqueous solution. Panel A shows a nucleophilic taurine attacking the electrophilic carbonyl of glyoxal to form the mono-imine. Panel B shows a second nucleophilic taurine attacking the electrophilic carbonyl of the mono-imine to form the di-imine.

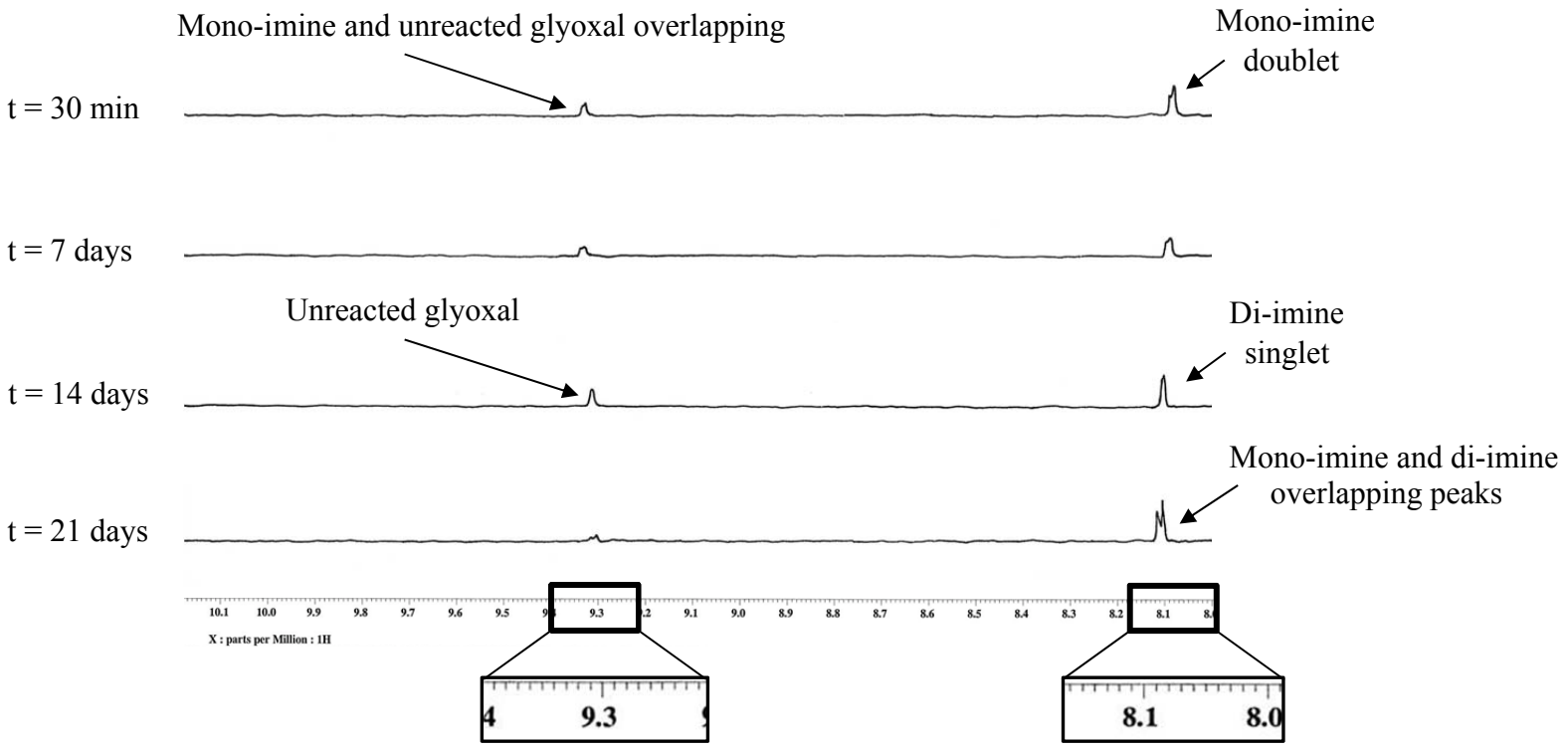


Figure 4. [1]HNMR spectra of the reaction between taurine and glyoxal in aqueous solution over time. The mono-imine appears as a doublet around 8.10 ppm, while the di-imine appears as a singlet around 8.13 ppm. Unreacted glyoxal appears as a singlet around 9.33 ppm.

Taurine's ability to permanently bond to and reduce (scrub) carbonyls would consist of taurine and glyoxal reacting to a di-imine product rapidly. Taurine ($R\text{-}NH_2$), acting as a nucleophile, would have to bond to the electrophilic carbonyls of glyoxal, rendering them inactive in terms of covalently bonding to a protein (Fig. 3, panels A and B). However, when taurine and glyoxal are mixed in aqueous solution, we see that the reaction to the di-imine is slow; it takes approximately 14 days to see evidence of its formation. Yet the reaction to mono-imine is rapid – we see its formation within 30 min (Fig. 4). These results suggest that taurine may be able to tie-up carbonyls, but the overall effect may be modest because the reaction to di-imine is slow.

Taurine only has nucleophilic character in its amine form ($R\text{-}NH_2$). One explanation as to why the reaction to di-imine is slow compared to mono-imine formation is that at pH 6 (the pH of the reaction between taurine and glyoxal), taurine exists primarily in the ammonium ion form ($R\text{-}NH_3^+$), due to the pKa of the amine being ~9.06. In aqueous solution, like in the reaction between taurine and glyoxal, 99.7% of the taurine will be in the ammonium ion form, and unable to react with glyoxal initially.

We wonder, is the reaction to the di-imine permanent, indicating taurine would truly scrub carbonyls? After 21 days, there is evidence that the taurine-glyoxal product reverted back to the mono-imine form and equilibrated between the mono-imine and di-imine until day 21 (Fig. 4). This equilibrium is slowly reached, which indicates that, although taurine is able to tie-up carbonyls, the

reaction is not permanent. This effect is understandable considering taurine's overall reduction in glycation is modest (~10% reduction in GHbA, Nandhini et al., 2004).

Question #3: Can taurine inhibit glucose ring-opening in solution, thus inhibiting reactive carbonyl species formation?

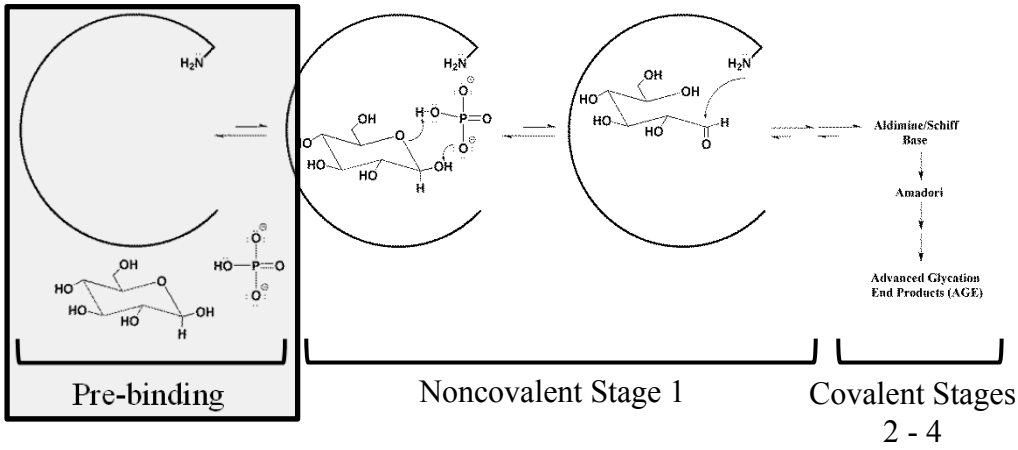


Figure 5. Nonenzymatic glycation (NEG) scheme with the temporal region of interest in question three highlighted in grey.

Inflection time studies were conducted and followed by ^{1}HNMR to determine if glucose, in the presence of taurine, has a reduced rate of ring-opening between α- and β-glucopyranose. When glucose is alone in aqueous solution at pH 7, it takes 270 min for inflection to be reached between α- and β-glucopyranose. When a strong base, dibasic phosphate, is added in a 1:1 molar

concentration with glucose, inflection of α-glucopyranose to β-glucopyranose is reached in less than 9 min at pH 8. This base enhances the ring-opening of glucose by acting as a bridge, to serve as both a base to deprotonate the anomeric OH, and an acid to protonate the hemiacetal O^- (Fig. 6).

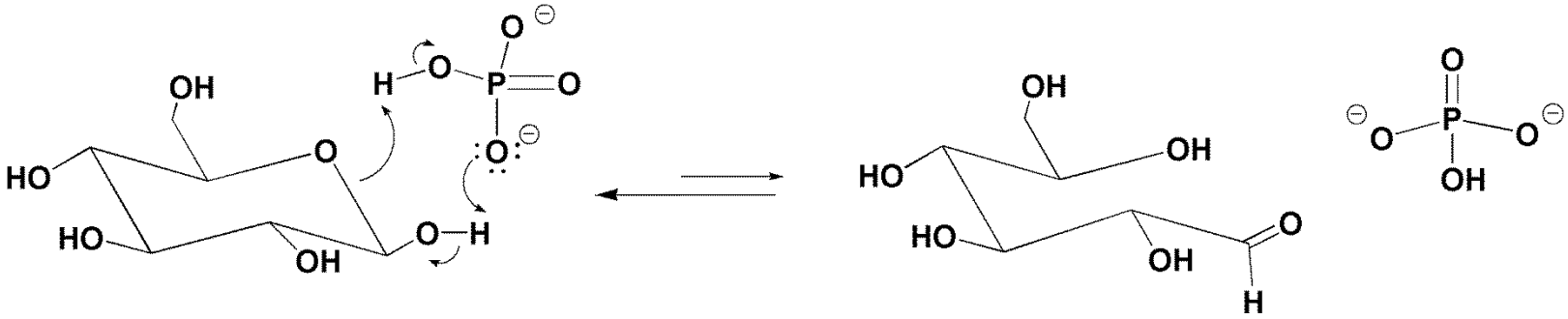


Figure 6. β-Glucopyranose ring-opening in aqueous solution via a dibasic phosphate (Pi) bridge. Dibasic Pi simultaneously acts as both a base to deprotonate the anomeric OH, and an acid to protonate the hemiacetal O^-.

When taurine is mixed with pure α-glucopyranose in D_2O (1:1), it takes approximately 150 min for α-glucopyranose to inflect at pH 6. This enhancement in the rate of glucose inflection time in the presence of taurine reveals that taurine may serve to increase the rate of ring-opening of glucose, rather than our prediction of inhibiting it. However, the effect of taurine on glucose inflection time, and thereby glucose ring-opening, is much more modest than a strong base, such as dibasic phosphate. Given the structure of taurine, a single taurine is not able to form a bridge to ring-open glucose like does phosphate, therefore two taurine molecules must work in tandem to ring-open glucose. One taurine (R-NH_2) must serve as a base to deprotonate the anomeric OH, while the other (R-NH_3^+) must serve as an acid to donate a proton to the hemiacetal O^- (Fig. 7). In this inflection experiment, the taurine and glucose solution was at pH 6. At this pH, taurine primarily exists in the ammonium ion form (pKa ~ 9.06). Therefore, taurine (R-NH_2) may not be in high concentration in solution, thus limiting the base available to ring-open glucose.

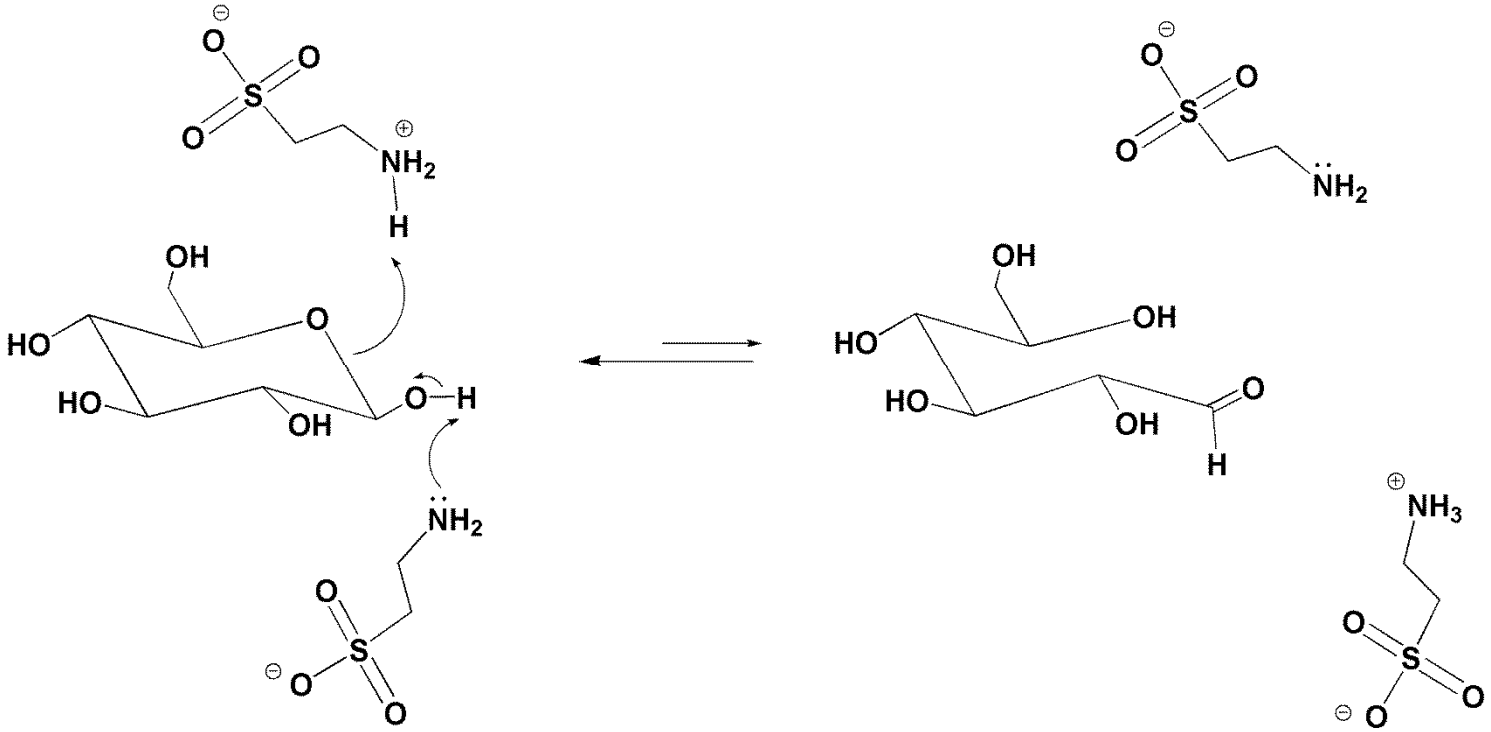


Figure 7. Proposed mechanism of β-glucopyranose ring-opening in aqueous solution via reaction with taurine (R-NH_2 and R-NH_3^+). The amine form of taurine acts as a base to deprotonate the anomeric OH, while a second taurine in the ammonium ion form acts as an acid to protonate the hemiacetal O^-.

Noncovalent Binding Questions:

Question #4: Does taurine theoretically compete with glucose binding in protein pockets, thus limiting NEG?

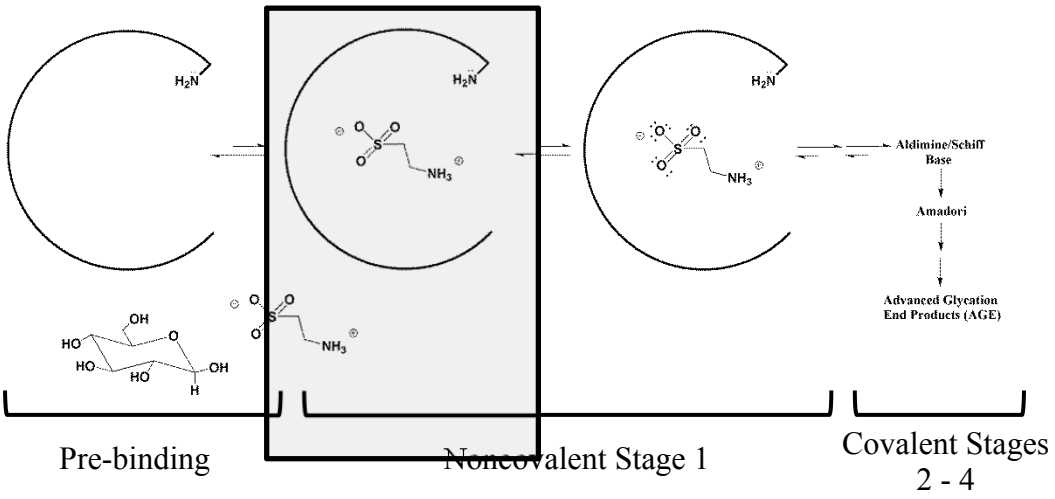


Figure 8. Nonenzymatic glycation (NEG) scheme with the temporal region of interest in question four highlighted in grey.

Table 1. The average binding exothermicities (ΔH) and common binding sites for taurine ($R\text{-}NH_2$, $R\text{-}NH_3^+$) and α- or β-glucopyranose to a single β-chain of hemoglobin (HbA). Taurine and glucopyranose were tethered to Val1 and all the lysine residues on HbA.

Substrate	**Average Binding Exothermicity* (kcal/mol)**	**Binding Sites on HbA (listed in decreasing order of occurrence)**
Taurine ($R\text{-}NH_2$)	-2.95	Val1, Lys82, Lys66, Lys17, Lys132
Taurine ($R\text{-}NH_3^+$)	-3.03	Val1, Lys82, Lys66, Lys61, Lys132, Lys65
α-glucopyranose	-3.39	Val1, Lys66, Lys82
β-glucopyranose	-3.19	Val1, Lys82, Lys66, Lys8, Lys144

*Averages were generated based on 50 poses

Both taurine ($R\text{-}NH_2$ and $R\text{-}NH_3^+$) and glucose (α- and β-glucopyranose) will bind to HbA in the same protein pockets with comparable exothermicities (Table 1). On average, glucose binds slightly better than taurine when it is alone in a protein pocket. We also found that taurine ($R\text{-}NH_2$ or $R\text{-}NH_3^+$) will concomitantly bind in the Val1/Lys82 protein pocket with an α- or β-glucopyranose with favorable exothermicities (Table 2). Binding with favorable exothermicity means that a species binds to a protein pocket better than a water molecule (~-1.5 kcal/mol). We use -2.5 kcal/mol as our cutoff for favorable binding as a conservative estimate. We found similar results with HSA, although the data is not shown.

Table 2. The average binding exothermicities (ΔH) for the second substrate binding in the sequential docking of taurine ($R\text{-}NH_2$, $R\text{-}NH_3^+$) and α- and β-glucopyranose to the Val1/Lys82 protein pocket of a single β-chain of hemoglobin (HbA).

First Substrate Bound	Second Substrate Bound	Average Binding Exothermicity* of the Second Substrate (kcal/mol)
α-glucopyranose	Taurine ($R\text{-}NH_2$)	-3.33
α-glucopyranose	Taurine ($R\text{-}NH_3^+$)	-3.30
β-glucopyranose	Taurine ($R\text{-}NH_2$)	-4.15
β-glucopyranose	Taurine ($R\text{-}NH_3^+$)	-3.57
Taurine ($R\text{-}NH_2$)	α-glucopyranose	-3.48
Taurine ($R\text{-}NH_3^+$)	α-glucopyranose	-3.53
Taurine ($R\text{-}NH_2$)	β-glucopyranose	-3.28
Taurine ($R\text{-}NH_3^+$)	β-glucopyranose	-3.12

*Averages were generated based on 50 poses

From our computational studies with taurine and glucose, we know that both taurine ($R\text{-}NH_2$ and $R\text{-}NH_3^+$) and α- and β-glucopyranose will independently bind to the same known glycation sites on HbA with similar, favorable exothermicities. This sets up the question of whether or not taurine can prevent glucose from entering the protein pocket, thus preventing the potential for NEG. We found that not only will taurine bind in the Val1/Lys82 pocket on HbA that is a glycation hotspot, but it will do so in the presence of glucose, with favorable exothermicities. The energies of taurine binding concomitantly with glucose are similar to those of taurine binding on its own (Table 2). Therefore, the presence of taurine within a protein pocket will not expel glucose from that same pocket. Additionally, if taurine is already bound in the Val1/Lys82 pocket of HbA, either form of glucose can concomitantly bind in that pocket (Table 2). Overall, our computational modeling of taurine and glucose binding to HbA revealed that taurine does not play a role as a binding inhibitor of glucose. Similar results were found when docking taurine and the glucopyranose isomers to HSA, although the data is not shown.

Question #5 & #6: Can taurine theoretically prevent glucose ring-opening in a protein pocket and thereby reduce NEG? Can taurine theoretically prevent a protein nucleophile from attacking a ring-opened glucose in a protein pocket?

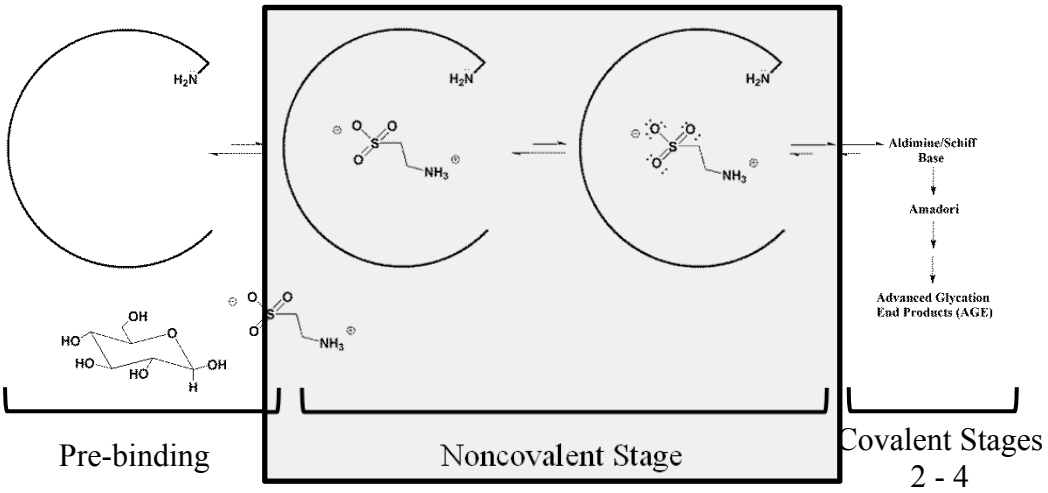


Figure 9. Nonenzymatic glycation (NEG) scheme highlighting the temporal region (i.e. the ring opening of glucose within a protein pocket and the nucleophilic attack of the protein on the ring-opened glucose) of interest in questions five and six.

At physiological pH (~ 7.4), approximately 99% of the taurine existing in solution (i.e., blood plasma or cytosol of cells) will be in the ammonium ion form. This is based on the pKa of taurine being ~9.06. Given the pKa's of certain amino acids, we can make predictions as to what acid/base interactions may be thermodynamically feasible within a protein pocket. For example, a terminal valine amine/ammonium ion has a pKa of approximately 7.1 on the β-chain of

HbA. Taurine may either deprotonate or protonate a terminal valine residue. Lysine, which has a pKa of approximately 10.54, may also be deprotonated by taurine, albeit not as well as a terminal valine residue. However, taurine will protonate a lysine better than a terminal valine residue. Additionally, taurine can deprotonate histidine, which has a pKa of approximately 6.04, much better than it will lysine or terminal valine, but it will not protonate a histidine residue.

An important limitation of using pKa values to make predictions within a protein pocket is that these pKa values describe the acid dissociation of a substrate in an aqueous environment. In aqueous environments the dielectric constant is roughly 78, whereas inside a protein pocket the dielectric constant is closer to 2, although different protein pockets may vary (Wyman, 1931). The dielectric constant represents the ratio of substrate permittivity to the permittivity of free-space, meaning that a higher dielectric constant supports electric charge better than a lower dielectric constant (Wyman, 1931). Given that protein pockets have a much lower dielectric constant than does an aqueous environment, the ability for charged species to exist in a protein pocket is much less favorable than in solution. This means that inside a protein pocket, an acid may have a lower pKa, and thus be more willing to give up its proton than in an aqueous environment. Additionally, bases inside protein pockets may be weaker bases and less likely to accept a proton. Therefore, when using solution phase pKa's to make predictions inside protein pockets, we are interested in the relative pKa of substrates and amino acid residues rather than the absolute values.

From our scheme of NEG (Fig. 9), three events must occur within a protein pocket in order for NEG to progress to the covalent stages. First, the ring-closed glucose isomer that initially binds must be ring-opened to form the electrophile. This ring-opening has two components, constituting the first two events that must occur in the pocket. A base must deprotonate the anomeric OH on glucose, and an acid must protonate the hemiacetal O^-. The roles of acid and base may be fulfilled by either intrinsic amino acid residues in the protein or effector reagents, like Pi, that bind in the pocket with glucose. The third event that must occur is the nucleophilic attack by the protein on the ring-opened glucose electrophile.

Based on theoretical acid/base reactions that may occur due to pKa difference between taurine and key amino acids, we predict that within a protein pocket, taurine in the amine form may play a few roles. First, it may increase NEG or NECPM by assisting in the formation of the nucleophile (Val1 or Lys) or base (Val1, Lys, or His). It does this by deprotonating these amino acid residues. Additionally, taurine ($R\text{-}NH_2$) may reduce NEG by deprotonating $His\text{-}H^+$ or $Lys\text{-}H^+$, preventing them from being capable of acting as acids to participate in the ring-opening of glucose. However, given that in solution approximately 1% of the taurine will be in the amine form, it is unlikely that these roles are significant in taurine's overall ability to reduce NEG. Additionally, taurine ($R\text{-}NH_3^+$) may reduce NEG or NECPM by inhibiting the nucleophile or base (Val1 or Lys). It

does this by protonating Val1 or Lys. Taurine's ammonium ion may also increase NEG by serving as the acid to ring-open glucose.

Table 3: Mechanistic options for taurine ($R\text{-}NH_2$) in the Val1/Lys82 pocket of human hemoglobin (HbA, X), and the Lys195/Lys199 protein pocket of human serum albumin (HSA, Z) based on proximity and orientation assessments of taurine using computational modeling. Glucose (α- or β-glucopyranose) is bound in the pocket first, followed in some cases by mono or dibasic Pi. Three baseline mechanism for ring-opening of glucose were assessed that result in the covalent attachment of glucose to an amino acid residue. Taurine's amine was hand-placed the pocket with glucose and Pi and energy minimized, then the geometry of the species in the pocket was assessed to determine whether proton transfer can occur to inhibit the electrophile formation (i.e. base or acid) or nucleophilic attack. Geometric inhibition occurs when glucose and amino acid residues move too far away from each other to react (>6Å). Glu, glucopyranose; Mono, monobasic; Di, dibasic; B, base; Nu, nucleophile.

			Chemical Inhibition			
			Electrophile		Nucleophile	
Sugar	**Pi**	**Baseline Mechanism**	**Indirectly Inhibits Base**	**Directly Inhibits Acid**	**Indirectly Inhibits Nu**	**Geometric Inhibition**
α-Glu		Naked		X, Z	X	Z(B)
β-Glu		Naked		X, Z		Z(Nu)
α-Glu	MonoPi	Bimolecular		Z		
β-Glu	MonoPi	Bimolecular		Z		
α-Glu	MonoPi	Bridge	Z	X		
β-Glu	MonoPi	Bridge	X	X, Z		X(B)
α-Glu	DiPi	Bimolecular		X, Z		
β-Glu	DiPi	Bimolecular	Z	X		
α-Glu	DiPi	Bridge	X, Z	X, Z		
β-Glu	DiPi	Bridge	X, Z	X	X	Z(Nu)

The potential role of taurine's amine inside the Val1/Lys82 pocket of HbA and the Lys195/Lys199 pocket of HSA is diverse, with actions that prevent the formation of the electrophile as well as the nucleophilic attack (Table 3). With respect to inhibiting the formation of the ring-opened glucose electrophile, taurine's ($R\text{-}NH_2$) role is primarily in directly deprotonating the acid, preventing it from donating a proton to the hemiacetal O^-. Although taurine ($R\text{-}NH_2$) can also protonate the base required for ring-opening, it first must accept a proton from a proton source (another base). Thus, it does not always achieve the geometry to act as a shuttle between a proton donor and the base involved in ring-opening of glucose.

Additionally in HSA, taurine's amine may achieve the proper geometry to protonate the nucleophile, however, the same issue arises as with its role in protonating the base. That is, taurine must first be protonated to the ammonium ion form, before it can protonate and inhibit the nucleophile (Fig. 10). Therefore, taurine ($R\text{-}NH_2$) rarely achieves the geometry in this pocket to act as this proton shuttle and interfere with the nucleophilic attack. Finally, taurine may geometrically inhibit the base, where the presence of taurine's amine in the protein pocket pushes the base too far away from the ring-closed glucose to react, although this is rarely observed.

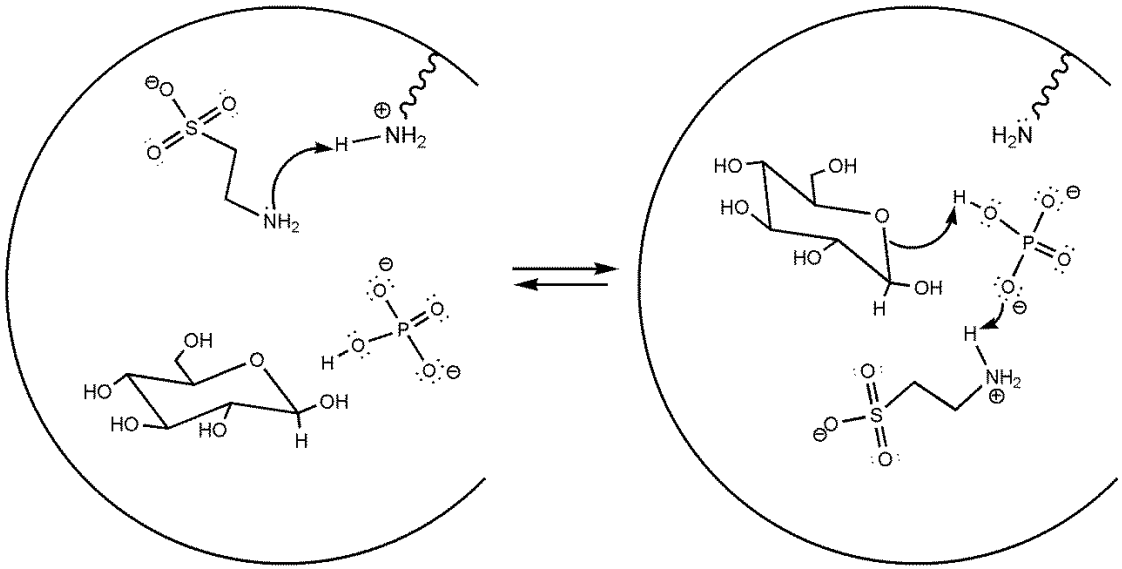


Figure 10. Taurine (R-NH_2) acting in a protein pocket to inhibit the base (dibasic Pi) from attacking glucose. Taurine (R-NH_2) must first be protonated by an amino acid residue, then it can protonate dibasic Pi, preventing dibasic Pi from deprotonating the anomeric OH on glucose.

Table 4: Mechanistic options for taurine ($R\text{-}NH_3^+$) in the Val1/Lys82 pocket of human hemoglobin (HbA, X), and the Lys195/Lys199 protein pocket of human serum albumin (HSA, Z) based on proximity and orientation assessments of taurine using computational modeling. Glucose (α- or β-glucopyranose) is bound in the pocket first, followed in some cases by mono or dibasic Pi. Three baseline mechanism for ring-opening of glucose were assessed that result in the covalent attachment of glucose to an amino acid residue. Taurine's amine was hand-placed the pocket with glucose and Pi and energy minimized, then the geometry of the species in the pocket was assessed to determine whether proton transfer can occur to inhibit the electrophile formation (i.e. base or acid) or nucleophilic attack. Geometric inhibition occurs when glucose and amino acid residues move too far away from each other to react (>6Å). Glu, glucopyranose; Mono, monobasic; Di, dibasic; B, base; Nu, nucleophile.

			Chemical Inhibition			
			Electrophile		Nucleophile	
Sugar	**Pi**	**Baseline Mechanism**	**Directly Inhibits Base**	**Indirectly Inhibits Acid**	**Directly Inhibits Nu**	**Geometric Inhibition**
α-Glu		Naked			X	Z(B)
β-Glu		Naked			X, Z	
α-Glu	MonoPi	Bimolecular	Z	X	Z	X(Nu, B)
β-Glu	MonoPi	Bimolecular	Z	X	X, Z	
α-Glu	MonoPi	Bridge	X	X	Z	
β-Glu	MonoPi	Bridge	X	X, Z	X, Z	
α-Glu	DiPi	Bimolecular	X, Z	X	X, Z	
β-Glu	DiPi	Bimolecular	X, Z	X	X	
α-Glu	DiPi	Bridge	X, Z		X, Z	
β-Glu	DiPi	Bridge	X, Z	X		Z(Nu)

Like taurine's amine form, the ammonium ion form also may achieve the proper geometry in the Val1/Lys82 pocket of HbA and Lys195/Lys199 pocket of HSA to inhibit the ring-opening of glucose as well as the protein's nucleophilic attack. In contrast to taurine's amine form, the ammonium ion is more reactive against the nucleophile and the base, yet reacts similarly against the acid. Taurine in the ammonium ion form achieves the proper geometry to protonate the nucleophile and the base far more frequently than the amine form of taurine, because the ammonium ion can directly donate a proton to the nucleophile or base. Taurine's amine must first be protonated, before it can donate a proton to the nucleophile or base, acting a shuttle. Because the ammonium ion can directly inhibit the nucleophile and base, it is not surprising that it does so better than the amine form.

Additionally, the ammonium ion form of taurine may achieve the correct geometry to inhibit the acid component of glucose ring-opening, although taurine ($R\text{-}NH_3^+$) must first be deprotonated before it can deprotonate the acid (Fig. 11, Table 4). This deprotonation of the ammonium ion occurs far more frequently in HbA than in HSA, so taurine's ability to inhibit the acid is greater in HbA than it is in HSA. Finally, taurine's ammonium ion may also be capable of geometric inhibition of the base and nucleophile, where the presence of taurine within the protein pocket pushes the base and nucleophile too far away from glucose to react, although this is rarely observed.

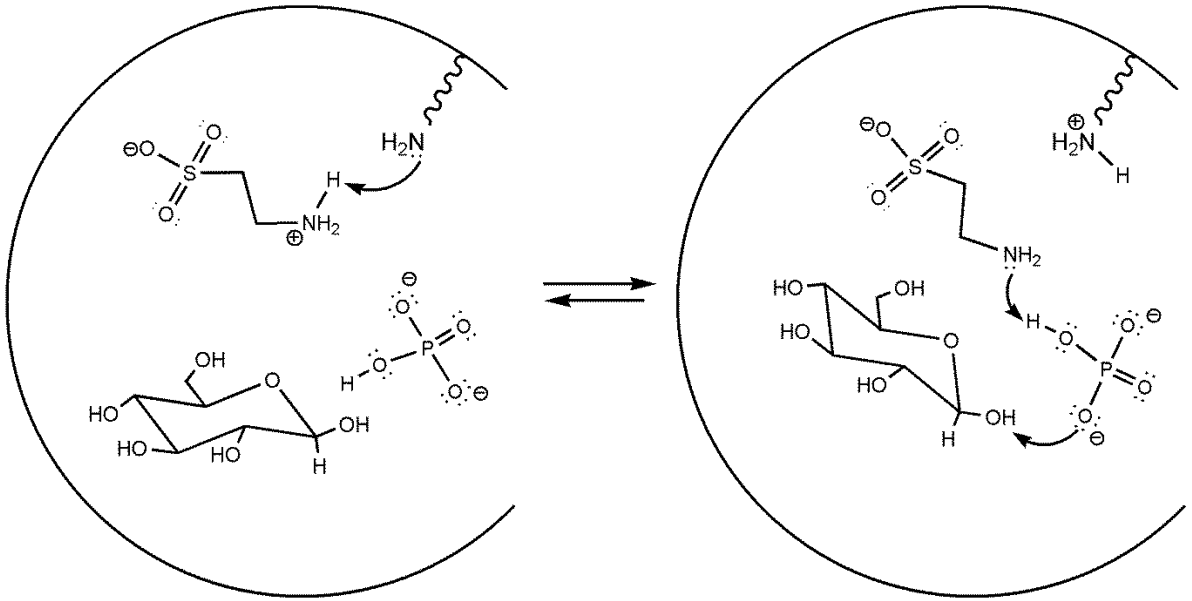


Figure 11. Taurine ($R\text{-}NH_3^+$) acting in a protein pocket to inhibit the acid (dibasic Pi) from attacking glucose. Taurine ($R\text{-}NH_3^+$) must first be deprotonated by an amino acid residue, then it deprotonates dibasic Pi, preventing dibasic Pi from protonating the hemiacetal O^- on glucose.

There does not appear to be a significant difference between taurine reducing NEG when either α- or β-glucopyranose is the sugar of interest in the protein pocket. However, there is a difference in the reactivity of taurine in reducing NEG when monobasic Pi or dibasic Pi is involved in ring-opening. Regardless of which phosphate is in the protein pocket, taurine ($R\text{-}NH_2$ and $R\text{-}NH_3^+$) can inhibit the electrophile formation by inhibiting the acid component of ring-opening. When monobasic Pi is in a protein pocket, taurine is only about half as capable at inhibiting the base component of ring-opening in HbA and HSA as when dibasic Pi is present. Additionally, taurine is equally able to inhibit the acid component of ring-opening in HbA when either phosphate is present, but in HSA it inhibits the acid better in the presence of monobasic Pi. Taurine is also better at inhibiting the nucleophile in HSA in the presence of monobasic Pi, whereas taurine in HbA it is better in the presence of dibasic Pi. Finally, taurine only exerts a geometric effect ("pushing" glucose >6Å from the base and nucleophile) against glucose in HbA in the presence of monobasic Pi, while in HSA only does so in the presence of dibasic Pi.

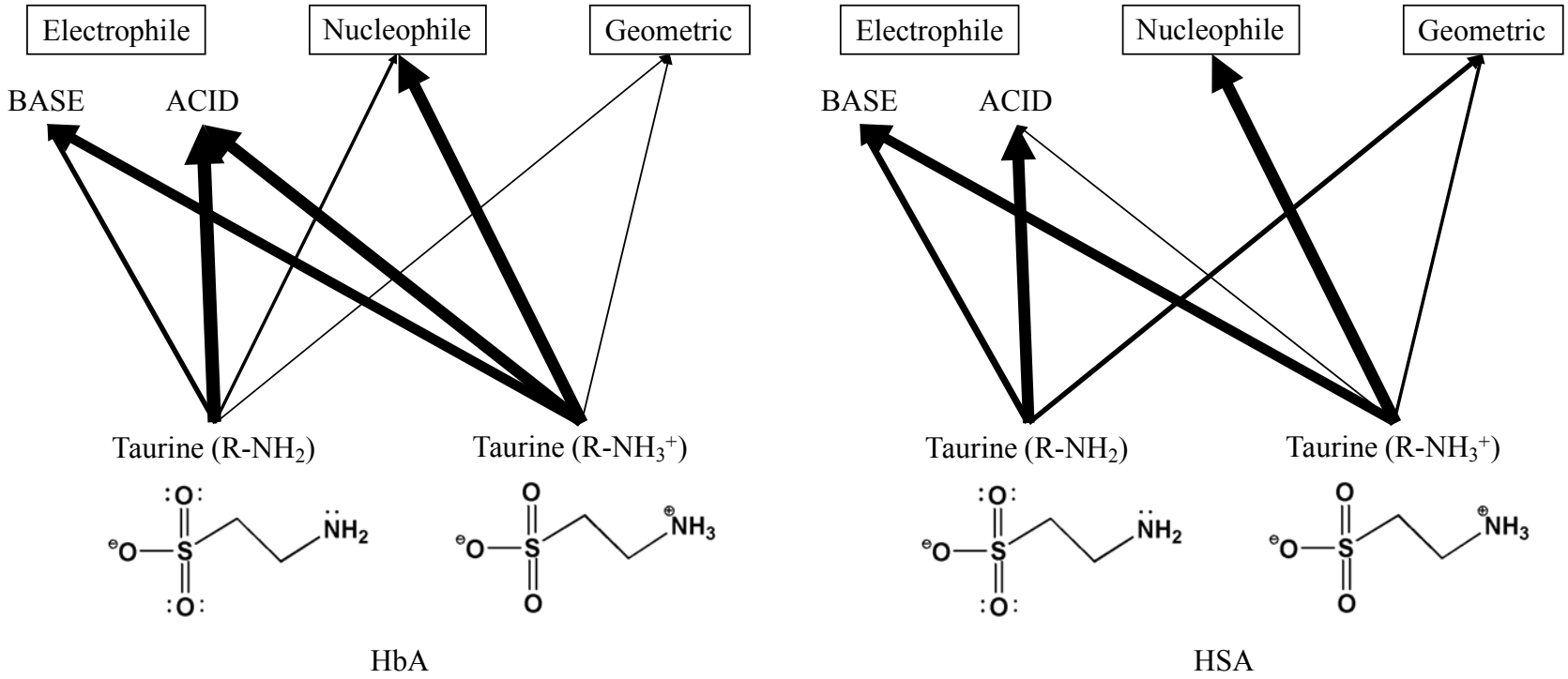


Figure 12. Taurine (R-NH_2 and R-NH_3^+) targets of inhibition in the noncovalent stage of nonenzymatic glycation (NEG) of hemoglobin (HbA, left) and albumin (HSA, right). The thickness of the arrows indicates a greater inhibitory effect of taurine (R-NH_2 or R-NH_3^+) on the target structure.

Both taurine ($R\text{-}NH_2$) and taurine ($R\text{-}NH_3^+$) are able to concomitantly bind in an intracellular and extracellular protein pocket with either α- or β-glucopyranose, and either monobasic or dibasic Pi. Not only can taurine ($R\text{-}NH_2$) and taurine ($R\text{-}NH_3^+$) concomitantly bind with the sugar and phosphate, but they both obtain the proper proximity and orientation to inhibit the ring-opening and nucleophilic attack of the protein in three baseline mechanisms of NEG (naked, bimolecular, and bridge). However, when we consider the relative concentration of taurine's amine form in solution (~1%), the effect of the amine is likely much less important than the effect of the ammonium ion. Fig. 12 shows that while both forms of taurine are able to obtain the geometries within the Val1/Lys82 pocket of HbA to inhibit electrophile formation, taurine ($R\text{-}NH_3^+$) is slightly better at inhibiting the base than is taurine ($R\text{-}NH_2$), but both are approximately equally good at inhibiting the acid. Additionally, both forms of taurine can inhibit the nucleophile, but the ammonium ion form is far superior. Finally, taurine ($R\text{-}NH_2$) and taurine ($R\text{-}NH_3^+$) both exert a small geometric inhibition in a protein pocket, pushing reacting centers too far away from each other.

These same general trends apply for taurine ($R\text{-}NH_2$ and $R\text{-}NH_3^+$) in the Lys195/Lys199 pocket of HSA, with two exceptions. First, in HSA the amine form of taurine is not able to inhibit the nucleophile, while it is able to in HbA. Second, the ammonium ion form of taurine is rarely able to inhibit the acid, while in HbA the ammonium ion readily inhibits the acid component of ring-opening.

Although the specific mechanisms in which taurine in the two forms inhibit either the generation of the electrophile or the nucleophilic attack by the protein differ slightly depending on the isomer of sugar bound, the type of phosphate bound, and the protein pocket, common themes are shared. These themes include donating a proton to a base or to a nucleophile, while accepting a proton from an acid, highlighting the multiple mechanistic options of taurine (Fig. 12).

2.4 Conclusion

Data from model reactions followed by ^{1}HNMR and computational modeling revealed that taurine may function to reduce NEG in several different temporal regions. Although taurine was able to enhance glucose ring-opening in solution, it was not able to covalently bind up ring-opened glucose. Taurine was, however, able to form adducts with glyoxal. Taurine reacted with glyoxal to form a mono-imine and di-imine, which was evident in ^{1}HNMR, suggesting that taurine may play a role in limiting NECPM in the early, pre-binding stage by binding to reactive carbonyl species.

Additionally, through computational modeling we found that taurine was able to bind to HbA and HSA at known glycation sites with favorably exothermicity. It will also concomitantly bind to these site with either α- or β-glucopyranose. Although the presence of taurine in a protein pocket does not prevent glucose from entering the same pocket, taurine may still be able to interfere with NEG in the noncovalent temporal region. We found that taurine obtained the geometry within the Val1/Lys82 pocket of HbA and Lys195/Lys199 pocket of HSA to inhibit the generation of the ring-opened glucose electrophile. Taurine can inhibit the process indirectly by either protonating the necessary base (i.e., amino acid residue or Pi) or deprotonating the required acid (i.e. amino acid residues or Pi).

Finally, taurine may also inhibit the nucleophilic attack on the ring-opened glucose electrophile, preventing the forward progression of NEG. It does this by protonating amino acid residues so that the residue is no longer nucleophilic.

References

1. Cuisinier, C., Michotte De Welle, J., Verbeeck, R. K., Poortmans, J. R., Ward, R., Sturbois, X., Francaux, M. (2002) Role of taurine in osmoregulation during endurance exercise. *Eur J Appl Physiol* 87, 489–495.
2. Brede, C., Hop, B., Jørgensen, K., Skadberg, Ø. (2016) Measurement of glycated albumin in serum and plasma by LC-MS/MS. *Scand J Clin Lab Invest* 76, 195–201.
3. Brutsaert, Erika. Diabetes Mellitus (DM) - Endocrine and Metabolic Disorders. *Merck Manuals Professional Edition*. N.p., Feb. 2017. Web. 04 May 2017. http://www.merckmanuals.com/professional/endocrine-and-metabolic-disorders/diabetes-mellitus-and-disorders-of-carbohydrate-metabolism/diabetes-mellitus-dm.
4. Delpierrre, G., Vertommen, D., Communi, D., Rider, M. H., Schaftingen, E. V. (2004) Identification of fructosamine residues deglycated by fructosamine-3-kinase in human hemoglobin. *J Biol Chem* 279, 27613–27620.
5. Ganea, E., Rixon, K. C., Harding, J. J. (1994) Binding of glucose, galactose and pyridoxal phosphate to lens crystallins. *Biochim Biophys Acta* 1226, 286-290.
6. Gardner, D. G., Shoback, D. (2011) Chapter 17: Pancreatic hormones & diabetes mellitus. *Greenspan's basic & clinical endocrinology* (9th ed.).

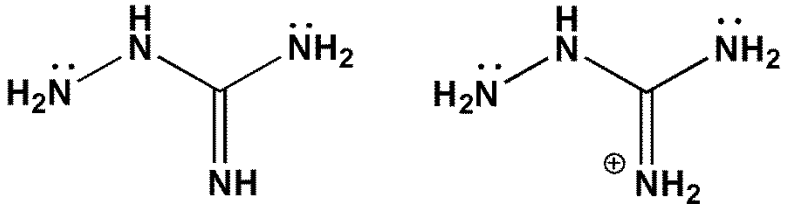
New York: McGraw-Hill Medical. ISBN 0-07-162243-8. OCLC 613429053.

7. Huxtable, R. J. (1992) Physiological actions of taurine. *Physiol Rev* 72, 101–163.
8. Melmed, S., Polonsky, K. S., Larsen, P., Reed, Kronenberg, H. M. *Williams textbook of endocrinology.* (12th ed.). Philadelphia: Elsevier/Saunders. pp. 1371–1435. ISBN 978-1-4377-0324-5.
9. Porter, D. W., Martin, W. G. (1992) Taurine flux in chicken erythrocytes. *Comp Biochem Physiol Comp Physiol* 102, 117–122.
10. Sen, S., Kar, M., Roy, A., Chakraborti, A. S. (2004) Effect of nonenzymatic glycation on functionl and structural properites of hemoglobin. *Biophys Chem* 113, 289-298.
11. Smith, R. J., Koenig, R. J., Binnerts, A., Soeldner, J. S., Aoki, T. T. (1982) Regulation of hemoglobin A1c formation in human erythrocytes in vitro. *J Clin Invest* 69, 1164-1168.
12. Wang, Y., Yu, H., Shi, X., Luo, Z., Lin, D., Huang, M. (2013) Stuctural mechanism of ring-opening reaction of glucose by human serum albumin. *J Biol Chem* 288, 15980-15987.
13. Wyman, J. (1931) Studies on the dielectric constants of protein solutions. *J Biol Chem* 90, 443-476.
14. Zuck, J., Borges, C. R., Braun, E. J., Sweazea, K. L. (2017) Chicken albumin exhibits natural resistance to glycation. *Comp Biochem Physiol Biochem Mol Biol* 203, 108–114.

Chapter 3. Taurine, Metformin and Aminoguanidine: How Do They Compare?

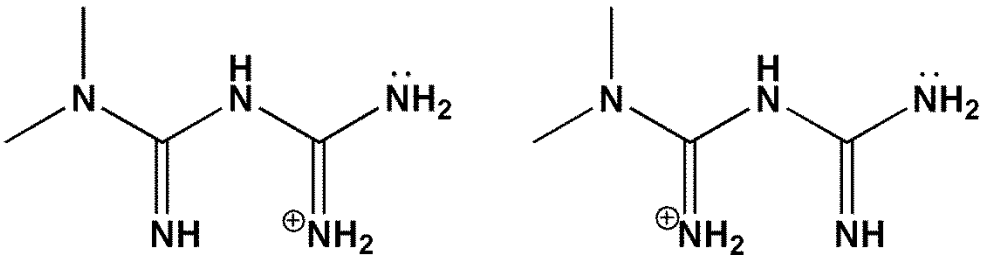
3.1 Introduction

Metformin (Fig. 1) and aminoguanidine (Fig. 2) are two types of drugs that have been given clinically to treat individuals with type 2 diabetes mellitus. Although aminoguanidine is no longer given to patients to treat diabetes because of its adverse side effects (Waz et al., 1997), metformin is currently the most commonly prescribed drug to treat type 2 diabetes. Metformin, which is given as a 500 mg pill once/day, works primarily as an antihyperglycemic agent, reducing the amount of glucose that is synthesized by the body. Its effects to lower blood glucose levels target the synthesis of glucose, rather than increase glucose uptake into cells, like insulin. Thus, the use of metformin decreases the likelihood of experiencing hypoglycemia compared to when insulin is administered (Viollet et al., 2012).



AG1 AG2

Figure 1. Aminoguanidine structures showing the two most stable protonation states (Bharatam et al., 2004).



MF1 MF2

Figure 2. Metformin structures showing the two most stable protonation states (Hernández et al., 2015).

The most common side-effects of metformin include gastrointestinal distress, vitamin B12 malabsorption, and lactic acidosis, in severe cases (Brutsaert, 2017). Aminoguanidine, although useful in the prevention of AGE formation, also inhibits diamine oxidase (DAO) and NO synthase. Inhibiting DAO can lead to the accumulation of histamine in the blood and ultimately cause dangerous vascular and respiratory effects, while inhibiting NO synthase affect the immune response and neuronal transmission (Nilsson, 1999).

Unlike taurine, metformin and aminoguanidine have been used clinically to treat diabetes mellitus. However, their effects against nonenzymatic glycation of proteins was not fully appreciated when metformin and aminoguanidine first came on the market. Both aminoguanidine and metformin have been investigated extensively in their action to scavenge dicarbonyls, such as glyoxal, methylglyoxal and 3-deoxyglucosone (3-DG) (Ruggiero-Lopez, 1999;

Beisswenger, 2003). Like taurine, metformin and aminoguanidine have reactive amine groups that allow the potential for multiple mechanistic roles in limiting NEG, beyond the scavenging of carbonyls.

The purpose of this chapter is to compare and contrast the mechanistic options elucidated for taurine against NEG in the previous chapter with metformin and aminoguanidine. Looking to both the literature and our current computational studies, we attempt to contextualize the importance of the actions of taurine by providing information about drugs that have been, and are currently being used to treat type 2 diabetes mellitus.

3.2 Methods

General Methods:

Nuclear Magnetic Resonance (NMR) - The NMR data was collected on a JEOL-EM300 at room temperature (~20°C). The NMR data obtained consisted of both ^{1}HNMR as well as ^{31}PNMR. All reactions were performed dissolved in D_2O obtained from Sigma-Aldrich (lot# MKBV2445V). The metformin was obtained from Calbiochem (lot# 2733457), and aminoguanidine was obtained from Sigma-Aldrich (lot# MKBT1749V). Glyoxal (40% w/w aq. soln.) was obtained from Alfa Aesar (lot# 10171751). Dibasic phosphate was obtained from Fisher Scientific (lot# 081308) and monobasic phosphate was obtained from Fisher Scientific (lot# 974537). Pure α-glucopyranose was obtained from Sigma-Aldrich (lot# MKBH5500V). Standards for all chemical were obtained using ^{1}HNMR, expect dibasic phosphate, and monobasic phosphate, which were obtained using ^{31}PNMR. Default settings were used, with the exception of number of scans run, which varied with experiment.

Computational Modeling – The non-covalent binding data was obtained in the program Molecular Operating Environment (MOE, ver. 2015.1001, Chemical Computing Group Inc., Montreal, Canada). The proteins used in the docking procedure were obtained from Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) and included: 1) A single β-chain of human hemoglobin (HbA) isolated from the fully oxygenated HbA crystal tetramer, 1B86 (obtained from the RCSB PDB (http:// www.pdb.org/pdb/home/home.do)) using Deep View-Swiss PDB Viewer (WINE,

http://spdv.vital-it.ch/wine.html), and 2) Human Serum Albumin (rHSA, 1AO6, Wang et al., 2013). The substrates that were used in the docking procedure included: 1) the α-glucopyranose and β-glucopyranose isomers were obtained from Heterocompound Information Centre, Uppsala, (HIC-UP, http:// xray.bmc.uu.se/hicup/), 2) metformin, taurine and aminoguanidine multiple protonation forms were built in MOE, and 3) HPO_4^{2-}, and $H_2PO_4^-$ were built with The PyMOL Molecular Graphics System (ver. 1.5.0.4 Schrödinger, LLC, http://www.pymol.org/).

All docking was done under default parameters with the exception of the temperature being set to a physiological value (310 K), induced fit setting being used, and energy minimization before each docking. For all dockings a distance of 5Å was used as a cutoff for effective geometry in protein pockets, and an energy of -2.5 kcal/mol.

Specific Methods:

Methods for Question #2: Does metformin or aminoguanidine inhibit glucose ring-opening in solution, thus RCS formation?

Inflection time of α-glucopyranose in the presence of taurine was obtained by ^{1}HNMR, measuring the percent of α- and β-glucopyranose generated as a function of time. The inflection time is defined as the time it takes for the concentration of α-glucopyranose and β-glucopyranose to be approximately 50%.

This is determined by measuring the integration of the doublets at 5.05 ppm (α-glucopyranose) and 4.46 ppm (β-glucopyranose).

Methods for Question #3: Does metformin or aminoguanidine compete with glucose binding in protein pockets, thus limiting NEG?

The HbA PDB file was altered in MOE so that Val1 and all histidine residues were deprotonated to amines ($R\text{-}NH_2$). Conversely, all lysine residues were protonated to ammonium ions ($R\text{-}NH_3^+$) in both HbA and HSA. The docking done to HbA was tethered to the Val1 and all lysine residues, due to these residues being the most commonly glycated sites on HbA (Delpierre et al., 2004). Independently, α-glucopyranose, β-glucopyranose, and metformin or aminoguanidine (MF1, MF2, AG1, and AG2) were docked with 50 poses produced. For each pose, we examined the substrates geometry and calculated an average exothermicity (ΔH).

In order to assess if metformin or aminoguanidine competes with glucose binding in a protein pocket, sequential docking was done with metformin (MF1 and MF2) or aminoguanidine (AG1 and AG2) and either α-glucopyranose or β-glucopyranose. The sequential docking was done by first docking one substrate, then saving a single pose and docking the next substrate to that pose. All 8 docking combinations were done. We observed if the second substrate would dock given the presence of the first substrate in the Val1/Lys82 protein pocket of

HbA and the Lys195/Lys199 protein pocket of HSA. The exothermicities (ΔH) were assessed for the docking of the second substrate.

Methods for Questions #4 & #5: Does metformin or aminoguanidine inhibit glucose ring-opening while bound and thereby inhibit NEG? Does metformin or aminoguanidine inhibit NEG by preventing the nucleophilic amino acid residues from attacking ring-opened glucose in a protein pocket?

Computational modeling was used to determine if the presence of metformin (MF1 and MF2) or aminoguanidine (AG1 and AG2) in the Val1/Lys82 pocket of HbA or Lys195/Lys199 pocket of HSA can prevent the acid or base from ring-opening a bound glucose or nucleophilic attack on a ring-opened glucose. Using MOE, we compared three potential mechanisms of ring-opening. The baseline mechanisms we used were the naked mechanism, bimolecular mechanism and bridge mechanism. Starting from each of the three baseline mechanisms (each using both α-glucopyranose and β-glucopyranose, and the bimolecular and bridge using both monobasic and dibasic Pi), we hand placed a metformin (MF1 and MF2) or aminoguanidine (AG1 and AG2) into the pocket and energy minimized. We then assessed the baseline mechanism again with the drug in place to see if the drug obtains the proper geometry to interfere with either the acid or base, by measuring the distance between reactive centers (i.e. nitrogen of amine groups on protein residues or metformin/aminoguanidine, and the anomeric carbon of the glucopyranoses). We also assessed the baseline

mechanisms to determine if metformin or aminoguanidine had the correct proximity and orientation to glucose to transfer a proton to the amino acid nucleophile, thus inhibiting the nucleophile. We measured the distance between the reactive centers on glucose, the protein and metformin or aminoguanidine, using a cutoff for reaction distance of 6Å.

3.3 Results and Discussion

Question #1: Does metformin or aminoguanidine prevent glucose from ring-opening in solution?

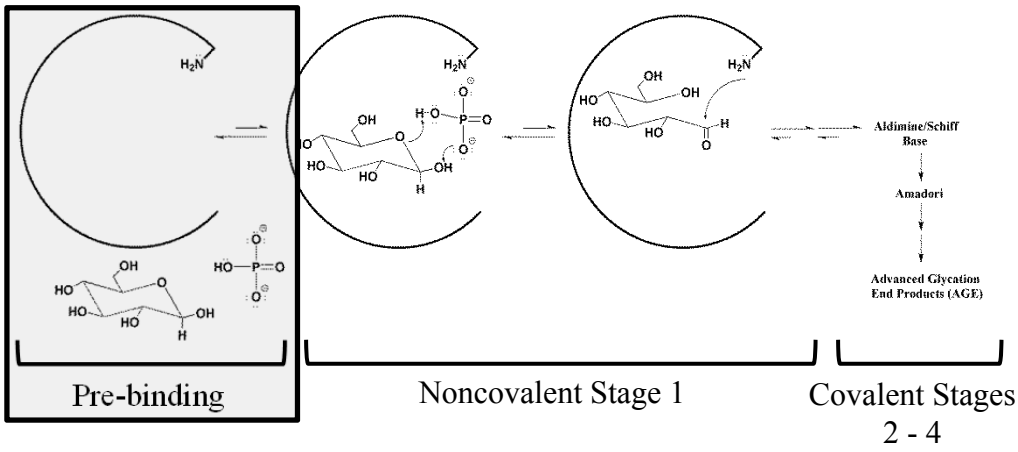


Figure 3. Nonenzymatic glycation (NEG) scheme with the temporal region of interest in question one highlighted in grey.

In the presence of metformin, the inflection of α-glucopyranose to β-glucopyranose in D_2O occurs in 230 min, while in the presence of aminoguanidine, glucose infects in D_2O in 250 min. Thus, neither of these drugs increase the inflection time of glucose, which inflects alone in D_2O in 270 min. In contrast, taurine causes a decrease in the inflection time of glucose, lowering it to 150 min.

Although taurine is not a great base in solution to ring-open glucose, it is able to ring-open glucose slightly better than both metformin and aminoguanidine. The conclusion we drew for taurine in chapter 2 is that, because taurine is able to modestly increase the ring-opening of glucose, it's function against NECPM likely does not include preventing RCS formation in solution. Remember that the first step of RCS formation in solution requires a ring-closed glucose isomer to ring-open. Therefore, because glucose can ring-open better in the presence of taurine, taurine may not inhibit the first step of RCS formation in solution. However, when we look at metformin and aminoguanidine in contrast to taurine, the drugs have a much smaller decrease in the inflection time of glucose. Although the decrease in glucose inflection time in the presence of the drugs is smaller than in the presence of taurine, a similar conclusion can be drawn with the drugs. That is, metformin and aminoguanidine do not prevent glucose from ring-opening in solution and therefore may not inhibit the first step of RCS production in solution.

Overall, taurine and the drugs differ in their reactivity toward glucose inflection time in solution, but are similar in that none of them may inhibit NECPM by inhibiting the production of RCS in plasma or the cytosol of cells.

Question #2: Does metformin or aminoguanidine compete with glucose binding in a protein pocket, thus limiting NEG?

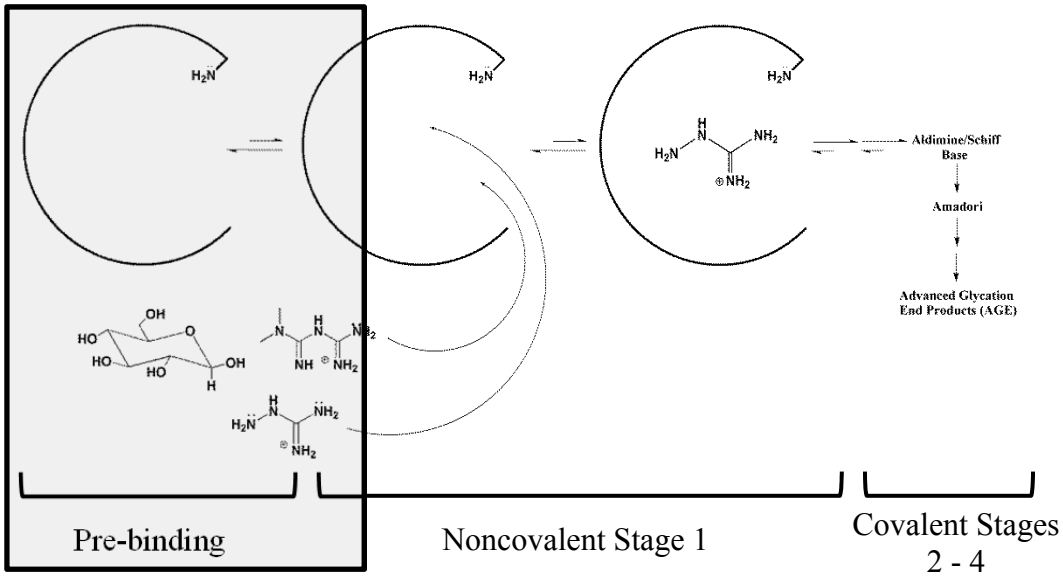


Figure 4. Nonenzymatic glycation (NEG) scheme with the temporal region of interest in question two highlighted in grey.

Table 1. The average binding exothermicities (ΔH) and common binding sites for aminoguanidine (AG1 and AG2) and α- or β-glucopyranose to the β-chain of hemoglobin (HbA). Aminoguanidine and glucopyranose were tethered to Val1 and all the lysine residues on HbA.

Substrate	Average Binding Exothermicity* (kcal/mol)	Binding Sites on HbA (listed in decreasing order of occurrence)
AG1	-2.61	Lys132, Val1/Lys66, Lys59/Lys144, Lys82
AG2	-2.64	Lys66, Lys59, Val1/Lys82, Lys95, Lys65
α-glucopyranose	-3.39	Val1, Lys66, Lys82
β-glucopyranose	-3.19	Val1, Lys82, Lys66, Lys8, Lys144

*Averages generated based on 50 poses

Table 2. The average binding exothermicities (ΔH) for the second substrate binding in the sequential docking of aminoguanidine (AG1 and AG2) and α- and β-glucopyranose to the Val1/Lys82 protein pocket of the β-chain of hemoglobin (HbA).

First Substrate Bound	Second Substrate Bound	Average Binding Exothermicity* of the Second Substrate (kcal/mol)
α-glucopyranose	AG1	-2.61
α-glucopyranose	AG2	-2.42
β-glucopyranose	AG1	-2.69
β-glucopyranose	AG2	-2.49
AG1	α-glucopyranose	-3.17
AG2	α-glucopyranose	-3.31
AG1	β-glucopyranose	-3.19
AG2	β-glucopyranose	-3.39

*Averages were generated based on 50 poses

Whereas taurine ($R\text{-}NH_2$ and $R\text{-}NH_3^+$) binds to the β-chain of HbA with relatively the same exothermicity as either α- or β-glucopyranose, aminoguanidine tends to bind to HbA with slightly lower exothermicity than the two glucose isomers (Table 1). However, AG1 and AG2 can both bind to the Val1/Lys82 glycation hotpot that the glucose isomers bind (Table 1). When the concomitant binding of glucose and aminoguanidine was assessed, the presence of either AG1 or AG2 in the Val1/Lys82 pocket of HbA did not prevent either α- or β-glucopyranose from binding favorably (Table 2). Favorable binding is defined as bind with an exothermicity of -2.5 kcal/mol or lower. AG1 was also able to bind favorably in the protein pocket with either glucose isomer already present. However, when α- or β-glucopyranose was in a protein pocket, AG2 was not able to favorable bind in that same pocket (Table 2). Although not shown, AG1 and AG2 were also able independently and concomitantly bind to HSA in the Lys195/Lys199 pocket with either α- or β-glucopyranose.

Table 3. The average binding exothermicities (ΔH) and common binding sites for metformin (MF1 and MF2) and α- or β-glucopyranose to the β-chain of hemoglobin (HbA). Metformin and glucopyranose were tethered to Val1 and all the lysine residues on HbA.

Substrate	Average Binding Exothermicity* (kcal/mol)	Binding Sites on HbA (listed in decreasing order of occurrence)
MF1	-2.61	Lys66
MF2	-2.64	Lys66, Lys65, Lys144
α-glucopyranose	-3.39	Val1, Lys66, Lys82
β-glucopyranose	-3.19	Val1, Lys82, Lys66, Lys8, Lys144

*Averages based on 50 poses generated

Table 4. The average binding exothermicities (ΔH) for the second substrate binding in the sequential docking of metformin (MF1 and MF2) and α- and β-glucopyranose to the Val1/Lys82 protein pocket of the β-chain of hemoglobin (HbA).

First Substrate Bound	Second Substrate Bound	Average Binding Exothermicity* of the Second Substrate (kcal/mol)
α-glucopyranose	MF1	-2.87
α-glucopyranose	MF2	-2.93
β-glucopyranose	MF1	-3.03
β-glucopyranose	MF2	-3.03
MF1	α-glucopyranose	-3.09
MF2	α-glucopyranose	-3.22
MF1	β-glucopyranose	-3.03
MF2	β-glucopyranose	-3.09

*Averages based on 50 poses generated

Like aminoguanidine, the metformin isomers will bind to the β-chain of HbA slightly less favorably than either glucopyranose (Table 3). Although metformin (MF1 and MF2) binds to similar sites as glucose (Lys66, Lys144) neither MF1 nor MF2 prefer to bind in the Val1/Lys82 pocket of HbA which is a known glycation hotspot (although both MF1 and MF2 will bind favorably to this glycation site if tethered to only Val1 and Lys82). Like taurine ($R\text{-}NH_2$ and $R\text{-}NH_3^+$), metformin (MF1 and MF2) and α- and β-glucopyranose are able to concomitantly bind in the Val1/Lys82 pocket of HbA with similar exothermicities to individual binding. Both metformin isomers bind slightly better when α- or β-glucopyranose inhabits the pocket, while the glucose isomers bind slightly worse when MF1 or MF2 inhabits the pocket, although each combination of concomitant binding is exothermically favorable (Table 4). Metformin was also able to bind independently and concomitantly to the Lys195/Lys199 pocket of HSA with either α- or β-glucopyranose (data not shown).

Although neither metformin nor aminoguanidine bind to HbA as well as taurine, all three compounds are common in that none of them will prevent a glucopyranose isomer from binding in the Val1/Lys82 pocket of HbA or Lys195/Lys199 pocket of HSA, nor will they expel glucose from these pockets.

Questions #3 and #4: Can metformin or aminoguanidine theoretically prevent glucose ring-opening in a protein pocket and thereby reduce NEG? Can metformin or aminoguanidine theoretically prevent a protein nucleophile from attacking a ring-opened glucose in a protein pocket?

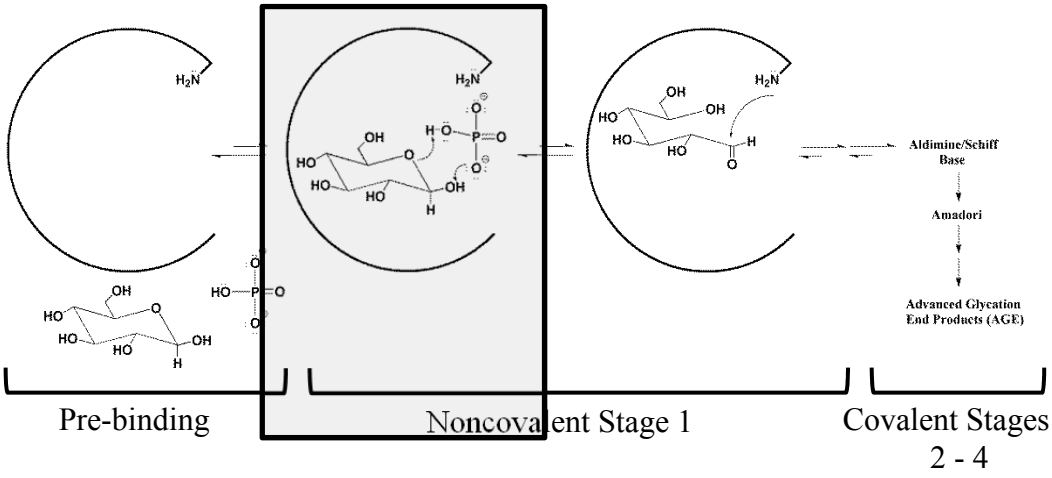


Figure 5. Nonenzymatic glycation (NEG) scheme highlighting the temporal region (i.e., the ring opening of glucose within a protein pocket and the nucleophilic attack of the protein on the ring-opened glucose) of interest in questions three and four.

AMINOGUANIDINE:

Table 5. Mechanistic options for AG1 in the Val1/Lys82 pocket of human hemoglobin (HbA, X), and the Lys195/Lys199 protein pocket of human serum albumin (HSA, Z) based on proximity and orientation assessments of AG1 using computational modeling. Glucose (α- or β-glucopyranose) is bound in the pocket first, followed in some cases by mono or dibasic Pi. Three baseline mechanism for ring-opening of glucose were assessed that result in the covalent attachment of glucose to an amino acid residue. AG1 was hand-placed the pocket with glucose and Pi and energy minimized, then the geometry of the species in the pocket was assessed to determine whether proton transfer can occur to inhibit the electrophile formation (i.e. base or acid) or nucleophilic attack. Geometric inhibition occurs when glucose and amino acid residues move too far away from each other to react (>6Å). Glu, glucopyranose; Mono, monobasic; Di, dibasic; B, base; Nu, nucleophile.

			Chemical Inhibition			
			Electrophile		Nucleophile	
Sugar	**Pi**	**Baseline Mechanism**	**Indirectly Inhibits Base**	**Directly Inhibits Acid**	**Indirectly Inhibits Nu**	**Geometric Inhibition**
α-Glu		Naked		X, Z		X(Nu), Z(B)
β-Glu		Naked		X, Z		Z(Nu)
α-Glu	MonoPi	Bimolecular	Z			Z(A)
β-Glu	MonoPi	Bimolecular		Z		Z(B)
α-Glu	MonoPi	Bridge	X, Z		Z	X(B, Nu), Z(B)
β-Glu	MonoPi	Bridge	Z	X, Z		
α-Glu	DiPi	Bimolecular	Z	X, Z		
β-Glu	DiPi	Bimolecular		X, Z		
α-Glu	DiPi	Bridge	Z	Z		
β-Glu	DiPi	Bridge	Z	X, Z		Z(Nu)

Table 6. Mechanistic options for AG2 in the Val1/Lys82 pocket of human hemoglobin (HbA, X), and the Lys195/Lys199 protein pocket of human serum albumin (HSA, Z) based on proximity and orientation assessments of AG2 using computational modeling. Glucose is (α- or β-glucopyranose) bound in the pocket first, followed in some cases by mono or dibasic Pi. Three baseline mechanism for ring-opening of glucose were assessed that result in the covalent attachment of glucose to an amino acid residue. AG2 was hand-placed the pocket with glucose and Pi and energy minimized, then the geometry of the species in the pocket was assessed to determine whether proton transfer can occur to inhibit the electrophile formation (i.e. base or acid) or nucleophilic attack. Geometric inhibition occurs when glucose and amino acid residues move too far away from each other to react (>6Å). Glu, glucopyranose; Mono, monobasic; Di, dibasic; B, base; Nu, nucleophile.

			Chemical Inhibition			
			Electrophile		Nucleophile	
Sugar	**Pi**	**Baseline Mechanism**	**Directly Inhibits Base**	**Indirectly Inhibits Acid**	**Directly Inhibits Nu**	**Geometric Inhibition**
α-Glu		Naked	X, Z			X(Nu), Z(B)
β-Glu		Naked	X, Z		X	X(Nu), Z(Nu)
α-Glu	MonoPi	Bimolecular	X		X	Z(Nu)
β-Glu	MonoPi	Bimolecular	X, Z		X	Z(Nu)
α-Glu	MonoPi	Bridge	X, Z			Z(Nu)
β-Glu	MonoPi	Bridge	X, Z			X(A, B)
α-Glu	DiPi	Bimolecular	Z	Z	X	
β-Glu	DiPi	Bimolecular	X, Z		Z	
α-Glu	DiPi	Bridge	Z		X, Z	
β-Glu	DiPi	Bridge	X, Z			Z(Nu)

Based on computational studies, aminoguanidine (AG1 and AG2) is able to obtain the geometry inside an intracellular protein pocket (HbA) and extracellular protein pocket (HSA) to inhibit both the generation of the ring-opened glucose electrophile and nucleophilic attack. However, there is a marked difference between the action of AG1 and AG2. When we compare AG1 and AG2 inside HbA, we notice that AG1 is rarely able to inhibit the base component of ring-opening (Table 5), while AG2 almost always attained the geometry to inhibit the base (Table 6). An explanation for this could be that AG1, a neutral species, must first accept a proton before it can donate to the base to inhibit ring-opening, like taurine's amine form. This indirect inhibition of the base does not occur very often in HbA; however, it does occur the majority of the time in HSA. In contrast, AG2 is already protonated, thus can directly inhibit the base component of ring-opening in HbA and HSA, like taurine's ammonium ion (Table 6).

When we compare AG1 and AG2 in terms of inhibiting the acid component of ring-opening, we now reverse the roles as seen with inhibiting the base. That is, AG1 is able to directly deprotonate the acid, and does so frequently in HbA and HSA (Table 5), while AG2 must first be deprotonated itself before it can deprotonate the acid. AG2's inhibition of the acid is similar to AG1's inhibition of the base: they both require an additional step (deprotonation, and protonation respectively). Our results suggest that AG2 is never able to indirectly inhibit the acid in HbA and very rarely able in HSA (Table 6).

To inhibit the nucleophile, AG1 experiences the same issue as it had with inhibiting the base – it must first be protonated. Because of this AG1 is never able to indirectly inhibit the nucleophile in HbA and very rarely is able to inhibit it in HSA (Table 5). AG2 is often able to directly inhibit the nucleophile in HbA, yet rarely achieves the geometry to inhibit the nucleophile in HSA (Table 6).

Unlike taurine, both forms of aminoguanidine frequently have a geometric inhibition. AG1 and AG2 may exert a geometric inhibition on the formation of the electrophile or nucleophile, but both AG1 and AG2 have this effect more often in HSA than in HbA (Table 5, 6).

Additionally, similar to taurine there does not appear to be a vast difference in the mechanism of inhibition of aminoguanidine based on what glucopyranose isomer is present in the protein pocket. The largest difference occurs with aminoguanidine's effect against the acid, which is more pronounced when β-glucopyranose is present than when α-glucopyranose is present.

When we parse out the effect of aminoguanidine to observe if there is a Pi specific effect, we see that mechanisms containing either monobasic or dibasic Pi can be inhibited against. When monobasic Pi is involved in the Pi effect, aminoguanidine (AG1 and AG2) is better able to inhibit the base in HbA than when dibasic phosphate is involved in the Pi effect. The effect of aminoguanidine (AG1 and AG2) against the base are relatively equal for both phosphate isomers in HSA. Additionally, aminoguanidine is much better at inhibiting the acid in both HbA and HSA when dibasic Pi is in the pocket than when monobasic Pi is in the

pocket. Aminoguanidine's effect against the nucleophile is approximately equal regardless of the phosphate isomer and protein pocket. Finally, aminoguanidine is much more able to exert a geometric inhibition in HSA when monobasic Pi is present. In HbA, when dibasic Pi is involved in the Pi effect, aminoguanidine does not have a geometric inhibition of ring-opening or nucleophilic attack. Overall, there seems to be more mechanistic specificity for aminoguanidine based on which phosphate isomer is in the protein pocket than was observed with taurine.

Although aminoguanidine is able to attain the proper geometry within a protein pocket to inhibit both ring-opening and nucleophilic attack, are these reactions thermodynamically feasible? When we assessed taurine, we used the pKa of taurine and amino acid residues to make predictions as to whether taurine may be able to engage in acid/base reactions with amino acids within a protein pocket. Because taurine has a pKa of ~9.06, which is similar to lysine it may be able to abstract or donate a proton to some of these residues. When we look at aminoguanidine (AG2), the pKa of this drug is ~11.5 in aqueous solution (Bharatam et al., 2004). Under physiological conditions (pH ~7.4), aminoguanidine will essentially always exist in the protonated form (AG2). Because of the high pKa value, aminoguanidine is a good base, but will rarely give up its proton at a physiological pH. This means that although it is geometrically feasible for aminoguanidine to inhibit ring-opening of glucose (i.e.

acid or base) and the nucleophilic attack on a ring-opened glucose electrophile (Table 5, 6), we predict that it is thermodynamically very unlikely to actually donate a proton or exist in a neutral state, as AG1.

METFORMIN:

Table 7. Mechanistic options for MF1 in the Val1/Lys82 pocket of human hemoglobin (HbA, X), and the Lys195/Lys199 protein pocket of human serum albumin (HSA, Z) based on proximity and orientation assessments of MF1 using computational modeling. Glucose (α- or β-glucopyranose) is bound in the pocket first, followed in some cases by mono or dibasic Pi. Three baseline mechanism for ring-opening of glucose were assessed that result in the covalent attachment of glucose to an amino acid residue. MF1 was hand-placed the pocket with glucose and Pi and energy minimized, then the geometry of the species in the pocket was assessed to determine whether proton transfer can occur to inhibit the electrophile formation (i.e. base or acid) or nucleophilic attack. Geometric inhibition occurs when glucose and amino acid residues move too far away from each other to react (>6Å). Glu, glucopyranose; Mono, monobasic; Di, dibasic; B, base; Nu, nucleophile.

			Chemical Inhibition			
			Electrophile		Nucleophile	
Sugar	**Pi**	**Baseline Mechanism**	**Inhibits Base**	**Inhibits Acid**	**Inhibits Nu**	**Geometric Inhibition**
α-Glu		Naked	X, Z		X	X(A, Nu), Z(B, Nu)
β-Glu		Naked	Z		X	X(A, Nu), Z(B, Nu)
α-Glu	MonoPi	Bimolecular	Z	X	Z	
β-Glu	MonoPi	Bimolecular	X	X, Z	X, Z	
α-Glu	MonoPi	Bridge	X, Z		Z	
β-Glu	MonoPi	Bridge	X, Z	Z	Z	X(B, Nu)
α-Glu	DiPi	Bimolecular	X, Z	Z		X(Nu)
β-Glu	DiPi	Bimolecular	X, Z	X, Z		Z(Nu)
α-Glu	DiPi	Bridge		X		X(Nu), Z(Nu)
β-Glu	DiPi	Bridge	X	X		Z(Nu)

Table 8. Mechanistic options for MF2 in the Val1/Lys82 pocket of human hemoglobin (HbA, X), and the Lys195/Lys199 protein pocket of human serum albumin (HSA, Z) based on proximity and orientation assessments of MF2 using computational modeling. Glucose (α- or β-glucopyranose) is bound in the pocket first, followed in some cases by mono or dibasic Pi. Three baseline mechanism for ring-opening of glucose were assessed that result in the covalent attachment of glucose to an amino acid residue. MF2 was hand-placed the pocket with glucose and Pi and energy minimized, then the geometry of the species in the pocket was assessed to determine whether proton transfer can occur to inhibit the electrophile formation (i.e. base or acid) or nucleophilic attack. Geometric inhibition occurs when glucose and amino acid residues move too far away from each other to react (>6Å). Glu, glucopyranose; Mono, monobasic; Di, dibasic; B, base; Nu, nucleophile.

			Chemical Inhibition			
			Electrophile		Nucleophile	
Sugar	**Pi**	**Baseline Mechanism**	**Inhibits Base**	**Inhibits Acid**	**Inhibits Nu**	**Geometric Inhibition**
α-Glu		Naked	X	X		Z(Nu)
β-Glu		Naked		X		X(Nu), Z(Nu)
α-Glu	MonoPi	Bimolecular	Z	X		
β-Glu	MonoPi	Bimolecular		X	X	Z(A, Nu)
α-Glu	MonoPi	Bridge	X, Z		Z	
β-Glu	MonoPi	Bridge	Z	X	X	Z(A)
α-Glu	DiPi	Bimolecular	X, Z	X, Z		
β-Glu	DiPi	Bimolecular	X, Z	Z		
α-Glu	DiPi	Bridge	X	X	Z	Z(Nu)
β-Glu	DiPi	Bridge			X	Z(Nu)

Just like with taurine and aminoguanidine, metformin (MF1 and MF2) is geometrically capable of inhibiting the ring-opening of glucose and nucleophilic attack on a ring-opened glucose within an intracellular and extracellular protein pocket. One of the biggest differences between metformin and aminoguanidine or taurine is that metformin, MF1 and MF2, both contain one protonated and one neutral primary imine. Because of this, we don't see as much diversity between protonation states with metformin as we do with taurine and aminoguanidine. For example, when we compare MF1 and MF2, we see that both are approximately equivalent at inhibiting the base component of ring-opening in HSA and HbA (Table 7, 8). This could be because both forms of metformin are able to directly inhibit the base by donating a proton. In contrast, AG1 and taurine ($R\text{-}NH_2$) both were required to abstract a proton from a proton source before they were able to inhibit the base, making it less likely that we would observe the proper geometry for these molecule to inhibit the base.

However, there is some diversity between MF1 and MF2 when it comes to inhibiting the acid required for ring-opening α-glucopyranose. In HbA, both forms of metformin are able to inhibit the acid roughly equally, yet in HSA MF2 is only about half as capable at inhibiting the acid as MF1 (Table 7, 8). This same pattern occurred with both taurine and aminoguanidine, where one protonation state (MF1, AG1 and taurine $R\text{-}NH_2$) was better able inhibit the acid in HSA than the other protonation state. Additionally, MF1 and MF2 are equally capable of

inhibiting the nucleophile in HbA, but once again MF1 is slightly better than MF2 in HSA.

Finally, we observed a geometric inhibition with both MF1 and MF2. With MF1, this geometric inhibition was equal in both HbA and HSA. However, MF2 had a much greater geometric inhibition in HSA than it did in HbA (Table 8). Like aminoguanidine, metformin could inhibit the ring-opening of glucose or the nucleophilic attack in a protein pocket by "pushing" atoms too far away to react.

To observe if metformin's effect on ring-opening or nucleophilic attack was isomer specific, we parsed the data into α- and β-glucopyranose. Metformin (MF1 and MF2) were similar at inhibiting the base component of ring-opening in HbA and HSA for α- and β-glucopyranose. MF1 and MF2 were also equally capable of inhibiting the acid for α- and β-glucopyranose in HbA, but in HSA, metformin was better at inhibiting the acid when β-glucopyranose was present. Additionally, when α-glucopyranose is present, metformin is able to inhibit the nucleophile better in HSA, and when β-glucopyranose is present, metformin inhibits the nucleophile better in HbA. Although metformin will exert a geometric effect against either glucopyranose isomer equally well in HbA, in HSA this effect is stronger against β-glucopyranose. Like taurine, metformin seems to have slight isomeric preference for glucose in different mechanisms of inhibition.

Metformin (MF1 and MF2) also seem to have a slight difference in action base on which phosphate is present in the pocket. The biggest differences

are seen in HSA, where metformin inhibits the acid better, and has a stronger geometric inhibition in the presence of dibasic Pi than monobasic Pi. Also, in both HSA and HbA, metformin is much better at inhibiting the nucleophile in the presence of monobasic Pi.

Data from Tables 7 and 8 show that both forms of metformin are able to obtain the proper proximity and orientation within an intracellular (HbA) and extracellular (HSA) protein pocket to inhibit the ring-opening of glucose, and the nucleophilic attack leading to covalent modification of the protein. However, like with aminoguanidine, it may not be thermodynamically feasible for metformin to interact with protein residues to accept or donate a proton. Metformin has two pKa values (~3.1 and ~13.8). It's pKa_1 (~3.1) represents the dissociation of a proton from a metformin that has two protonated imines to form a mono-protonated metformin. The second pKa_2 (~13.8) represents the dissociation of a proton from the mono-protonated form (i.e. MF1 and MF2) to form a neutral metformin. This means that it is unlikely that MF1 or MF2 will accept a proton to form the di-protonated form, nor donate a proton to form the neutral form of metformin at physiological pH. Just like with aminoguanidine, it may be geometrically possible for metformin to inhibit glucose ring-opening or nucleophilic attack on glucose in a protein pocket (Fig. 6), but it may not be thermodynamically favorable. This could mean that both metformin and

aminoguanidine are unable to inhibit NEG in the noncovalent stage, whereas taurine may play a role against NEG in this temporal region.

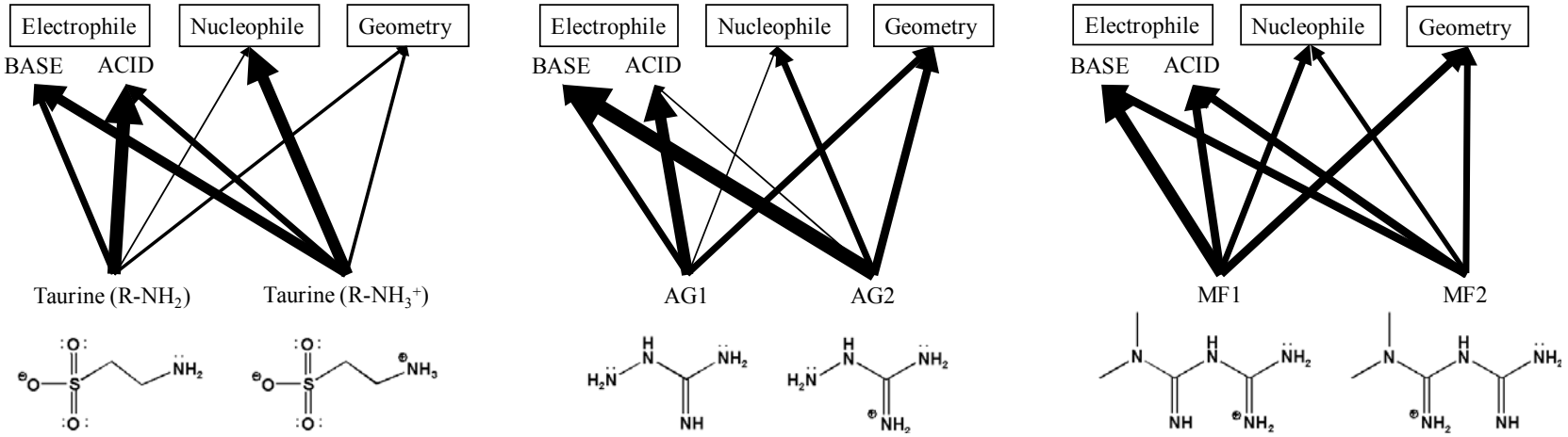


Figure 6. Comparison between the potential targets of inhibition for taurine, metformin and aminoguanidine within a protein pocket. The thickness of the arrows represents relative level of inhibition for that target and are averaged across HbA and HSA. Arrows are reflective of the chemical and geometric possibilities for each compound found using computational modeling.

3.4. Conclusion

When we assessed the action of metformin and aminoguanidine against NEG in the pre-binding temporal region, we found that, like taurine, neither of the drugs were able to bond with ring-open glucose in solution to reduce the effective glucose concentration. Additionally, we observed during our inflection time studies that the drugs had little effect on the ring-opening of glucose in solution. These findings indicate that the drugs likely do not interact with glucose in the pre-binding stage of NEG, however many studies have shown that these drugs can interact with dicarbonyls in solution (Hirsh et al., 1992; Solis-Calero et al., 2015; Kinsly, et al., 2016). Therefore, the drugs, like taurine, may function in the pre-binding stage of glycation to reduce the effective concentration of certain RCS, but not glucose specifically.

Our computational modeling studies revealed that both metformin and aminoguanidine are able to bind to glycation hotspots on HbA and HSA with favorable exothermicities. Not only could both drugs bind to these proteins favorably, but they could concomitantly bind in the presence of α- or β-glucopyranose. With aminoguanidine, however, the order of binding mattered, as AG2 was unable to bind favorably in a protein pocket as the second substrate. Once metformin or aminoguanidine was bound in a protein pocket with glucose and Pi, both drugs were able to achieve the geometry necessary to inhibit the electrophile formation (i.e. acid or base component of glucose ring-opening) and the nucleophilic attack. Compared to taurine and metformin, aminoguanidine was less able to inhibit the electrophile formation and nucleophilic attack. AG1 was

unable to inhibit the nucleophilic attack in HbA, and AG2 was unable to inhibit the acid component of ring-opening in HbA. However, both aminoguanidine and metformin were more capable than taurine to have a geometric inhibition in HbA and HSA, where the presence of the drugs pushed glucose too far away from the protein or Pi to ring-open glucopyranose and for the nucleophile to attack the ring-opened glucose electrophile.

Although metformin and aminoguanidine were both able to achieve the geometry in a protein pocket to inhibit the electrophile formation and nucleophilic attack, based on their high pKa's (~13.1 for metformin, and ~11.5 for aminoguanidine) it is unlikely that these drugs would be able to exist in the deprotonated form at physiological pH (7.4). This means that the extent of the drugs interference within a protein pocket may be limited to their geometric inhibition rather than chemical inhibition.

References

1. Besswenger, P., Ruggiero-Lopez, D. (2003) Metformin inhibition of glycation processes. *Diabetes Metab* 29, 95-103.
2. Bharatam, P. V., Iqbal, P., Malde, A., Tiwari, R. (2004) Electron delocalization in aminoguanidine: a computational study. *J Phys Chem A* 108, 10509–10517.
3. Brutsaert, Erika. Diabetes Mellitus (DM) - Endocrine and Metabolic Disorders. *Merck Manuals Professional Edition*. N.p., Feb. 2017. Web. 04 May 2017.
4. Delpierrre, G., Vertommen, D., Communi, D., Rider, M. H., and Schaftingen, E. V. (2004) Identification of fructosamine residues deglycated by fructosamine-3-kinase in human hemoglobin. *J Biol Chem* 279, 27613–27620.
5. Hernández, B., Pflüger, F., Kruglik, S. G., Cohen, R., Ghomi, M. (2015) Protonation–deprotonation and structural dynamics of antidiabetic drug metformin. *J Pharm Biomed Anal* 114, 42–48.
6. Hirsh, J., Petrakova, E., Feather, M. (1992) The reaction of some dicarbonyls sugars with aminoguanidine. *Carbohydr Res* 232, 125-130.
7. Kinsky, O. R., Hargraves, T. L., Anumol, T., Jacobsen, N. E., Dai, J., Snyder, S. A., Monks, T. J., Lau, S. S. (2016) Metformin scavenges methylgloxal to form a novel imidazolinone metabolite in humans. *Chem Res Toxicol* 29, 227-234.

8. Nilsson, B. O. (1999) Biological effects of aminoguanidine: an update. *Inflamm. Res* 48, 509–515.
9. Ruggiero-Lopez, D., Lecomte, M., Moinet, G., Patereau, G., Lagarde, M., Wiernsperger, N. (1999) Reaction of metformin with dicarbonyls compounds. Possible implications in the inhibition of advanced glycation end product formation. *Biochem Pharmacol* 58, 1765-1773.
10. Solis-Calero, C., Ortega-Castro, J., Frau, J, Munoz, F. (2015) Scavenger mechanism of methylgloxal by metformin. A dft study. *Theor Chem Acc* 134, 47-60.
11. Viollet, B., Guigas, B., Sanz Garcia, N., Leclerc, J., Foretz, M., Andreelli, F. (2012) Cellular and molecular mechanisms of metformin: an overview. *Clin Sci* 122, 253–270.
12. Wang, Y., Yu, H., Shi, X., Luo, Z., Lin, D., Huang, M. (2013) Stuctural mechanism of ring-opening reaction of glucose by human serum albumin. *J Biol Chem* 288, 15980-15987.
13. Waz, W. R., Van Liew, J. B., Feld, L. G. (1997) Nitric oxide-inhibitory effect of aminoguanidine on renal function in rats. *Kidney Blood Press Res* 20, 211–217.

Chapter 4. Unifying Conclusion

Currently, over 29 million Americans suffer from diabetes mellitus, and this number is only increasing. Type 2 diabetes mellitus is very expensive to treat: over 1.3 billion dollars are spent annually on treating those with diabetes mellitus, most of which are patients with type 2 diabetes (American Diabetes Association, 2012). If untreated, this disease can progress and cause further damage in the body by leading to the development of nephrology, vascular complications, blindness and even death (Brownlee et al., 1984; Rondeau and Bourdon, 2011). Understanding the mechanism by which persistent hyperglycemia (high blood glucose), as a result of diabetes, structurally modifies intra and extracellular proteins is of importance. The nonenzymatic glycation of proteins describes the process by which glucose permanently bond to and modifies a proteins structure. This process is of clinical importance because the amount of glycated hemoglobin, the most abundant protein found in red blood cells, can be measured to track the diabetic condition over long periods of time because the process of NEG occurs over weeks to months, whereas blood glucose levels can change within hours (Brownlee et al., 1984). The HbA1c test shows the amount of glycated hemoglobin. Although protein modification by glucose is a normal process in healthy individuals, HbA1c levels above 6.5% are characteristic of diabetes. The target HbA1c level for individuals with diabetes is at or below 7% (American Diabetes Association, 2012).

Understanding the mechanism by which glucose covalently binds to proteins is important because it reveals key players 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 and the nucleophilic attack on an electrophilic glucose by an amino acid residue (Bunn et al., 1978). 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 a host of advance glycation end-products (AGE) (Cho et al., 2007).

Although significant work has been done to understand the mechanism of the covalent stages of NEG, little has been presented about the initial, noncovalent stages. In the last few years, students in our lab have developed an increased understanding and application of the early noncovalent stages, highlighting the importance of inorganic phosphate toward generating the ring-opened glucose electrophile while bound in a protein pocket (Clark et al., 2013). This work has expanded the understanding of the noncovalent stage of NEG and has shown that it is more complex and diverse than previously thought, with multiple mechanisms, both including and excluding phosphate, leading to a covalently bound glucose (Fig. 1).

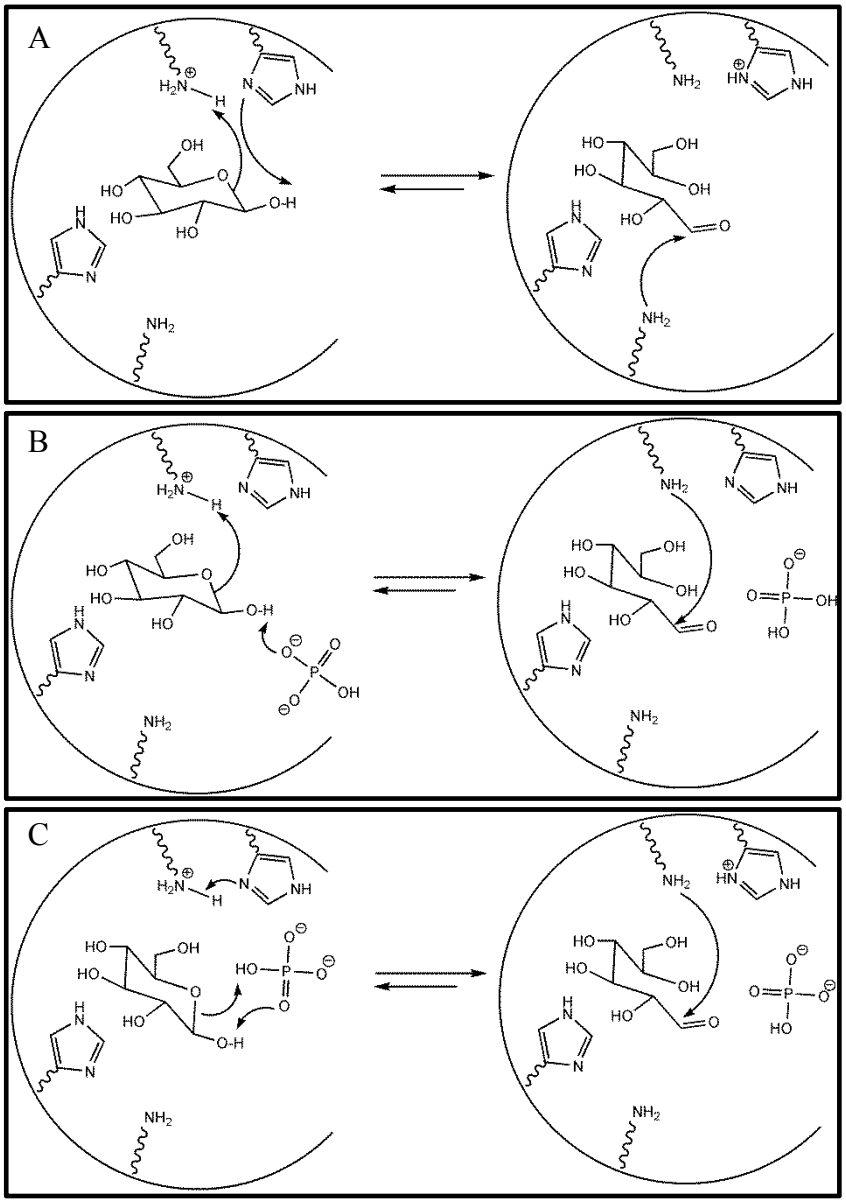


Figure 1. The three baseline mechanisms of glucose ring-opening in hemoglobin (HbA). Panel A represents the naked mechanism, where His143 acts as the base and Lys82 acts as the acid to ring-open β-glucopyranose. Val1 attacks a ring-opened glucopyranose as the nucleophile. Panel B represents the bimolecular mechanism, where dibasic Pi acts as the base and Lys82 acts as the acid to ring-open β-glucopyranose. Lys82 then attacks a ring-opened glucopyranose as the nucleophile. Panel C represents the bridge mechanism, where dibasic Pi acts as both the base and acid to ring-opened β-glucopyranose, while His143 prepares Lys82 as the nucleophile. Lys82 then attacks a ring-opened glucopyranose as the nucleophile.

Just as understanding how NEG progresses is of clinical importance, understanding how NEG can be inhibited is also significant. Certain pharmaceuticals have been developed to treat diabetes, thereby reducing the amount of glycated protein, both directly and indirectly. For example, metformin is the most common drug given to diabetics and is designed to target the liver to reduce the production of glucose (Besswenger and Lopez, 2003). This antihyperglycemic drug also reduces the rate of NEG, a concentration driven process, by reducing blood glucose levels (Bailey and Turner, 1996). Aminoguanidine, a pharmaceutical which is no longer given clinically, is able to inhibit NEG by interacting with 3-DG to prevent the formation of AGE in the later stages of NEG (Hirsch et al., 1992). Even reagents that are not given to treat diabetes may have an important role in limiting the progression of NEG. For example, taurine, a common additive to energy drinks, has been implicated as an antiglycation agent after it was shown to reduce the level of glycated hemoglobin formed when incubated with both normal and hyperglycemic levels of glucose in vitro (Nandhini et al., 2003). Interestingly, some species of birds have very high plasma concentrations of taurine (mM range) yet don't experience significant extracellular protein glycation (Zuck et al., 2017). In humans, the concentration of taurine varies greatly throughout the body. Typically, plasma levels are low (μM range) while intracellular taurine in cardiac muscle cells and in neurons in the CNS can be much greater (mM range) (Lourenco and Camilo, 2002). Contrary to birds, humans experience high levels of glycated protein in the plasma as well as intracellularly in red blood cells (Brownlee et al., 2005).

Additionally, the function of taurine as an antioxidant in the human body has been well characterized. As an antioxidant, taurine is able to covalently bind up reactive oxygen species (ROS) (Hosseini and Abdollahi, 2013). In the late stages of NEG, ROS are known to be produced from glycated proteins and contribute to the formation of AGE (Baynes et al., 1989). Taurine can bind up these ROS, and consequently has been implicated as a physiological antiglycation agent. However, little work has been done to understand if taurine can play a direct role in inhibiting NEG early in the mechanistic scheme (i.e., the noncovalent stages). This temporal range of the NEG scheme may be a likely target for taurine based on the finding that taurine can bind to glucose and other aldehydes to form complexes and it does this better than certain amino acids like glycine and alanine (Ogasawara et al., 1993).

Based on these findings with taurine, we predicted that taurine plays a mechanistic role in the early, noncovalent stages of NEG to limit the progression of glycation. This thesis follows a question driven approach to assess if taurine can interfere at specific steps of the pre-binding and early, noncovalent binding of glucose to both human hemoglobin (HbA) and human serum albumin (HSA). Using model reactions in D_2O, followed by characterization with ^{1}HNMR, we assess the pre-binding stage of NEG. This allowed us to model reactions that may take place in aqueous solution, such as in the blood plasma or cytosol of cells. In the pre-binding stage, we ask: *can taurine covalently bond with glucose in aqueous solution to effectively reduce glucose concentration and thereby reduce*

the extent of NEG?; can taurine covalently bond to permanent RCS glyoxal in aqueous solution to effectively reduce glyoxal concentration and thereby reduce the extent of NECPM?; and *can taurine inhibit glucose ring-opening in solution, thus RCS formation?* We also implement computational modeling using Molecular Operating Environment (MOE) to assess the taurine's interaction in the noncovalent stage of NEG. In regards to the noncovalent stage we ask: *can taurine theoretically compete with glucose to bind in a protein pocket?; can taurine theoretically prevent glucose ring-opening in a protein pocket?;* and *can taurine theoretically prevent the protein nucleophile from attacking a ring-opened glucose in a protein pocket*? MOE allows us to determine the energy of substrates binding to protein as well as to assess the geometry and through-space distances of substrates and the protein.

The overall finding of this thesis is that taurine plays multiple mechanistic roles at different temporal regions to inhibit the progression of NEG. Our experiments in the pre-binding stage revealed that taurine will not reduce the effective concentration of glucose by bonding with the ring-opened form in solutions. However, taurine will bind up glyoxal by forming both a mono-imine and di-imine product. An equilibrium exists between the mono- and di-imine products after 21 days, suggesting that taurine may not permanently scavenge glyoxal. However, the interaction between taurine and glyoxal may be pH dependent. When taurine and glyoxal are reacted at pH 6, taurine is overwhelming in the ammonium ion form, due to the pKa of the amine being ~ 9.06. As a result,

the ammonium ion form of taurine is not able to react as a nucleophile and bind glyoxal. The reaction between glyoxal and taurine can be enhanced if additional reagents are around, such as dibasic Pi, that can deprotonate the ammonium ion of taurine and make more nucleophilic amine available to react with glyoxal.

Additionally, taurine will not prevent glucose from ring-opening in solution, but rather enhance it by a factor of 2. One of the requirements for glucose ring-opening is the presence of an acid to protonate the hemiacetal oxygen and a base to deprotonate the anomeric hydroxyl group. Taurine in its ammonium ion form can act as an acid, while the amine form can act as a base. Therefore, when two molecules of taurine are present in aqueous solution with one molecule of glucose, ring-opening can occur. Although this reaction in the plasma may not lead to enhanced glycation because glucose only exists in the ring-opened form transiently and binds to the protein in a ring-closed form, it does lead us to wonder if taurine can have a different role toward ring-opening glucose once inside a protein pocket, thus limiting the early, noncovalent stage of NEG?

Our computational experiments with MOE, targeted at the noncovalent stage of NEG, initially revealed that both forms of taurine ($R\text{-}NH_2$ and $R\text{-}NH_3^+$) can bind in a protein pocket with either glucopyranose isomer. This shows that taurine does not compete with glucose, but rather will exist concomitantly in many protein pockets (both on HbA and HSA) with glucose. Additionally, monobasic and dibasic Pi will bind favorable in the same pocket that hold a

taurine and glucose molecule, setting the stage for the next two questions we pursued.

Using MOE, we assessed if taurine, inside the Val1/Lys82 pocket of HbA and the Lys195/199 pocket of HSA, will prevent glucose from ring-opening. By starting with three baseline mechanisms of ring-opening (naked, bimolecular and bridge) we hand placed taurine in select positions, then energy minimized to see if taurine will stay in the pocket with the correct proximity and orientation to interfere with the mechanism of glucopyranose ring-opening. This data revealed that taurine, in its both its amine and ammonium ion form can inhibit the ring-opening of glucose by protonating the base or deprotonating the acid. When the amine form of taurine inhibits the base, taurine must first be protonated. Similarly, when the ammonium ion of taurine inhibits the acid, taurine must first be deprotonated. We also used MOE to assess if taurine will prevent the protein nucleophile from attacking a ring-opened glucose inside a protein pocket. Once again, we found that both forms of taurine are able to inhibit the nucleophile from attacking the ring-opened glucose (however, the amine form of taurine must first be protonated inside the protein pocket).

It is noteworthy that we did observe some slight differences in taurine's mechanistic ability to inhibit ring-opening of glucose or nucleophilic attack in different proteins (HbA and HSA). These differences may indicate that the actions of taurine are diverse and depend on the protein environment in which taurine resides, including which other reagents (i.e., glucose isomer or phosphate) are

bound. Because the effectiveness of taurine may differ in intracellular (HbA) versus extracellular (HSA) protein pockets, we propose taurine may also be a factor that accounts, in part, for the glycation gap. The glycation gap refers to the difference in glycated protein when measured via HbA1c and fructosamine. Ideally, HbA1c and fructosamine would indicate the same level of glycation in an individual, however this is not always the case (Gould et al., 1997). Taurine's role against NEG and the differences we observed in HbA and HSA could explain a portion of the glycation gap, although further studies are needed in order to test this postulate.

By comparing the mechanism(s) of action of taurine within a protein pocket with metformin and aminoguanidine, it is clear that the pharmaceuticals are slightly better at achieving the necessary geometry within HbA and HSA to participate in acid/base chemistry. This means that the drugs, like taurine, may interfere with glucose-ring-opening and the nucleophilic attack. However, these drugs do not initially bind to HbA or HSA as favorably as taurine. Also, if we consider the pKa's of metformin (~13.38, and 3.1) and aminoguanidine (~11.5), it is unlikely that these drugs will be able to lose or gain a proton from amino acid residues or phosphate in a protein pocket. Taurine, which has a pKa of ~9.06, is thermodynamically more likely to participate in acid/base chemistry with lysine amino acid residues and phosphate in a protein pocket than are metformin or aminoguanidine. Therefore, although the drugs and taurine all may achieve the proper geometry to inhibit glucose ring-opening or nucleophilic attack within

HbA and HSA, taurine may be far more reactive than the drugs at inhibiting NEG in this temporal region due to its ability to lose and gain protons.

Overall, this work contributes toward the understanding of NEG from the perspective of inhibition. While much of the work with taurine focuses on the later, covalent stages of NEG, this work fills in some of the gaps regarding taurine's role in the initial, noncovalent stage. However, this work is far from finished. While computational modeling with MOE allows us peak into a protein pocket, it has its limitations. One of the main assumptions of this work is that taurine, glucose and Pi will remain in a protein pocket for an equal, extended period of time. Future studies should focus on the retention time of taurine within a protein pocket. Because the process of NEG, and the inhibition of NEG is a concentration driven process, and that taurine levels are much lower than glucose levels in the plasma and red blood cells of humans, the retention time of taurine within HbA and HSA is an important consideration. If taurine is able to remain in a pocket for much longer than glucose and Pi, the concentration differential may be less important and taurine may have a greater impact against NEG within a protein pocket. However, it is important to remember that the overall impact of taurine against glycated hemoglobin is moderate (~10% reduction) (Nandhini et al., 2004).

Additionally, our work focuses on computational modeling to establish potential interactions within the protein pocket. In the future, it would be worthwhile to perform incubations with taurine, glucose or glucose derivatives

and Pi to determine the overall effect on glycated proteins. In previous incubations with taurine, glucose and protein, one aspect that is often not controlled for is the concentration of Pi. Most studies use very high concentrations of Pi (~100 mM). Our work shows that taurine has a direct role in a protein pocket against Pi from acting as a facilitating reagent to ring-open glucose. Because these reactions are concentration dependent, it is important to consider the concentration of Pi being used during incubations.

Finally, our work with taurine is limited to the interactions between taurine's amine or ammonium ion and other molecules. It may be of interest to determine if the sulfonate end of taurine interacts with proteins or effector reagents in such a way that NEG is limited. Studies of this nature could be extended using computational modeling as well as model reactions in solution.

References

1. American Diabetes Association. (2017) Classification and Diagnosis of Diabetes, Diabetes Care 40 (Suppl. 1), S11-S24.
2. Bailey, C. J., Turner, R. C. (1996) Drug therapy: metformin. *N Engl J Med* 334, 574-579.
3. Baynes, J. W., Watkins, N. G., Fisher, C. I. (1989) The Amadori product on protein: structure and reactions. *Prog Clin Biol* 304, 43-67.
4. Besswenger, P., Ruggiero-Lopez, D. (2003) Metformin inhibition of glycation processes. *Diabetes Metab* 29, 95-103.
5. Brownlee, M., Vlassara, H., Cerami, A. (1984) Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med* 101, 527-37.
6. Brownlee, M. (2005) The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54, 1615-1625.
7. Bunn, H. F., Haney, D. N., Gabbay, K. H., Gallop, P. M. (1975) Further identification of the nature and linkage of the carbohydrate in hemoglobin A1c. *Biochem Biophys Res Comm* 67, 103-109.
8. Cho, S. J., Roman, G, Yeboah, F. (2007) The road to advanced glycation end products: a mechanistic perspective. *Curr Med Chem* 14, 1653-1671.
9. Clark, S. L., Santin, A. E., Bryant, P. A., Holman, R. W., Rodnick, K. J. (2013) The initial noncovalent binding of glucose to human hemoglobin in nonenzymatic glycation, *Glycobiol* 23, 1250-1259.

10. Hirsh, J., Petrakova, E., Feather, M. (1992) The reaction of some dicarbonyls sugars with aminoguanidine. *Carbohydr. Res* 232, 125-130.
11. Hosseini, A., Abdollahi, M. (2013) Diabetic neuropathy and oxidative stress: therapeutic perspectives. *Oxid Med Cell Longev* 10.1155/2013/168039.
12. Lourenco, R. and Camilo, M. E. (2002) Taurine: a conditionally essential amino acid in human? An overview of health and disease. *Nutr Hosp* 17, 262-270.
13. Nandhini, T. A., Anuradha, C. V. (2003) Inhibition of lipid peroxidation, protein glycation and elevation of membrane ion pump activity by taurine in RBC exposed to high glucose. *Clin Chim Ac* 336, 129-135.
14. Rondeau, P., Bourdon, E. (2011) The glycation of albumin: structural and functional impacts. *Biochimie* 93, 645–658.