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Insights into the Progression of Labile Hb A_{1c} to Stable Hb A_{1c} via a Mechanistic Assessment of 2,3-Bisphosphoglycerate Facilitation of the Slow Nonenzymatic Glycation Process

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Abstract

Nonenzymatic glycation (NEG) of human hemoglobin (Hb A) consists of initial non covalent, reversible steps involving glucose and amino acid residues, which may also involve effector reagent(s) in the formation of labile Hb A_{1c} (the conjugate acid of the Schiff base). Labile Hb A_{1c} can then undergo slow, largely irreversible, formation of stable Hb A_{1c} (the Amadori product). Stable Hb A_{1c} is measured to assess diabetic progression after labile Hb A_{1c} removal. This study aimed to increase the understanding of the distinctions between labile and stable Hb A_{1c} from a mechanistic perspective in the presence of 2,3-bisphosphoglycerate (2,3-BPG). 2,3-Bisphosphoglycerate is an effector reagent that reversibly binds in the Hb A_{1c} pocket and modestly enhances overall NEG rate. The deprotonation of C2 on labile Hb A_{1c} in the formation of the Amadori product was previously proposed to be rate-limiting. Computational chemistry was used here to identify the mechanism(s) by which 2,3-BPG facilitates the deprotonation of C2 on labile Hb A_{1c}. 2,3-Bisphosphoglycerate is capable of abstracting protons on C2 and the α-nitrogen of labile Hb A_{1c} and can also deprotonate water and/or amino acid residues, therefore preparing these secondary reagents to deprotonate labile Hb A_{1c}. Parallel reactions not leading to an Amadori product were found that include formation of the neutral Schiff base, dissociation of glucose from the protein, and cyclic glycosylamine formation. These heretofore under appreciated parallel reactions may help explain both the selective removal of labile from stable Hb A_{1c} and the slow rate of NEG.

Keywords

Amadori product; conjugate acid of Schiff base; Hb A_{1c}; 2,3-bisphosphoglycerate (2,3-BPG); nonenzymatic glycation (NEG); water

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Introduction

The covalent attachment of glucose to proteins is a well-known posttranslational modification that proceeds *via* a slow process called nonenzymatic glycation (NEG) [1–3]. The most clinically-relevant model protein for the study of the NEG process in *vivo* is human hemoglobin (Hb A) [1]. The initial stage of the NEG process (Figure 1) is the noncovalent binding of a glucopyranose ring (structure 1), which then ring opens, while bound to protein, to a more reactive ring-opened aldehydic form of glucose (structure 2), that is still non covalently bound [4]. After several mechanistic steps, (structure 2) can proceed to a covalently-attached conjugate acid of the Schiff base (structure 3). Specifically, when the conjugate acid of the Schiff base (structure 3) is formed at one (or both) of the N-terminal Val1 residues on the two β chains of the Hb A tetramer [5,6], the resulting species is clinically referred to as labile Hb A_{1c} or pre Hb A_{1c}. Labile Hb A_{1c} (structure 3) can then proceed *via* multiple mechanistic steps to generate a covalently-attached Amadori product (structure 6), clinically referred to as Hb A_{1c} or stable Hb A_{1c} [7,8]. Stable Hb A_{1c} can then proceed *via* multiple mechanistic steps to an array of advanced glycation end (AGE) products [9].

The overall NEG process results in covalent modification of Hb A by glucose (referred to as glycation). The specific structures for covalently-modified Hb A are a composite of structures 3 (formed reversibly), 4a, 4b and 6 (formed with modest reversibility), and AGE (formed non reversibly). Covalent modification of a protein by glucose can alter protein function [10] and is thought to be linked to the chronic complications of diabetes mellitus [11]. This glucose concentration-dependent process is accelerated in diabetic patients due to persistent hyperglycemia.

The clinical measurement of stable Hb A_{1c} (structure 6), reflects average glycemia and the continuous process of NEG occurring over approximately 3 months and highlights the slow time frame for the glycation of intracellular proteins [1,2,4,12] compared to enzymatic processes. Stable Hb A_{1c} also has a strong predictive value for microvascular complications and cardiovascular disease in populations with diabetes [11]. A key initial step in the measurement of stable Hb A_{1c} (structure 6) is the elimination its precursor, labile Hb A_{1c} (the conjugate acid of the Schiff base, structure 3) in *vitro* [13]. However, recent studies by Hempe *et al.* [14] suggest that labile Hb A_{1c} is not a single chemical structure but consists of multiple structures with interrelated dissociation kinetics. It is also apparent that multiple effector reagents may facilitate the formation of structure 6 in *vivo*.

An effector reagent is a small molecule that can concomitantly bind and thus reside in the same protein pocket with a glucopyranose. Effector reagents can either facilitate or inhibit covalent bond making or bond breaking [15]. The organic phosphate 2,3-bisphosphoglycerate (2,3-BPG) is a physiological species that can be an effector reagent. 2,3-Bisphosphoglycerate exists as a mixture of multiple anionic forms in a pH-dependent dynamic equilibrium. The major organic phosphate in human erythrocytes is 2,3-BPG, and, at a normal concentration of 4.0–5.0 mM, is comparable to Hb A on a molar basis [16]. 2,3-Bisphosphoglycerate is known to bind in the N-terminal Val1 pocket between the β chains of Hb A [17]. 2,3-Bisphosphoglycerate is also thought to accelerate Hb A glycation modestly

in the conversion of structure 3 (labile Hb A_{1c}) to the Amadori product (structure 6) (stable Hb A_{1c}) [12]. Specifically, under aerobic and anaerobic conditions, the rate of Hb A_{1c} formation *in vitro* was 30.0 and 50.0% higher, respectively, at high (~9.0 mM) vs. low (0.7–0.8 mM) 2,3-BPG concentrations in human erythrocytes.

The focal point of the current study is framed around three interrelated questions. First, can a more complete understanding of why the overall NEG process is relatively slow arise from a mechanistic analysis of the interplay between labile- and stable-Hb A_{1c}? Second, how do the clinical assay methods utilized for removing labile Hb A_{1c} work and what are the chemical structures and mechanistic pathways involved? Finally, how is 2,3-BPG involved in the interplay between labile- and stable-Hb A_{1c}? Computational methods were employed to follow the progression from labile Hb A_{1c} formation to the generation of stable Hb A_{1c}. Specific emphasis was placed on chemical bond making/bond breaking participation by 2,3-BPG in this transformation. In addition, an assessment of possible parallel mechanistic pathways (here defined as reactions) that do not directly proceed from labile-to-stable Hb A_{1c}, was developed and discussed in relation to the NEG process. This study is intended to contribute to a more accurate and complete depiction of the NEG process that produces stable Hb A_{1c}.

Materials and methods

A fully oxygenated Hb A crystal tetramer PDB 1GZX, a partially oxygenated PDB 1B86 Hb A tetramer with tetrabasic 2,3-BPG bound, and a fully deoxygenated Hb A crystal tetramer PDB 1BZ0 were utilized (from <http://www.rcsb.org/>). Both dianionic and tetraanionic 2,3-BPG structures were generated from a tetraanionic 2,3-BPG structure (RCSB PDB) and energy minimized within molecular operating environment (MOE, 2013.08; Chemical Computing Group ULC, Montreal, Québec Province, Canada). Both forms of 2,3-BPG were utilized because the intrinsic pK_a values for protons associated with the phosphate groups bound to C2 and C3 of 2,3-BPG are 6.99 and 7.28, respectively [18], meaning that both dianionic and tetraanionic 2,3-BPG will be physiologically present. Starting from the original RCSB PDB crystal structure, the N-terminal Val1 of the β chains was modified to a conjugate acid of the Schiff base (structure 3) and geometry minimized in MOE (computational modification of geometry to arrive at the minimal energy structure conformation). Further modification of RCSB PDB crystal structures included protonation or deprotonation of specific amino acid residues followed by geometry optimization to generate proteins in different charge states. Specifically, each Hb A structure was modified within MOE such that one N-terminal Val1 was modified to the conjugate acid of the Schiff base and the other was either a nucleophilic NH₂ amine or a NH₃⁺ ammonium cation. All lysine R groups of the Hb A tetramer were ammonium cations (NH₃⁺). Histidine R groups were R-N neutral species and aspartic acid and glutamic acid R groups were R-COO⁻ anion species. Water was already present within all the crystal structures analyzed.

Single effector reagents (2,3-BPG or water) were then placed in the Val1 pocket between the β chains of 1BZ0 and 1GZX (whether modified or not) and geometry optimized. The energy-minimized structures were then visualized and evaluated for potential mechanisms. A conservative cutoff distance of 5 Å between potential reactive centers (distances between

acidic protons and basic lone pairs of electrons; distances between nucleophilic and electrophilic atoms, *etc.*) was employed [19,20]. The reactive centers considered included reactive atoms (or lone pairs of electrons) on effector reagents (non covalently bound 2,3-BPG and/or water), the non covalently bound glucose, and resident amino acids in the Val1 pocket. From these measurements, potential mechanistic pathways were identified. Mechanistic pathways that involved deprotonation of the C2 structure 3 (Figure 1) in forward progression to the Amadori product (structure 6) were considered. In addition, all potential mechanistic pathways from structure 3 whether they led to structure 6 or not, were assessed.

Results

Table 1 lists the species involved in geometrically-possible reactions of labile Hb A_{1c} [which is the conjugate acid of the Schiff base (structure 3) in the Hb A_{1c} pocket] both without and with 2,3-BPG present. For crystals without 2,3-BPG pre bound (1BZ0 and 1GZX), the neighboring His2 residue was the only residue within the 5 Å cutoff distance that could serve as a potential base towards the C2 hydrogen of structure 3. This reaction is designated as 'C2 deprotonation' (Table 1, column 1) and is shown as mechanistic pathway A in Figure 2. The proper geometry for this reaction was achieved in only 12.5% of the conformations observed as energetic minima (Table 1, column 6). Other amino acids (*e.g.* Lys8, Asp79 and Lys82 on the same β chain) were all at distances greater than 5 Å from the proton of interest on the C2 of structure 3. Thus, in the absence of 2,3-BPG in the Hb A_{1c} pocket, based upon our criteria (5 Å or less), His2 is the only amino acid residue capable of C2 deprotonation and formation of the Amadori product structure 6 (stable Hb A_{1c}, Figure 1), albeit such a reaction is predicted to be a low probability event based on geometry optimized structures.

It is noteworthy that for the few structures that are energetic minima where the C2 of structure 3 can be deprotonated by His2 it is also possible to deprotonate the α-nitrogen of structure 3 (which is an ammonium ion). When the α-nitrogen of structure 3 is deprotonated, the neutral Schiff base structure 3a is formed (Table 1, row 2; Figure 1, mechanistic pathway II). This option is possible because the α-nitrogen of structure 3 is just 2.5 Å from C2. This reaction is designated 'N-deprotonation' (Table 1, column 1). Proper geometry for this reaction occurred in 25.0% of the energy-minimized conformations. When N-deprotonation occurs, no direct progression to stable Hb A_{1c} is possible.

A summary of the possible fates of the conjugate acid of the Schiff base structure 3 in the Val1 pocket of Hb A in the presence of 2,3-BPG is provided in Figure 1. Starting at the conjugate acid of the Schiff base, structure 3 (Figure 1), formation of the stable Amadori product structure 6 at Val1 of the β chains of Hb A requires the deprotonation of the C2 on structure 3 (Figure 1, mechanistic pathway I; Figure 2, mechanistic pathway B). For any of the energy-minimized crystals containing a bound 2,3-BPG, at least one of the basic groups (one carboxylate and/or one or more of the phosphates of either tetraanionic or dianionic 2,3-BPG) was within reacting distance (~2–5 Å) to deprotonate C2 of structure 3. Therefore, Amadori formation *via* structure 5, and then structure 6 (Figure 1, mechanistic pathway I and Table 1, with 2,3-BPG, row 1) is geometrically possible. Proper geometry for this

reaction was achieved in essentially all (98.0%) of the conformations observed as energetic minima.

In addition, 2,3-BPG can function indirectly by deprotonating nearby water and/or native amino acid residues, preparing these secondary reagents to deprotonate the conjugate acid of the Schiff base structure 3 (Figure 2, mechanistic pathway C). Therefore, a computational investigation of the degree of potential water and amino acid residue participation was conducted. In the Hb A_{1c} pocket in the absence of 2,3-BPG, water was present but was not in range (5Å) to deprotonate the C2. Thus, water participation in the absence of 2,3-BPG is unlikely. In pockets with 2,3-BPG present, there were anywhere from 1–6 water molecules within 5Å of the C2 proton that are also within range for 2,3-BPG to facilitate water basicity. Thus, water participation as a secondary species acting as a base facilitated by 2,3-BPG is predicted to occur with high probability (Table 1, with 2,3-BPG, rows 3–10).

Bound 2,3-BPG can facilitate C2 deprotonation in yet another indirect mechanistic pathway. The inclusion of 2,3-BPG in the Hb A_{1c} pocket can change the conformation of the Hb A_{1c} pocket and move certain basic amino acid residues closer to the acidic proton on structure 3. This conformational change, a proximity effect, brings previously unavailable basic amino acid residues (<5Å) close enough to deprotonate the C2 of structure 3 without any direct 2,3-BPG participation (Figure 2, mechanistic pathway D). Specifically, the presence of bound 2,3-BPG induced conformational change such that the basic sites on Asp73, Asp79 and/or His2, were moved to within 5Å of the proton of the C2 of structure 3 (note that none of these amino acid residues were close enough to the C2 proton in pockets without 2,3-BPG present).

The α-nitrogen of structure 3 can also be deprotonated forming the neutral Schiff base structure 3a (Table 1 and Figure 1, mechanistic pathway II). Potential bases in this reaction are 2,3-BPG, Asp79, His2, or water facilitated by 2,3-BPG. This diverse set of reactions falls under the 'N deprotonation' designation (Table 1, with 2,3-BPG, rows 10–14). Proper geometry for these reactions occurred in the vast majority (95.0%) of the energy-minimized structures and involved the conjugate acid of the Schiff base on the β1Val1 and β2Val1 of Hb A. Regardless of which basic species is involved in the mechanism, structure 3a is the resulting species generated and has no direct connection to structures 5 or 6 without first reverting back to structure 3 (Figure 1).

Based upon computations from MOE, the reversion from structure 3 to a detached glucose structure 2 and the unmodified βVal1 is geometrically viable (Table 1, second to last row). Reversible formation of cyclic glycosylamines structure 4a (α and β forms of six-membered rings), and structure 4b (α and β forms of five-membered rings) were computationally predicted to occur *via* pathways IV and V, respectively. The reversible formation of the cyclic glycosylamines does not afford a direct path to structure 6 without first reverting back to structure 3.

Discussion

In landmark early investigations, both Higgins and Bunn [21] and Lowrey *et al.* [22], established that the formation of labile Hb A_{1c} (which was then referred to as pre-Hb A_{1c}) was reversible and not rate-limiting in the overall NEG process (*i.e.* formation of labile Hb A_{1c} is a relatively fast step). It should be noted that in most extant schemes in the literature, including [21,22], labile Hb A_{1c} was rendered as a neutral Schiff base (an imine; structure 3a in Figure 1) when, in fact, the initially-formed labile Hb A_{1c} is the conjugate acid of the Schiff base (an iminyl ion). A more complete depiction of labile Hb A₁ formation has been forwarded by Clark *et al.* [4], Rodnick *et al.* [15] and Park *et al.* [23], which includes the recently-discovered detail that the initial glucose species that binds to Hb A is not ring-opened but is rather a cyclic glucopyranose that ring-opens while bound as a necessary precondition before it leads to structure 3, the conjugate acid of the Schiff base that is labile Hb A_{1c}. Gil *et al.* [24] recognized and highlighted that structure 3 is the initially-formed covalent species when a nucleophilic amino acid residue (*e.g.* the amine form of the N-terminal Val1 on the two β chains of Hb A) reacts with the electrophilic ring-opened form of glucose (structure 2). Moreover, several studies have shown that the rate of structure 3 formation is influenced by effector reagents such as inorganic phosphate and water serving as acids and/or bases [3,20,25,26].

Lowrey *et al.* [22] suggested and Gil *et al.* [24] demonstrated that the formation of stable Hb A_{1c} structure 6 from labile Hb A_{1c} structure 3 is largely (or entirely) irreversible and slow, constituting the rate-determining step of NEG for Hb A in the presence of 2,3-BPG. Smith *et al.* [12], extending from their data, posited that the rate of conversion from the conjugate acid of the Schiff base (labile Hb A_{1c}, structure 3) to stable Hb A_{1c} (structure 6, the Amadori product; Figure 1, mechanistic pathway I) increases under the influence of nearby 2,3-BPG. Gil *et al.* [24], using kinetic isotope effect (KIE) analysis, definitively demonstrated that the mechanistic step that determines the rate of stable Hb A_{1c} formation in the presence of 2,3-BPG is the abstraction of the C2-proton on structure 3, by a base (Figure 1, mechanistic pathway I, with the abstraction of the proton that is rendered in blue). The KIE method of Gil *et al.* [24] did not, however, enable the identification of the specific base (or bases) involved. Our intent is to discern what the base(s) is/are that deprotonate the C2 proton on labile Hb A_{1c} structure 3 in the progression towards stable Hb A_{1c} structure 6 and to determine whether other acid/base and/or nucleophile/electrophile reactions can occur that alter the rate of progression from structures 3 to 6. The intent is to elucidate why stable Hb A_{1c} formation is so slow (on the order of weeks) and to better understand the structural/mechanistic chemistry associated with the clinical assay methods utilized to eliminate labile Hb A_{1c} structure 3 prior to the measurement of stable Hb A_{1c} structure 6.

Our computational assessment predicts that His2 is the only amino acid residue capable of C2 deprotonation in the formation of the Amadori product structure 6 (stable Hb A_{1c}, Figure 1) in the absence of 2,3-BPG. This singular mechanistic pathway is predicted to occur with low probability (only 12.5% of the predicted conformations that were energetic minima had suitable geometry to react). Despite the molecular proximity of His2 to the C2 of structure 3 in the Hb A_{1c} pocket, a significant geometric constraint for the reaction exists. This may ultimately help explain the low probability of His2 participation in C2 deprotonation of the

conjugate acid of the Schiff base (structure 3). In addition, for all geometric energy minima where the C2 of structure 3 can be deprotonated by His2, the α -nitrogen of structure 3 (an ammonium ion) can also be deprotonated. N-deprotonation of structure 3 is a viable reaction with twice the geometric probability as C2 deprotonation (Table 1). Moreover, the proton on N (Figure 1 rendered in green; pKa ~14) is far more acidic and is therefore much easier to deprotonate than is the proton on C2 (pKa ~20; Figure 1, rendered in blue). Furthermore, N-deprotonation of structure 3 generating structure 3a, has no direct connection to structures 5 or 6 without first reverting back to structure 3. Thus, stable Hb A_{1c} formation in the absence of 2,3-BPG and any other effector reagent should be extremely slow. However, it should be noted from earlier studies that physiological inorganic phosphate (existing at concentrations of 0.5–0.8 mM in the erythrocyte) [27] can bind with glucose in the Hb A_{1c} pocket and serve as an effector reagent, increasing the rate of Hb A glycation [26]. Moreover, it is estimated that 67.0% of all Hb A_{1c} pockets in erythrocytes possess a bound 2,3-BPG [28]. The salient point not highlighted in previous studies is that without an effector reagent, the rate of conversion from structure 3 to structure 6 would be exceedingly slow.

The carboxylate and/or one of the phosphates of either tetraanionic 2,3-BPG or dianionic 2,3-BPG, were close enough (~2–5Å) to directly deprotonate C2 of structure 3 in 98.0% of all energetic minima conformations. Unlike the geometrically-constrained His2 with a single lone pair of electrons on nitrogen capable of serving as a reactive base, 2,3-BPG is less constrained and has as many as five oxygen atoms with lone pairs of electrons that can potentially serve as a base. Furthermore, 2,3-BPG has many more conformations with the requisite geometry to deprotonate the C2 of structure 3 than does His2. In addition, 2,3-BPG can function indirectly by deprotonating nearby water and/or native amino acid residues, preparing these secondary reagents to deprotonate the conjugate acid of the Schiff base structure 3 (Supplementary Figure 1, mechanistic pathway C). The presence of numerous water molecules (with conformational freedom) associated with 2,3-BPG in the Hb A_{1c} pocket and the ability of water to serve as a base when deprotonated by 2,3-BPG highlights another underappreciated potential pathway for 2,3-BPG to deprotonate structure 3 and proceed to structure 6. Bound 2,3-BPG can also facilitate C2 deprotonation in yet another indirect mechanistic pathway. The inclusion of 2,3-BPG in the Hb A_{1c} pocket can change the conformation of the Hb A_{1c} pocket and move the surrounding amino acid residues closer to structure 3 (Supplementary Figure 1, mechanistic pathway D). This conformational change constitutes a proximity effect and brings previously unavailable basic amino acid residues (<5Å) close enough to deprotonate the C2 of structure 3 (without any direct 2,3-BPG participation).

An assessment of the reactive possibilities available to labile HbA_{1c} (structure 3, the conjugate acid of the Schiff base) is key in discerning why NEG is so slow. Five nonenzymatic fates for structure 3 exist. Only mechanistic pathway I produces stable Hb A_{1c} directly. The 2,3-BPG and other potential bases such as His2, Asp79, or water can also directly or indirectly deprotonate the α -nitrogen of structure 3 forming the neutral Schiff base structure 3a, a species that cannot go to stable Hb A_{1c} structure 6 without first returning to structure 3 (Figure 1, mechanistic pathway II). The reversion from structure 3 to a detached glucose structure 3 and the unmodified β Val1 is shown as mechanism pathway III (Figure 1). From this, the glucose can entirely dissociate from the protein and thus this

mechanistic pathway equates to reversion to starting species. Lastly, reversible formation of cyclic glycosylamines structure 4a (α and β forms of six-membered rings), and structure 4b (α and β forms of five-membered rings) were computationally predicted to occur *via* pathways IV and V, respectively (Figure 1).

Mechanistic pathways (II-V) are all reversible pathways and, when manifest, will compete against stable Hb A_{1c} structure 6 formation (Figure 1, mechanistic pathway I). Because there are so many geometrically viable reactions from labile Hb A_{1c} structure 3 that do not proceed to stable Hb A_{1c} structure 6 the formation of structure 6 should be limited. Furthermore, C2 deprotonation is already known to be the rate-limiting step in the formation of stable Hb A_{1c} [24], albeit without mechanistic justification. Given that the only mechanistic pathway that does lead to structure 6 must involve C2 deprotonation (Figure 1), our proposed mechanism supports the original posit that deprotonation of the C2 proton should be rate-limiting. It is important to note that the degree of reversibility for these competing equilibria are unknown.

A common procedure utilized to eliminate labile Hb A_{1c} structure 3 from the clinical assay of stable Hb A_{1c} structure 6, is to add acid to a pH of *ca.* 5–6 [29]. How is this method efficacious for the selective removal of structure 3? When acid is added, both the deprotonation of the C2 proton and the proton on the nitrogen of structure 3 is disfavored, thus mechanistic pathways I and II (Figure 1) become less competitive. Because mechanistic pathway I is the only path from labile Hb A_{1c} structure 3 to stable Hb A_{1c} structure 6 and this pathway becomes less competitive with acid addition, little (or perhaps none) of the structure 3 that exists in a blood sample will proceed to structure 6. The only outlet that is productive (that leads to something other than the reversible formation of structure 3) will be mechanistic pathway III (Figure 1) that will lead, ultimately, to the detachment of a glucose molecule from the Hb A protein [for the mechanism for this process, see Supplementary Figure 1(a)]. At the same time that structure 3 is depleted upon acid addition (exiting as glucose), any stable Hb A_{1c} structure 6 present in the clinical mixture will react with acid and merely undergo reversible protonation/deprotonation [for the mechanism for this process, see Supplementary Figure 1(b)] that cannot detach the glucose from the protein. Thus, acid addition to a mixture of structures 3 and 6 will selectively eliminate the labile Hb A_{1c} structure 3 (as detached glucose) from the stable Hb A_{1c} structure 6, while stable Hb A_{1c} structure 6 concentration will be unaffected. This mechanistic extrapolation provides a plausible explanation for why the addition of acid to a blood sample will enable an accurate measurement of stable Hb A_{1c} structure 6 free of labile Hb A_{1c} structure 3.

A second clinical laboratory method for the removal of labile from stable Hb A_{1c} involves the addition of phosphate at a high concentration [13]. This methodology may be explained by the Le Chatelier and Braun principle [30], which states that if a reactant's concentration is decreased in a dynamic reversible equilibrium, the equilibrium will shift to the left (towards starting materials). Our computational data predicts that as phosphate is added, it binds HbA in competition with a glucopyranose [26]. In some clinical assays of stable Hb A_{1c}, phosphate has been added to hemolyzing solutions at levels well above physiological values (*e.g.* 100 mM [31]), which should therefore reduce the effective concentration of glucopyranose. This procedure will shift the equilibrium in mechanistic pathway III (Figure

1) toward detached glucose, likely resulting in the removal of structure 3 from the Hb A_{1c} pocket. The addition of phosphate will not affect the concentration of structure 6, because the formation of structure 6 is largely irreversible and thus, for structure 6, the Le Chatelier and Braun principle [30] is not operative. As such, addition of phosphate to a mixture of structures 3 and 6 will likely slowly remove the labile Hb A_{1c} structure 3 (as detached glucose) without affecting the concentration of stable Hb A_{1c} structure 6, enabling an accurate measure of stable Hb A_{1c} structure 6 free of complicating structure 3. A related strategy to remove labile Hb A_{1c} from blood samples is the lowering of the concentration of extracellular glucose [13]. Once again, depending on the incubation temperature, glucose concentration, and time, the Le Chatelier and Braun principle [30] will likely be operative and selective for the elimination of labile Hb A_{1c} prior to clinical assays of stable Hb A_{1c}.

In summary, starting from labile Hb A_{1c} structure 3, mechanistic pathways II, III, IV and V are all computationally viable and yet do not lead directly to stable Hb A_{1c} structure 6. The only direct path from labile Hb A_{1c} structure 3, to stable Hb A_{1c} structure 6 is mechanistic pathway I. Thus, with respect to the formation of structure 6, mechanistic pathways II, III, IV and V are unproductive and occur in competition with stable Hb A_{1c} formation. Therefore, as the extent of II, III, IV and/or V increases relative to I, the amount of stable Hb A_{1c} structure 6 formation is reduced. These multiple unproductive fates for labile Hb A_{1c} structure 3 formed in the NEG of human Hb A have not been reported before and are the likely reason for the slow overall progression of NEG. The addition of 2,3-BPG increases the rate of both structures 3 and 6 formation (*via* mechanistic pathway I). Finally, the described mechanistic pathways and effects of the Le Chatelier and Braun principle [30] can help explain how the successful strategies to selectively remove labile Hb A_{1c} structure 3 from clinical laboratory assays of stable Hb A_{1c} structure 6 studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The five fates of labile Hb A_{1c}, structure 3, the conjugate acid of a Schiff base (located in the rectangle in the middle of the figure) that is generated in the NEG process for glucose in the Val1 pocket of Hb A in the presence of 2,3-BPG. Note that the relative lengths of arrows on reversible steps for all the mechanistic pathways (except mechanistic pathway I) are drawn with equal length. This does not suggest that the equilibrium in both directions is equal. Rather, the degree of forward vs. reverse equilibrium varies with conditions and thus are rendered in the figure without respect to the relative magnitude of the equilibrium. For

mechanistic pathway I, the reverse process out-competes the forward process under all conditions. In the noncovalent stages of NEG, a glucopyranose molecule structure 1, must first enter the Hb A_{1c} protein pocket and then be modified to ring-opened species structure 2 while in the pocket, so it can become sufficiently reactive [4]. Structure 2 can then undergo multiple reactions to covalently bond to the N-terminal valine and become structure 3, the conjugate acid of the Schiff base which is labile Hb A_{1c} [26]. Mechanistic pathway I: in structure 3 (labile Hb A_{1c}), the asterisk (*) denotes the C2 atom. The proton on the C2 atom is blue and, in this study, is referred to as the C2 proton. The C2 proton can be deprotonated by a base, specifically the lone pair of electrons on the oxygen of a phosphate or a lone pair of electrons on the oxygen of the carboxylate of tetraanionic 2,3-BPG. This reversibly generates structure 5, which, by multiple steps (not shown), can then irreversibly go to structure 6, stable Hb A_{1c}. This pathway is referred to as 'C2 deprotonation' in Table 1 and constitutes the direct formation of Hb A_{1c}. Mechanistic pathway II: in structure 3, (labile Hb A_{1c}), the green proton on the nitrogen is abstracted by a base. The abstraction generates structure 3a. Note that structure 3a is what has been mistakenly referred to in many other articles as labile Hb A_{1c}. Structure 3a has no direct connection to structure 6, stable Hb A_{1c}, without firstly reverting back to structure 3. This pathway referred to as 'N deprotonation' in Table 1. Mechanistic pathway III: here a nucleophilic water molecule (not shown in the figure) attacks the C1 carbon of structure 3, and, after multiple mechanistic steps (not shown), the result is reversion to structure 2, that is noncovalently-bound in the protein pocket, which then can go to structure 1. This constitutes a complete detachment of structure 1 (ring-closed glucose) from Hb A. This is referred to as 'H₂O nucleophilic attack'. Note that 2,3-BPG is not necessarily involved in this mechanistic pathway. However, 2,3-BPG can potentially facilitate H₂O nucleophilicity, which would mean that 2,3-BPG can inhibit Hb A_{1c} formation by this pathway. Mechanistic pathways IV and V: both pathways IV and V involve the electrophilic C1 of structure 3 (designated with a ^) undergoing an intramolecular nucleophilic attack by a lone pair of electrons on an oxygen atom of an OH on either C5 (generating structure 4a) or C4 (generating structure 4b). Neither structure 4a or 4b has a direct connection to structure 6, stable Hb A_{1c}, without first reverting back to structure 3. These mechanistic pathways are referred to collectively as 'intramolecular OH nucleophilic attack'.

Figure 2.

A representation of four energetic minima calculated from MOE depicting geometrically-possible abstractions of the C2 proton from structure 3 in the Hb A_{1c} pocket under certain conditions. Each is rendered as a partial mechanism, just showing the initial abstraction, and are thus given forward arrows only (though the overall processes are reversible). In the figure BPG represents 2,3-bisphosphoglycerate.

Table 1.

A summary of the computational of geometrically-possible reactions of labile Hb A_{1c} structure 3, without and then with a 2,3-bisphosphoglycerate present in the Hb A_{1c} pocket. The results here are limited to mechanistic pathways I and II.

Without 2,3-BPG				
Geometrically-Possible Reactions ^a	Potential Bases ^b	Species Formed ^c	Direct Progression to Amadori ^d	Proper Geometry Achieved ^e
C2 deprotonation (I) ^f	His2	structures 5 then 6	yes	12.5%
N deprotonation (II) ^f	His2	structure 3a	no	25.0%
With 2,3-BPG				
Geometrically-Possible Reactions ^a	Potential Bases ^b	Species Formed ^c	Direct Progression to Amadori ^d	Proper Geometry Achieved ^e
C2 deprotonation (I) ^g	2,3-BPG ^h	structures 5 then 6	yes	98.0%
C2 deprotonation (I) ^{f,i}	2,3-BPG ^h ; H ₂ O	structures 5 then 6	yes	98.0%
C2 deprotonation (I) ⁱ	2,3-BPG ^j ; H ₂ O	structures 5 then 6	yes	98.0%
C2 deprotonation (I) ^g	2,3-BPG ^h ; H ₂ O; His2	structures 5 then 6	yes	98.0%
N deprotonation (II) ^g	2,3-BPG ^h ; H ₂ O	structure 3a	no	95.0%
N deprotonation (II) ^g	2,3-BPG ^h ; Asp79	structure 3a	no	95.0%
N deprotonation (II) ^{f,i}	2,3-BPG ^h ; H ₂ O	structure 3a	no	95.0%
N deprotonation (II) ⁱ	2,3-BPG ^k ; H ₂ O	structure 3a	no	95.0%
N deprotonation (II) ^g	2,3-BPG ^h ; H ₂ O; His2	structure 3a	no	95.0%
N deprotonation (II) ⁱ	2,3-BPG ^l ; H ₂ O; His2	structure 3a	no	95.0%

2,3-BPG: 2,3-bisphosphoglycerate.

^aThe geometrically possible reaction designated as 'C deprotonation' involves the initial abstraction of the hydrogen on the C2 of the protonated Schiff base (structure 3) by a nearby (less than 5 Å) base. The designation of 'N deprotonation' involves the initial abstraction of the hydrogen on the nitrogen of structure 3 by a nearby base.

^bThe listed species are potential bases based upon a distance between the abstracted proton and the lone pair on the indicated species of less than 5 Å. All listed amino acid residues were on the same β chain as the Schiff base. Note that for H₂O to act as a suitable base, it must be deprotonated by another base such as 2,3-BPG or an amino acid residue as it deprotonates the designated target species. Note that no distinction between the dianionic and tetraanionic forms of 2,3-BPG are made with respect to these measurements.

^cThese species are indicated in Figure 1. Structure 3a is a Schiff base deprotonated at the terminal N, structure 5 is an enol, and structure 6 is an Amadori product.

^dYes means that direct forward progress to Amadori product structure 4 (and hence, formation of Hb A_{1c} and AGEs) is possible. A No means the indicated structure (2 or 3a) must revert (*via* reversible reaction) back to the protonated Schiff base structure 3 to then go on to structure 5 and ultimately to structure 6 in the formation of Hb A_{1c}.

^eThe percentages listed indicate the percentage of conformations that were calculated as geometry-optimized minima that meet the criteria for potential reaction (*e.g.* those that meet the less than 5 Å distance between reaction centers for the mechanism to occur) vs. the total number of conformations calculated as geometry-optimized minima.

f Starting PDB crystal: 1BZ0.

g Starting PDB crystal: 1B86.

h 2,3-BPG phosphate groups only.

i 2,3-BPG phosphate groups and carboxyl group.

j Starting PDB crystal: 1GZX.

k 2,3-BPG phosphate group 1.

l 2,3-BPG phosphate group 2.