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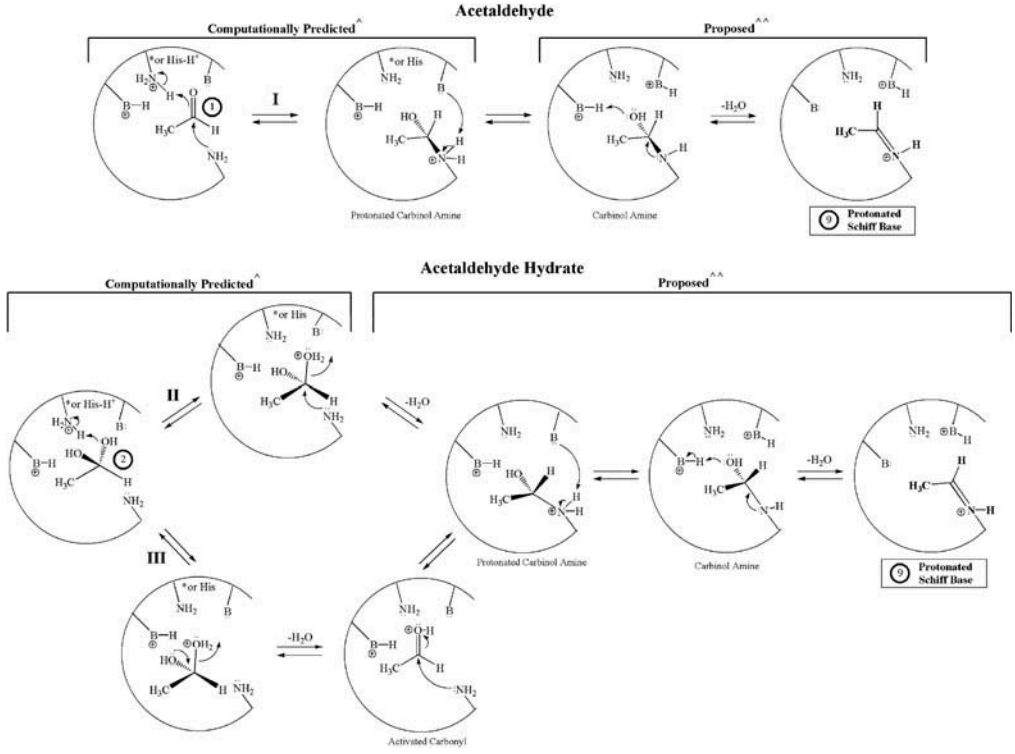

BIOCHEMISTRY & STRUCTURAL BIOLOGY | RESEARCH ARTICLE

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New evidence for the diversity of mechanisms and protonated Schiff bases formed in the non-enzymatic covalent protein modification (NECPM) of HbA by the hydrate and aldehydic forms of acetaldehyde and glyceraldehyde

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Abstract: Acetaldehyde is a physiological species existing in blood. Glyceraldehyde is a commonly used surrogate for glucose in studies of nonenzymatic glycation. Both species exist in dynamic equilibrium between two forms, an aldehyde and a hydrate. Nonenzymatic covalent protein modification (NECPM) is a process whereby a protein is covalently modified by a non-glucose species. The purpose here was to elucidate the NECPM mechanism(s) for acetaldehyde and glyceraldehyde with human hemoglobin (HbA). For the first time, both aldehydic and hydrate forms of acetaldehyde and glyceraldehyde were considered. Computations and model reactions followed by ^{1}H NMR were employed. Results demonstrated that the aldehyde and hydrate forms of acetaldehyde bind and covalently-modify Val1 of HbA via different chemical mechanisms, yet generated an identical protonated Schiff base (PSB). The aldehyde and hydrate of glyceraldehyde also covalently

ABOUT THE AUTHOR

Justin Lewis Our interdisciplinary research group consists of a physiologist (KJR) and an organic chemist (RWH) at Idaho State University. Our research group also typically consists of students from the Biological Sciences and Chemistry. Our research focus is to better understand the bioorganic mechanisms associated with the nonenzymatic glycation of proteins. Our primary tools include computational methodology based upon crystal structures and model reactions followed by ^{31}P and ^{1}H nuclear magnetic resonance spectroscopy (NMR). Our goals are to predict what physiological reagents and chemical effectors might be involved in the early stages of the nonenzymatic covalent protein modification (NECPM) and to elucidate the mechanisms for the corresponding reactions. This work focused on elucidating the NECPM mechanism(s) for acetaldehyde and glyceraldehyde with human hemoglobin (HbA). The findings suggest that the generated protonated Schiff base structures may be central to pathophysiological outcomes because they determine the structure of the stable covalent protein adducts formed.

PUBLIC INTEREST STATEMENT

Alcohol toxicity, defined as severe detrimental physiological implications from excessive alcohol consumption, is an increasingly important issue in our culture. Acetaldehyde is a molecule that forms from the metabolism of ingested ethanol and has been shown to modify certain proteins and cause deleterious protein function. The purpose of this study was for the first time to elucidate nonenzymatic covalent protein modification (NECPM) mechanisms for *how* acetaldehyde and structurally-related glyceraldehyde interact with a model protein, human hemoglobin (HbA). Key structures identified in the acetaldehyde and glyceraldehyde modification of HbA were protonated Schiff bases (PSB). Structure of the PSB may be central to pathophysiological outcomes because they ultimately determine the structure of the stable modified protein product formed. Moreover, a comparison of the PSB for acetaldehyde with those for glyceraldehyde provides a potential explanation for why acetaldehyde is connected to alcohol toxicity and not connected to diabetic complications whereas glyceraldehyde *is* connected to diabetic complications but not to alcohol toxicity.

modified Val1 via mechanisms distinct from one another, yet generated an identical PSB. It is noteworthy that the PSB from acetaldehyde and glyceraldehyde were different structures. The PSB from acetaldehyde is proposed to proceed to covalent adducts that have been implicated in alcohol toxicity. Conversely, the PSB generated from glyceraldehyde can form an Amadori which has been implicated in diabetic complications. Thus, the PSB structure generated from acetaldehyde versus glyceraldehyde may be central to pathophysiological outcomes because it determines the structure of the stable covalent adduct formed.

Subjects: Biochemistry; Organic Chemistry; Computational and Theoretical Chemistry

Keywords: acetaldehyde; hemoglobin; glyceraldehyde; Schiff base; nonenzymatic covalent protein modification (NECPM)

1. Introduction

Acetaldehyde exists in a dynamic equilibrium between an aldehydic form, which is formally a reactive carbonyl species (RCS), and a hydrate form which is not (Figure 1, structures **1** and **2**; and, for a mechanistic perspective, Supplementary Figure 1). The hydrate **2** is the predominant form at 65% (or more) in aqueous media, depending upon pH. These species are physiologically relevant and exist in erythrocytes (Dipadova, Alderman, & Lieber, 1986) and in the plasma at normal concentrations of ca. 10–20 μM and at 50 μM in chronic alcoholics (Korsten, Matsuzaki, Feinman, & Leiber, 1975).

A primary source of circulating acetaldehyde in certain humans can be the metabolism of ingested ethanol. Additional exogenous sources are extremely diverse and include cigarettes, motor vehicle exhaust, wood burning sources, food additives, cleaning agents, and the production process for many commercial chemicals (O'Brien, Siraki, & Shangari, 2005). Acetaldehyde is also produced endogenously in intermediary metabolism (O'Brien et al., 2005) and by colonic bacteria (Vispää, Tillonen, & Salaspuro, 2002).

Acetaldehyde has been shown to covalently-modify certain proteins, including HbA (Gaines, Salhany, Tuma, & Sorrell, 1977; Roede, Stewart, & Petersen, 2010; Tuma, Hoffman, & Sorrell, 1991; Tuma, Jennett, & Sorrell, 1987; Tuma, Newman, Donahue, & Sorrell, 1987). Moreover, multiple proteins that have undergone covalent modification by acetaldehyde have exhibited altered function (Mauch, Donohue, Zetterman, Sorrell, & Tuma, 1986), particularly lysine-dependent enzymes (Tuma et al., 1987). In this earlier work, the diversity of structures of the initially bound species and the corresponding nature and magnitude of reactivity were not fully developed. Based upon our literature survey, the differential binding and follow-up chemistry between the aldehydic form of acetaldehyde vs. the hydrate of acetaldehyde has not been considered. Because the concentration of the hydrate **2** in aqueous solution relative to that of the aldehyde **1** is high (65% hydrate, unbuffered at RT), it critically matters whether the hydrate can bind and whether, once bound, there are available mechanisms for covalent modification of HbA. For this work, binding refers to non-covalent, reversible interactions (intermolecular forces of attraction) between a substrate and amino acid residues of a protein within a protein pocket. Bonding refers to the formation of a stable covalent bond between a bound substrate and the amino acid residue of a protein. Such bonding is typically irreversible but may be reversible.

The prevailing mechanistic scheme for RCS nonenzymatic covalent protein modification is referred to as NECPM (Figure 2, Rodnick, Holman, Ropski, Huang, & Swislocki, 2017).

Specifically, an amine NH_2 group of an amino acid residue (e.g., a lysine R group or an N-terminal amino acid) can act as a nucleophile attacking a bound substrate that serves as

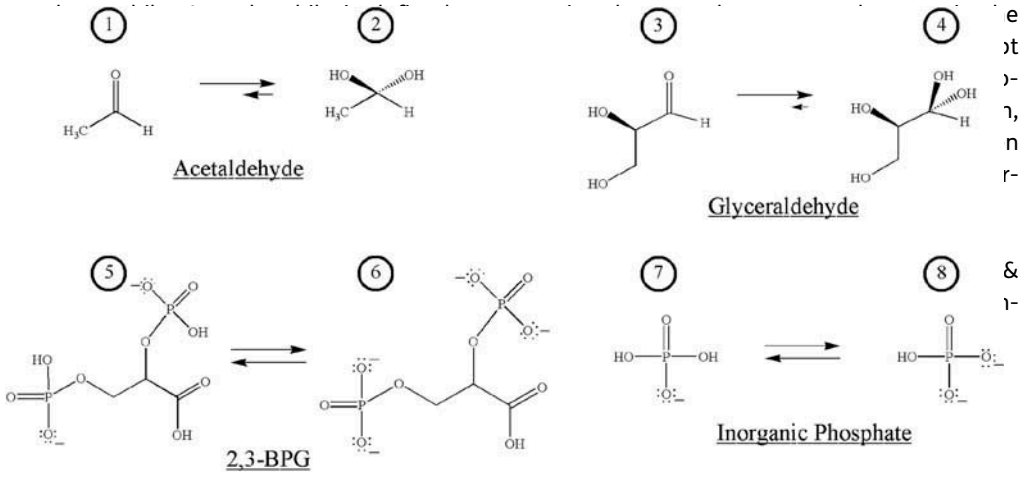

Figure 1. Structures of salient species in the assessment of the nonenzymatic covalent protein modification of HbA by acetaldehyde or glyceraldehyde, with potential effector reagents 2,3-bisphosphoglycerate (2,3-BPG) and inorganic phosphate (Pi).

Structures **1** and **2** represent the aldehydic and hydrate forms of acetaldehyde. The relative length of the equilibrium arrows indicate the relative equilibrium concentrations of each form in aqueous solution (33% aldehyde, 66% hydrate). Structures **3** and **4** represent the aldehydic and hydrate forms of glyceraldehyde that exist in equilibrium (4% aldehyde, 96% hydrate). Structures **5** and **6** represent di-anionic and tetra-anionic 2,3-bisphosphoglycerate (2,3-BPG) that exist in equilibrium based upon pH. Structures **7** and **8** represent two forms of inorganic phosphate: mono-anionic Pi, **7**, and di-anionic Pi, **8**, both of which exist under physiological conditions.

covalent binding takes place. However, the following mechanistic details regarding the binding of acetaldehyde are yet unknown: a) which species bind(s), the aldehyde **1**, the hydrate **2**, or both, b) the binding exothermicity of the(se) binding species relative to one another and relative to that for the glucopyranoses (Clark, Santin, Bryant, Holman, & Rodnick, 2013), c) what might be the role of inorganic phosphate (Pi) (Smith et al., 2018), and d) what is/are the structures of the intermediates involved. For comparative purposes, glyceraldehyde is also of interest because glyceraldehyde is structurally similar to acetaldehyde. Both species are physiological electrophiles and both exist as a dynamic equilibrium between an aldehydic RCS form and a non-RCS hydrate (for glyceraldehyde structures **3** and **4**, for acetaldehyde structures **1** and **2**; Figure 1). For glyceraldehyde, the hydrate **4** exists at ca. 90+% of total equilibrium mixture in aqueous media at physiological pH. Like acetaldehyde, glyceraldehyde is known to bind to HbA (Nacharaju & Acharya, 1992). Like acetaldehyde, previous work on glyceraldehyde does not detail the potential mechanistic differences between the aldehydic and hydrate forms.

Several central questions prompted this investigation. Do both the aldehydes and their hydrates bind HbA? If so, do the intermediates and mechanism(s) for the aldehyde differ from those for the hydrate? Do they both proceed in NECPM and, if so, is progression in NECPM via similar intermediates and mechanistic pathways? Finally, how does the NECPM process for the acetaldehyde manifold (**1** and **2**) compare to that for glyceraldehyde (**3** and **4**)? Can the physiological differences between acetaldehyde and glyceraldehyde potentially be better understood in view of the NECPM mechanistic differences between **1** and **2** as a manifold versus that for **3** and **4**?

To develop a more complete answer to these questions, a computational assessment was conducted for the binding characteristics and exothermicities of both forms of acetaldehyde (structures **1** and **2** in independent computations) and both forms of glyceraldehyde (structures **3** and **4** in independent computations) in the HbA_{1c} pocket. Specific emphasis was placed on evaluating the energy-minimized geometries of bound **1**–**4** (in independent computations) in the pocket to discern potential NECPM mechanisms that can theoretically lead to covalent modification. Given that the HbA_{1c} pocket often possesses either bound di-anionic or tetra-anionic 2,3-BPG (2,3-bisphosphoglycerate, Figure 1, structures **5** and **6**) or mono-anionic or di-anionic Pi (inorganic phosphate, Figure 1, structures **7** and **8**) the concomitant binding of these species with **1**–**4** were also evaluated. Concomitant binding was defined as two species reversibly undergoing noncovalent binding in the same protein pocket at the same time. Further, model reactions between lysine (as a surrogate for the nucleophilic amino acid residues on HbA) and the manifold of **1** and **2** in aqueous solution followed by ^{1}H nuclear magnetic resonance spectroscopy (NMR)

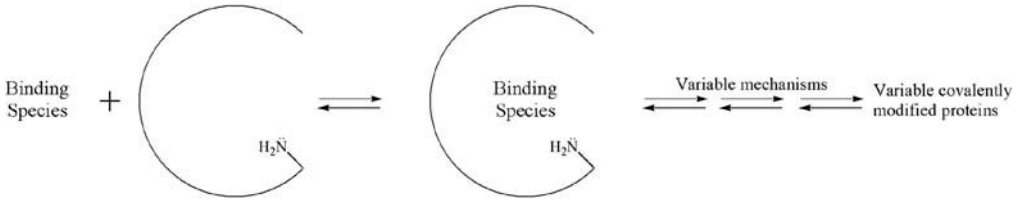

Figure 2. Generic scheme for the nonenzymatic covalent protein modification (NECPM) of a protein by a non-glucose binding species.

The semi-circle represents a generic protein pocket in which a non-glucose species binds. Here the scheme represents a protein pocket that includes the N-terminus. The NECPM process can proceed in internal protein pockets as well. Once bound in the pocket, the binding species, serving as an electrophile, can undergo nucleophilic attack by a lone pair on an amine group of an amino acid residue (shown here as an N-terminus amino acid) which ultimately results in covalent attachment to the protein via variable mechanisms.

were utilized to probe whether reactions do, in fact, occur and whether there is differential reactivity between **1** and **2**.

The goal of these experiments was to, for the first time, discern the structures of the initial covalently modified species formed and the NECPM mechanistic pathway(s) involved for **1–4**. Finally, a comparison of the NECPM mechanisms for the acetaldehyde manifold (**1** and **2**) with those for the glyceraldehyde manifold (**3** and **4**) was made to posit a potential explanation for why acetaldehyde is connected to alcohol toxicity and not connected to diabetic complications whereas glyceraldehyde *is* connected to diabetic complications but not to alcohol toxicity.

2. Results

In each of the 5 environments reflected in Table 1, aldehydic (**1**) and hydrate (**2**) forms of acetaldehyde bound in the HbA_{1c} pocket with similar exothermicities (−3.2 to −2.7 kcal/mol and −3.4 to −2.8 kcal/mol, respectively). Although the exothermicity of binding was nearly equal between the two species, the probability of meeting geometric requirements (a 5Å or less distance

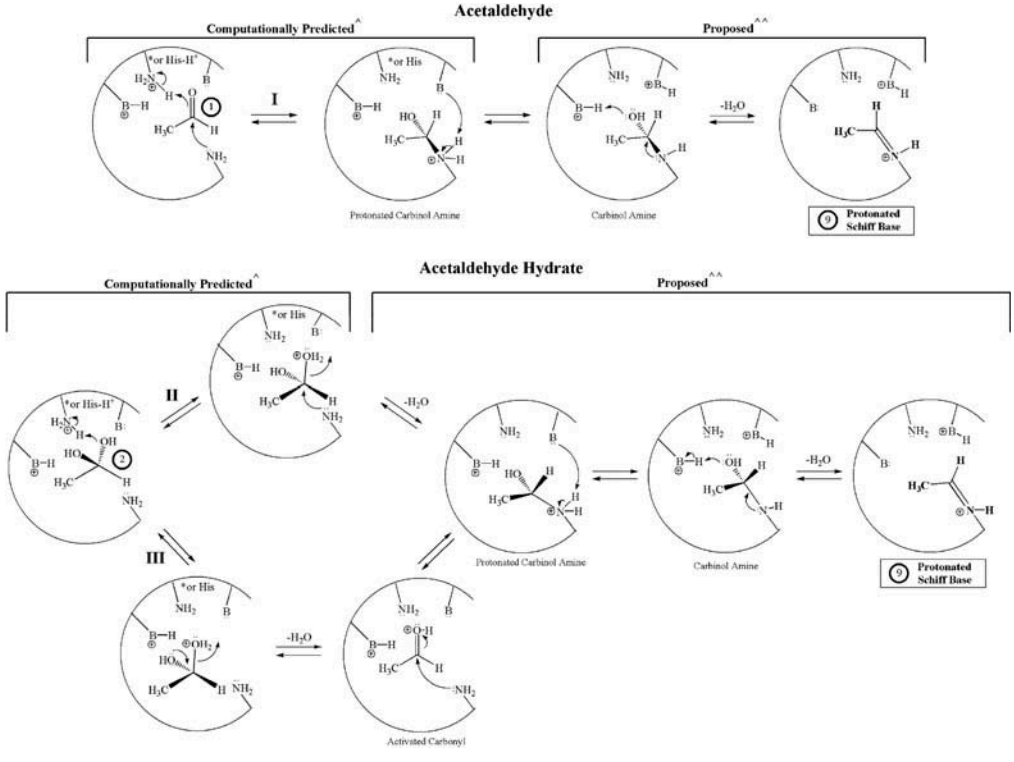

Figure 3. Potential NECPM mechanisms for covalent modification of Val1 of HbA that are geometrically possible for acetaldehyde *1* (Mechanism *I*) and the acetaldehyde hydrate *2* (Mechanisms *II* and *III*) based upon molecular modeling with MOE.[1]

These mechanisms were predicted to be geometrically able to proceed based upon energetic minima calculated from MOE. In the top box, mechanism **I**, species **1** binds and then the nucleophilic lone pair on the nitrogen of a terminal valine amino acid residue attacks the carbonyl carbon of **1**. This causes the π-bond of the carbonyl to break, enhancing the basicity of the carbonyl oxygen, which then abstracts a proton from a nearby acidic amino acid residue (Lys NH_3^+ or a protonated histidine). This forms the protonated carbinol amine. A basic amino acid residue can then deprotonate the amine, generating the carbinol amine. The lone pair on the nitrogen can then form a π-bond, as the alcohol group (hydroxyl group) abstracts

between reactive nucleophiles with electrophiles and acids with bases) for covalent modification of Val1 varied as a function of amino acid residue charge state in the HbA_{1c} pocket (Table 1, columns 4, 5). This was the case because the charge state of the amino acid residues dictated whether it is an acid, a base, an H-bond donor, or an H-bond acceptor. Thus, the potential mechanistic role of the amino acid residues involved in the mechanism also changed with environment.

a proton from a nearby acidic amino acid residue and departs as water. This forms the protonated Schiff base, **9**. Mechanisms **II** and **III** begin with the hydrate form of acetaldehyde, **2**, bound in the protein pocket. In Mechanism **II**, the oxygen of an alcohol group on **2** abstracts a proton from a nearby acidic amino acid residue. The lone pair on the nitrogen of a terminal valine then attacks as a nucleophile, causing water to depart as a leaving group. This generates the protonated carbinol amine. In Mechanism **III**, the oxygen of an alcohol group on **2** abstracts a proton from a nearby acidic amino acid residue. The lone pair on the oxygen of the remaining alcohol group of **2** forms a π-bond to carbon. This facilitates the loss of water as a leaving group and generates an activated carbonyl species. The carbonyl carbon is then attacked by a nucleophilic lone pair on the nitrogen of a terminal valine to form the protonated carbinol amine. Both Mechanisms **II** and **III** involve an identical protonated carbinol amine that leads to the formation of the same protonated Schiff base, **9**.

The computational results of the concomitant binding of **1**, the aldehydic form of acetaldehyde, in the HbA_{1c} pocket with 2,3-BPG (structures **5** and/or **6**) or inorganic phosphate (structures **7** and/or **8**) are also provided in Table 1. The salient features for concomitant binding of 2,3-BPG with acetaldehyde in the HbA_{1c} pocket were as follows: a) binding exothermicities were generally comparable to single species binding; b) di-anionic 2,3-BPG, **5**, facilitated covalent modification of Val1 more so than did tetra-anionic 2,3-BPG, **6**; c) the presence of 2,3-BPG diminished the geometric probability of acetaldehyde covalent modification of Val1 relative to the single-molecule binding and resulting modification by acetaldehyde alone (in the absence of 2,3-BPG). The salient features of the concomitant binding of Pi with acetaldehyde in the HbA_{1c} pocket were similar to those for the noncovalent binding of acetaldehyde in the HbA_{1c} pocket concomitant with 2,3-BPG, with two notable exceptions. There were no geometries for concomitant binding that can result in the covalent modification of Val1, such that the presence of mono- or di-anionic Pi would diminish the formation of HbA_{1c} relative to the binding of acetaldehyde alone.

When **2** underwent concomitant binding in the HbA1c pocket with 2,3-BPG the binding exothermicity was comparable to single species binding (Table 1). Thus, the exothermicity of the first binding event (binding of 2,3-BPG, **5** or **6**, in the HbA1c pocket) was comparable to the second event (binding of acetaldehyde, **1** or **2**, to the HbA1c pocket that already had a bound **5** or **6**). The aldehydic form of acetaldehyde **1** bound concomitantly with either form of 2,3-BPG or Pi in the HbA_{1c} pocket (Table 1). Acetaldehyde **1** achieved suitable geometry upon concomitant binding with 2,3-BPG to covalently modify Val1 (See Table 1, row 3, column 4). Suitable geometry for Val1 modification was not achieved when **1** bound in the HbA1c with Pi. Acetaldehyde hydrate **2** also bound concomitantly with either form of 2,3-BPG or Pi and achieved proper geometry for covalent modification of Val 1 in every case (Table 1, row 4, column 4). When 1 bound with either form of Pi, covalent modification of Val1 did not occur. Based on geometric arguments alone and single species binding, **1** was twice as likely to covalently modify Val1 compared with **2** (15.3% of binding events vs. 7.3%, Table 1, rows 1 and 2, column 4). In contrast, **1** was much less likely to covalently modify Val1 than was **2** in the presence of either 2,3-BPG or Pi (3.5% vs. 14.2%, Table 1, rows 3 and 4, column 4).

While computational modeling can be used to predict whether the proper geometry for reaction is possible, it does not confirm that a reaction can, in fact, occur. Evidence that reaction occurs between acetaldehyde and lysine (acting as a surrogate for the N-terminal valine residue in the HbA_{1c} pocket) in solution was provided by the disappearance of ^{1}H NMR spectroscopy signals associated with **1** (methyl group singlet at 2.05 ppm) and **2** (methyl group doublet at 1.1 ppm) and the appearance of new signals. The methyl group singlet associated with **1** was found to disappear more rapidly with time than the methyl group doublet associated with **2**. At 8 min the ratio of unreacted **1**-to-**2** was 41:59; at 165 min it was 35:65, indicating that the **1** and **2** reactions with lysine were faster than re-equilibration between **1** and **2** and that **1** reacts faster with lysine than does **2**. This emergence of new signals with time provided additional evidence that reaction occurs leading to protonated Schiff base, Schiff base and an enamine (that is produced from the protonated Schiff base and is consistent with what is typically observed from imine (Schiff base)/enamine chemistry). Specifically, the salient product signals were the methyl group of the protonated Schiff base at 1.8 ppm, the amine doublet at 9.2 ppm, and three alkene doublets of doublets between 6 and 7 ppm for the enamine (Table 2).

The composite results of the data from computations associated with glyceraldehyde (**3** and **4**) in the $HbA1_{1c}$ pocket (Table 2) led to the following salient points: a) the binding exothermicities for both the aldehydic and the hydrate forms of glyceraldehyde were similar to each other; b) the binding exothermicities of all four single-species bindings (**1**–**4**) were within a similar range, though glyceraldehyde bound with slightly greater exothermicity than does acetaldehyde; c) the geometric probability for covalent protein modification by **3** and **4** at Val1 was favored when the nearby Lys82 and His2 were protonated; and d) the probability of suitable geometry for covalent protein modification by **3** and **4** was comparable to that for **1** and **2**.

Table 1. Computational modeling of acetaldehyde (aldehyde *1* and hydrate *2*) binding in the HbA_{1c} pocket of human hemoglobin as both single-species binding and concomitant binding with potential effector reagents, 2,3-BPG (*5* or *6*) or Pi (*7* or *8*)

Bound Species	Second Species Bound	Environment based upon charge state of Amino Acid Residues	Binding Energies (kcal/mol)	% of Binding Events with Geometry Suitable for Reaction to Modify Val1	Identity of the Acid involved in Facilitating Covalent Modification
Acetaldehyde 1	–	1	−3.2 to −2.7	3.3%	Lys82
	–	2	−3.2 to −2.7	26.6%	Lys8 or Lys82 or His2
	–	3	−3.4 to −2.8	10.0%	Lys8 or Lys82
	–	4	−3.2 to −2.7	23.3%	Lys8 or Lys82 or His2
	–	5	−3.0 to −2.5	13.3%	Lys8 or Lys82
		AVG		**15.3%**	
Acetaldehyde Hydrate 2	–	1	−3.4 to −2.8	16.6%	Lys82 or Lys8
	–	2	−3.5 to −2.9	6.6%	Lys82 or Lys8 or His2
	–	3	−3.6 to −2.8	6.6%	Lys82 or Lys8
	–	4	−3.7 to −3.0	6.6%	Lys82 or His2
	–	5	−3.4 to −2.9	0.0%	N/A
		AVG		**7.3%**	
Acetaldehyde 1	2,3-BPG^{2-}	1	−3.0 to −2.6	7.4%	Lys8 or 2,3-BPG^{2-}
	2,3-BPG^{4-}	1	−3.3 to −2.7	6.7%	Lys82 or 2,3-BPG^{4-*}
	Pi^{-}	1	−3.2 to −2.7	0.0%	N/A
	Pi^{2-}	1	−3.0 to −2.7	0.0%	N/A
		AVG		**3.5%**	
Acetaldehyde Hydrate 2	2,3-BPG^{2-}	1	−3.5 to −2.8	26.6%	2,3-BPG^{2-} or Lys82 or Lys8
	2,3-BPG^{4-}	1	−3.8 to −2.9	16.6%	Lys8 or 2,3-BPG^{4-*} or Lys82
	Pi^{-}	1	−3.5 to −2.8	6.6%	Lys82 or Lys8
	Pi^{2-}	1	−3.5 to −2.9	6.6%	Lys82
		AVG		**14.2%**	

Table 1 contains data from the computational modeling of acetaldehyde (**1**) and its hydrate (**2**) binding in the HbA_{1c} pocket of human hemoglobin, both as single-species binding and with concomitant binding with either form of 2,3-BPG (**5** or **6**) or Pi (**7** or **8**). Column 1 describes which form of acetaldehyde (**1** or **2**) participates in binding. Column 2 describes which species (if any) was concomitantly bound in the pocket. Column 3 describes the environment of the HbA_{1c} pocket in which binding took place (charge states are defined at the end of Table 1 Notes). Column 4 describes the range of exothermicity (in kcal/mol) with which the substrate bound in the pocket. Note that for concomitant binding, the overall exothermicity is the sum of the two binding events. As an example, for **1** binding with 2,3-BPG^{2-} total exothermicity is (−3.2 to −2.7) + (−3.0 to −2.6) or ca. −6.2 to −5.3 kcal/mol. Column 5 describes the percentage of binding events with suitable geometry for nucleophilic attack by a Val1 residue (relative to the total number of binding events that were energetic minima). Column 6 describes the identity of the acid that facilitates the Val1 attack. Note that in column 6, the acids are listed in the order of decreasing % of binding events that meet the geometric requirements for participation. *Indicates that the species is acting as a proton shuttle (abstracting a proton off of an acidic residue and then acting as an acid).

Environment 1—Standard Environment: $Val(NH_2)$, $Lys(NH_3^+)$, $Glu/Asp(COO^-)$, His(Neutral; 146-H^+).
Environment 2—H-Bond Donating/Acidic Environment: $Val(NH_2)$, $Lys(NH_3^+)$, Glu/Asp(COOH), His(H^+).
Environment 3—H-Bond Accepting/Basic Environment: $Val(NH_2)$, $Lys(NH_3^+)$, $Glu/Asp(COO^-)$, His(Neutral).
Environment 4—Mixed Environment 1: $Val(NH_2)$, $Lys(NH_3^+)$, $Glu/Asp(COO^-)$, His(H^+).
Environment 5—Mixed Environment 2: $Val(NH_2)$, $Lys(NH_3^+)$, Glu/Asp(COOH), His(Neutral).

The exothermicities of concomitant binding for **3** and **4** in the presence of all four potential effector reagents (structures **5**–**8**, Table 2, column 4) were similar. The probability of suitable geometry for covalent modification of Val1 was dependent upon the charge state of the concomitantly bound species (Table 2, column 5). For the concomitant binding of acetaldehyde and glyceraldehyde with effector reagents, the hydrate forms had a greater probability of Val1 modification on average (Table 1, column 5 and Table 2, column 5).

3. Discussion

3.1. Single-species binding of acetaldehyde/acetaldehyde hydrate in HbA_{1c} pocket

Based upon the binding geometries determined from the results in Table 1 and model reactions followed by ^{1}H NMR spectroscopy, multiple mechanistic possibilities for acetaldehyde covalent modification of Val1 within the HbA_{1c} pocket are predicted (depicted in Figure 3, Mechanism **I** for **1** and Mechanisms **II** and **III** for **2**) and presented in detail.

In mechanism **I** (Figure 3), the aldehydic form of acetaldehyde **1** initially binds in the HbA_{1c} pocket and suitable geometries are predicted for the RNH_2 of Val1 to act as a nucleophile and attack the carbonyl carbon of **1**. At the same time, an RNH_3^+ of a nearby Lys residue (or His-H^+ residue) has suitable geometry to act as the conjugate acid to protonate the carbonyl oxygen as the nucleophilic attack proceeds. The result of this proposed mechanism is the production of a protonated carbinol amine. Upon the formation of the protonated carbinol amine, we propose that a base (a His or a Lys RNH_2 residue) abstracts the proton off the nitrogen of the protonated carbinol amine, generating the carbinol amine (with water as a leaving group) that can then proceed, ultimately, to the protonated Schiff base. There are suitable geometries for two additional possible mechanistic pathways for covalent modification of Val1 within the HbA_{1c} pocket by the acetaldehyde hydrate, **2** (Figure 3, Mechanisms **II** and **III**). In Mechanism **II**, one of the hydrate OH groups is protonated (by either an R-NH_3^+ of a nearby Lys or a protonated His), generating an electrophilic modified hydrate that is then attacked by a Val1 nucleophile (with water as a leaving group), generating a protonated carbinol amine. Interestingly, from the protonated carbinol amine, the likely path to the protonated Schiff base is the same as that for the reaction of **1** in the HbA_{1c} pocket. The second possible pathway for covalent modification by **2** (Figure 3, Mechanism **III**) starts with the loss of water from the protonated hydrate which generates an activated carbonyl, which is *then* attacked by the Val1 nucleophile. Once more, the subsequent proposed chemistry yields the same protonated Schiff base that is generated from initially bound acetaldehyde. Thus, it does not matter whether the initially bound substrate is acetaldehyde **1** or its hydrate **2**, in that the final structure for the covalently-modified protein is the same protonated Schiff base because they share the same protonated carbinol amine intermediate.

3.2. Concomitant binding of acetaldehyde/acetaldehyde hydrate in the HbA_{1c} pocket with 2,3-BPG or Pi

From a mechanistic perspective, the facilitation of covalent modification of Val1 by concomitant binding of acetaldehyde or its hydrate with either form of 2,3-BPG or Pi is categorically related to the proposed mechanisms depicted in Figure 3. The only distinction is that di-anionic 2,3-BPG, **5**, or mono-anionic Pi, **7**, is computationally-predicted to be able to play the role of the acid to protonate the oxygen of the initially bound species (the carbonyl oxygen of bound **1** or either of the hydroxyl oxygens of bound **2**) in the formation of the protonated carbinol amine. Further, any of **5**–**8** can potentially serve as a base and/or conjugate acid in the conversion of the protonated carbinol amine to the protonated Schiff base. Here again, an identical protonated Schiff base is formed irrespective of which of **1** or **2** initially bind and irrespective of whether 2,3-BPG or Pi are involved.

3.3. Comparison of acetaldehyde to glyceraldehyde

The interpretation of the binding characteristics of the two forms of glyceraldehyde (**3** and **4**) in the HbA_{1c} pocket (Table 2) lead to a bank of mechanistic predictions. First, like acetaldehyde, each of the glyceraldehyde forms **3** and **4** bind as single molecules and concomitantly with either form of

Table 2. Computational modeling of Glyceraldehyde/Hydrate binding to the HbA_{1c} pocket of human hemoglobin both as single-species binding and with concomitant binding with 2,3-BPG or with Pi

Bound Species	Second Species Bound	Environment based upon charge state of Amino Acid Residues	Binding Energies (kcal/mol)	% of Binding Events with Geometry Suitable for Reaction to Modify Val1	Identity of the Acid involved in Facilitating Covalent Modification
Glyceraldehyde 3	–	1	−3.6 to −3.1	46.7%	Lys82 or Lys8
	–	2	−3.5 to −3.3	13.3%	Lys8 or Lys82
	–	3	−3.0 to −2.9	3.3%	Lys8
	–	4	−3.7 to −3.4	26.6%	Lys8 or Lys82
	–	5	−3.6 to −3.2	0.0%	N/A
		AVG		**18.0%**	
Glyceraldehyde Hydrate 4	–	1	−3.6 to −3.1	30.0%	Lys82
	–	2	−3.8 to −3.2	0.0%	N/A
	–	3	−3.8 to −3.0	0.0%	N/A
	–	4	−4.0 to −3.3	33.3%	Lys8 or Lys82
	–	5	−3.7 to −3.3	0.0%	N/A
		AVG		**12.7%**	
Glyceraldehyde 3	2,3-BPG^{2-}	1	−3.8 to −2.8	26.7%	Lys8 or 2,3-BPG^{2-} or Lys82
	2,3-BPG^{4-}	1	−4.1 to −3.1	23.3%	Lys82 or 2,3-BPG^{4-*}
	Pi^{-}	1	−3.8 to −2.8	20.0%	Lys8 or Lys82
	Pi^{2-}	1	−3.7 to −3.0	0.0%	N/A
		AVG		**17.5%**	
Glyceraldehyde Hydrate 4	2,3-BPG^{2-}	1	−3.8 to −2.9	33.3%	Lys82 or 2,3-BPG^{2-} or Lys8
	2,3-BPG^{4-}	1	−4.2 to −3.2	10.0%	Lys82
	Pi^{-}	1	−3.9 to −2.8	13.3%	Lys82 or Lys 8
	Pi^{2-}	1	−3.9 to −2.8	0.0%	N/A
		AVG		**14.1%**	

Table 2 contains data from the computational modeling of glyceraldehyde (**3**) and its hydrate (**4**) binding to the HbA_{1c} pocket of human hemoglobin, both as single-species binding and with concomitant binding with either form of 2,3-BPG (**5** or **6**) or Pi (**7** or **8**). Column 1 describes which form of glyceraldehyde (**3** or **4**) participates in binding. Column 2 describes which species (if any) was concomitantly bound in the pocket. Column 3 describes the environment of the HbA_{1c} pocket in which binding took place (charge states are defined at the end of Table 1 Notes). Column 4 describes the range of exothermicity (in kcal/mol) with which the substrate bound in the pocket. Note that for concomitant binding, the overall exothermicity is the sum of the two binding events. As an example, for **3** binding with 2,3-BPG^{2-} total exothermicity is (−3.6 to −3.1) + (−3.8 to −2.8) or ca. −7.4 to −5.9 kcal/mol. Column 5 describes the percentage of binding events with suitable geometry for nucleophilic attack by a Val1 residue (relative to the total number of binding events that were energetic minima). Column 6 describes the identity of the acid that facilitates the Val1 nucleophilic attack. Note that in column 6, the acids are listed in the order of decreasing % of binding events that meet the geometric requirements for participation.

*Indicates that the species is acting as a proton shuttle (abstracting a proton off of an acidic residue and then acting as an acid).

Environment 1—Standard Environment: $Val(NH_2)$, $Lys(NH_3^+)$, $Glu/Asp(COO^-)$, His(Neutral; 146-H^+).
Environment 2—H-Bond Donating/Acidic Environment: $Val(NH_2)$, $Lys(NH_3^+)$, Glu/Asp(COOH), His(H^+).
Environment 3—H-Bond Accepting/Basic Environment: $Val(NH_2)$, $Lys(NH_3^+)$, $Glu/Asp(COO^-)$, His(Neutral).
Environment 4—Mixed Environment 1: $Val(NH_2)$, $Lys(NH_3^+)$, $Glu/Asp(COO^-)$, His(H^+).
Environment 5—Mixed Environment 2: $Val(NH_2)$, $Lys(NH_3^+)$, Glu/Asp(COOH), His(Neutral).

2,3-BPG or Pi. Both single molecule and concomitant bindings of **3** and **4** possess suitable geometries to generate species that can then go on to covalently modify Val1. Second, the geometries observed in these binding computations predict that multiple mechanisms for NECPM are possible (Figure 4, Mechanisms **I**, **II**, or **III**).

Third, just as is the case for the acetaldehyde species, the proposed mechanisms to arrive at a covalently modified protein at Val1 differ whether the initially bound species of glyceraldehyde is the aldehyde **3** or the hydrate **4**. That said, the mechanisms all converge to the same sequence of protonated carbinol amine to carbinol amine and then, ultimately, to an identical protonated Schiff base (Figure 4). Therefore, similarly to acetaldehyde, it does not matter which species (**3** or **4**) initially binds regarding the structure of the first major covalent species (a protonated Schiff base) formed. The protonated Schiff base generated from **1** or **2** (Figure 3, species **9**) is however different from that which is formed from initially-bound **3** or **4** (Figure 4, species **10**).

4. Summary

We show for the first time that both the aldehyde **1** and hydrate **2** forms of acetaldehyde bound as independent species in the HbA_{1c} pocket with suitable geometries to lead to the covalent modification of Val1. Both **1** and **2** were predicted to undergo concomitant binding in the same pocket in the presence of either form of 2,3-BPG or either form of Pi, again with suitable geometry for the covalent modification of Val1. The aldehydic form of acetaldehyde, **1**, had unique mechanistic pathways compared to the hydrate form, **2**, yet the mechanisms converged at an identical protonated Schiff base, **9**. Similar to acetaldehyde, the aldehydic and hydrate forms of glyceraldehyde (**3** and **4**) bound as independent species in the HbA_{1c} pocket with suitable geometries to lead to the covalent modification of Val1. Both **3** and **4** were predicted to undergo concomitant binding in the same pocket in the presence of 2,3-BPG or Pi. The aldehydic form **3** of glyceraldehyde had unique mechanistic pathways compared to the hydrate **4**, yet the mechanisms also converged at an identical protonated Schiff base, **10**. As a point of major emphasis, the Val1 protonated Schiff base **9**, generated from either form of acetaldehyde (**1** or **2**), was structurally different from the Val1 protonated Schiff base **10** generated from **3** or **4**.

5. Potential implications

In contextualizing these findings with what is known in the literature, our attention goes to the structure of known acetaldehyde/protein adducts (species generated *after* the protonated Schiff base). The covalent species that have been measured and have been implicated as central to alcohol toxicity are: a) an acetaldehyde enamine, a cyclic derivative 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative (MDHDC), and a modified aldehyde 2-formyl-3-(alkylamino)butanal (FAAB) (Tuma et al., 2001); b) a crotonaldehyde Schiff base (Tuma et al., 1991); and c) a cyclic imadazolidinone (De Benedetto & Fanigliulo, 2009). These five structures are here termed alcohol toxicity adducts (ATA). Rolla et al. (2000) have asserted that the formation of these five adducts correlates to the degree of alcoholic liver injury in patients with alcohol-induced liver disease. While the structures of these five acetaldehyde/protein adducts are known (Tuma et al., 2001), detailed mechanisms for their production are not. We posit that all five ATA species can arise from the protonated Schiff base, **9**. As a result, we propose that the protonated Schiff base, **9**, is a necessary precursor for the ATA that correlate to alcoholic liver disease.

The protonated Schiff base from acetaldehyde, **9**, however, *cannot* form an Amadori intermediate, therefore no advanced glycation end products, AGE, will be produced. Since it is AGE species that are implicated in diabetic complications (Brownlee et al., 2001; Singh, Barden, Mori, & Beilin, 2001) this is a likely reason why acetaldehyde has no direct connection to diabetic complications. These novel findings suggest that the protonated Schiff base determines the structure of the stable covalent adduct, and thus the structure of the protonated Schiff base formed in NECPM may be more important to pathophysiological outcomes than previously thought.

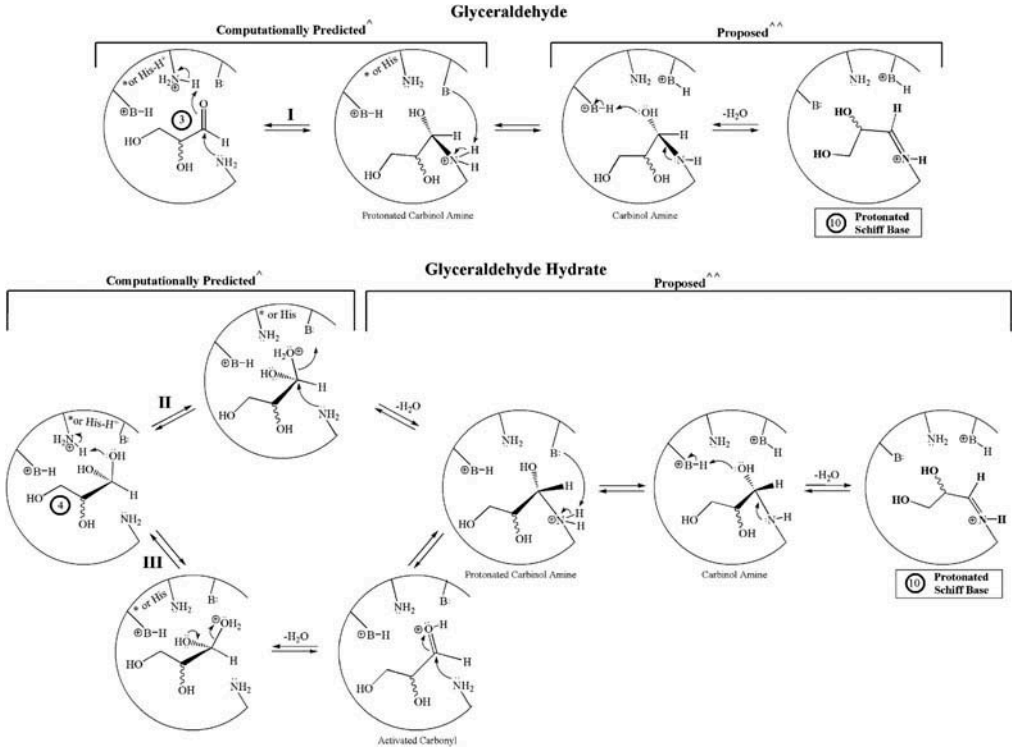

Figure 4. Potential NECPM mechanisms for HbA covalent modification that are geometrically possible for glyceraldehyde *3* (Mechanism *I*) and the glyceraldehyde hydrate *4* (Mechanisms *II* and *III*) based upon molecular modeling with MOE.[2]

These three mechanisms are predicted to be geometrically feasible based upon calculated energetic minima from computations conducted in MOE. In Mechanism **I**, the aldehydic form of glyceraldehyde, **3**, binds and the nucleophilic lone pair on the nitrogen of a terminal valine amino acid residue attacks the carbonyl carbon of **3**. This causes the π-bond to break and enhances the basicity of the carbonyl oxygen, which then abstracts a proton from a nearby acidic amino acid residue (Lys NH_3^+ or a protonated histidine). This generates the protonated carbinol amine. Once formed, a basic amino acid residue can deprotonate the positively charged amine to form the carbinol amine. The lone pair on nitrogen can then form a π-bond, increasing the basicity of the oxygen of the alcohol which can then abstract a proton from an acidic amino acid reside. The protonated alcohol group then departs as water. This forms the protonated Schiff base, **10**. Mechanisms **II** and **III** begin with the hydrate form of glyceraldehyde, **4**, bound in the protein pocket. In Mechanism **II**, the oxygen of an alcohol group on **4** abstracts a proton from a nearby acidic amino acid residue. The lone pair on the nitrogen of a terminal valine then attacks as a nucleophile, causing water to depart as a leaving group. This generates the protonated carbinol amine. In Mechanism **III**, the oxygen of an alcohol group on **4** abstracts a proton from

6. Experimental procedures

6.1. NMR experiments

To investigate whether computationally proposed reactions can occur (see below), acetaldehyde was placed into an aqueous solution containing lysine (acting as a surrogate for the nucleophilic N-terminal valine residue in the HbA_{1c} pocket) and allowed to react as a function of time and was followed by ^1H NMR spectroscopy. All ^1H NMR experiments were performed at room temperature on a JEOL ECX-300 spectrometer. Reagents were all purchased commercially and used without further purification. Reactions were followed as a function of time (ca. 8 min being the earliest time point based upon getting the NMR tube loaded, locked, and shimmed). From there, data was collected in time intervals out to 165 min. The solution pH of each of the samples subjected to NMR analysis was determined with a pH meter to be 7.9. The model reaction NMR tube consisted of 40 µl of acetaldehyde, 5 mg of lysine, and 700 µl of D_2O.

6.2. Computational substrates

Protein/substrate computations utilized Molecular Operating Environment (Molecular Operating Environment (MOE), 2015). The structures utilized in the computations for protein-substrate interactions were as follows: a) the fully oxygenated HbA crystal tetramer 1GZX was obtained from the RCSB PDB (http://www.pdb.org/pdb/home/home.do); b) the α- and β-glucopyranose isomers were obtained from Heterocompound Information Centre, Uppsala, (HIC-UP, http://xray.bmc.uu.se/hicup/); c) mono- and di-anionic Pi and water were built with The PyMOL Molecular Graphics System (ver. 1.5.0.4 Schrödinger, LLC, http://www.pymol.org/) or made within MOE; and d) the acetaldehyde and glyceraldehyde and their respective hydrates (**1**–**4**) were hand-built and optimized in MOE.

6.3. Computational modeling to assess the binding of substrates to HbA

Within MOE, the HbA β-chain utilized was modified into each of the respective environments via MOE geometry optimization and used as input structures for binding investigations (as specified in the captions for Tables 2 and 3). For concomitant binding studies, the initially bound species was tether-docked in the pocket of interest and then a second substrate was then inserted and energy minimized.

a nearby acidic amino acid residue. The lone pair on the oxygen of the remaining alcohol group of **4** forms a π-bond to carbon. This facilitates the loss of water as a leaving group and generates an activated carbonyl species. The carbonyl carbon is then attacked by a nucleophilic lone pair on the nitrogen of a terminal valine to form the protonated carbinol amine. Both Mechanisms **II** and **III** involve a common protonated carbinol amine species and the following chemistry from the common intermediate leads to the formation of the same protonated Schiff base, **10**.

All structures that were exothermic beyond the limit of −2.5 kcal/mol (the binding exothermicity of water) were analyzed, with a cutoff distance of 5Å established for effective reaction within protein pocket(s) (Bobadilla, Nino, & Narasimhan, 2005).

A computational assessment of the geometries for the bound forms of acetaldehyde (**1** and **2**) in the HbA_{1c} pocket was used as a model to elucidate the potential NECPM mechanisms that can theoretically lead to covalent modification of N-terminal Val1 on the β-chains. The covalent modification of Val1 was of particular interest because it is that which is clinically evaluated as HbA_{1c}. Specifically, **1** and **2** (in separate experiments) were bound to HbA in five different environments to assess exothermicity and the probability for bound species to assume the proper geometry for covalent modification of Val1. These five environments were used to reflect a range of physiological possibilities. Environments ranged from basic to acidic where amino acid residues of interest were either non-protonated bases or protonated conjugate acids (for the specific amino acid residues and their charge states in each environment, see the legend of Table 1). For comparative purposes, glyceraldehyde and its hydrate (**3, 4**) were computationally evaluated via the same approach as that taken for acetaldehyde and its hydrate.

Additional MOE computational modeling was conducted in order to determine whether **1** and **2** can undergo exothermic concomitant binding with either form of 2,3-BPG (structures **5** and/or **6**) or inorganic phosphate (structures **7** and/or **8**). Again, binding exothermicities were determined and geometries were assessed to discern whether the concomitantly-bound species have the proper orientation, while bound, to react in a mechanism that could theoretically lead to a covalently modified Val1 within the HbA_{1c} pocket.

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Competing Interests
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