Functional prediction from conformational dynamics of glycated and glutathionylated HbE and HbD Punjab

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Glycation and glutathionylation are important post-translational modifications (PTMs) of human haemoglobin that act as biomarkers of diabetes mellitus and oxidative stress. These PTMs perturb the function of normal haemoglobin. However, the structure–function correlation of these PTMs of genetically modified haemoglobin remained unexplored. Using hydrogen/deuterium exchange mass spectrometry, we studied the conformational dynamics of glycated and glutathionylated forms of two haemoglobin variants, HbE and HbD Punjab. Like glycated and glutathionylated normal haemoglobin, these PTMs of HbE were expected to have increased oxygen affinity. However, for HbD Punjab, glycation was predicted to have decreased oxygen affinity whereas glutathionylation to have increased oxygen affinity.

Keywords: Glutathionylation, HbE, HbD Punjab, glycation, post-translational modifications, structure–function correlation.

Post-translational modification (PTM) of proteins might alter their structure and functions. PTMs are involved in various cellular processes such as protein folding, regulation of metabolism and defense mechanism etc.¹,² They are also reported to be involved in several diseases such as diabetes, cancer, neurological disorders, etc³,⁴.

Glycation is a non-enzymatic PTM of proteins, where primary amino groups present in the N-terminus of the polypeptide chain and at the side chain functional group of free, accessible lysine residues of a protein, get covalently modified with glucose via Amadori rearrangement. Glycated haemoglobin formed by the modification of N-terminus ε-amino group of β-globin chain (HbA₁c) serves as a biomarker of average glycemic index for the past 120 days in patients with diabetes mellitus⁵. Glycation perturbs the functionality active structure of a protein and consequently its biological function. In glutathionylation, another non-enzymatic PTM of proteins – the free, accessible cysteine residues of proteins get covalently modified with oxidized glutathione (GSSG). In glutathionylation, Cys93 residue of β-globin chain of haemoglobin gets modified and it has been found to act as a marker of oxidative stress in various medical conditions such as chronic renal failure⁶, hyperlipidemia⁶, diabetes⁷,⁸, neurological diseases such as Friedreich’s ataxia⁹.

Glycation and glutathionylation of human haemoglobin A (HbA) have been reported to alter its function¹⁰,¹¹. Compared to HbA, glycated and glutathionylated haemoglobin have higher affinity for oxygen. As both these PTMs are specific to functional groups, possibility of the same PTMs exists for the genetic variants of human haemoglobin. However, neither structural nor functional changes of the post-translationally modified haemoglobin variants are reported to date. In this study, using hydrogen/deuterium exchange (H/DX) based mass spectrometry (MS) platform, we analysed the structural changes upon glycation and glutathionylation of two genetic variants of human haemoglobin, haemoglobin E (HbE) and haemoglobin D Punjab (HbD Punjab). Comparing the structure–function correlation of glycated HbA and glutathionylated HbA and the structural changes monitored through conformational dynamics of these post-translationally modified HbE and HbD Punjab, we predicted probable functional disorders associated with glycation and glutathionylation of HbE and HbD Punjab.

Materials and methods

Materials

Pepsin, D₂O (99.9%), sodium dithionite, carboxy methyl cellulose (CM-52), 2,2′-dithiopyridine (2-PDS), sephadex G-10 and ε-cyano-4-hydroxycinnamic acid (CHCA) matrix were obtained from Sigma (St Louis, USA). Trifluoroacetic acid (TFA) was obtained from Merck, Germany. LC/MS grade acetonitrile, water and formic acid (FA) were from Fluka, Germany. Polyethylene glycol

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(PEG) mix, the calibrator for mass spectrometer, was purchased from Waters, Milford, MA, USA. All other chemicals were of analytical grade.

Collection of sample and processing

After obtaining prior written consent, 2 ml venous blood was collected from patients carrying haemoglobin variants, HbE and HbD Punjab and from diabetic patients carrying these variants, into EDTA-coated vacutainers at Manipal Hospital, Bengaluru. The study was approved by Institutional Ethics Committee. Plasma was removed after centrifugation at 3000 rpm for 10 min at 25°C. The pellet containing erythrocytes was washed with 0.9% NaCl (aqueous) thrice and lysed with eight volumes of ice-cold distilled water. The hemolysate was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was used to measure absorbance at 548 nm. Concentration of haemoglobin was calculated using ε = 12.51 mM⁻¹ cm⁻¹ per heme unit.

Purification of HbE and HbD Punjab by ion-exchange chromatography

CM-52, used as cation-exchange material, was equilibrated with 10 mM potassium phosphate buffer of pH 6.8. Haemolysate, containing haemoglobin variant, was dialysed against the same buffer. Protein was purified by eluting with pH gradient using mobile phase A (10 mM potassium phosphate, pH 6.8) and mobile phase B (10 mM potassium phosphate, pH 8.3). Absorbance of collected fractions was measured at 548 nm. The purified haemoglobin variants, HbE and HbD Punjab, were characterized using mass spectrometer. Fractions containing purified variants were concentrated and dialysed against 50 mM NH₄HCO₃ buffer, pH 7.4. A part of each variant was subjected to glutathionylation.

Processing of glycated samples

Samples carrying HbA, HbE and HbD Punjab of diabetic patients were collected at Manipal Hospital, Bengaluru. Haemolysate, prepared as mentioned above, was dialysed against 50 mM NH₄HCO₃ buffer of pH 7.4. Due to limited access of diabetic patients carrying variants of haemoglobin, we conducted the conformational analysis in a mixed population comprising native haemoglobin variant and their glycated forms. Thus our analysis was restricted to only signature peptide of glycated haemoglobins.

In vitro glutathionylation of HbE and HbD Punjab

Glutathionyl HbE (GSHbE) and glutathionyl HbD Punjab (GSHbD Punjab) were synthesized in vitro using the protocol described elsewhere. In brief, haemoglobin was incubated with 2-PDS in the molar ratio of haemoglobin : 2-PDS = 1 : 10, in 50 mM NH₄HCO₃ buffer, pH 7.4, for 1 h at 0°C. The excess PDS was removed using Sephadex G-10 spin column. Synthesized thiopyridinyl haemoglobin was subjected to reaction with reduced glutathione (GSH) in a molar ratio of Hb : GSH = 1 : 40 at 0°C for 1 h. Unreacted GSH was removed using Sephadex G-10 spin column. The extent of glutathionylation was monitored by the mass analysis of intact globin chains.

Structural analysis of glycated and glutathionylated haemoglobin variants

Preparation of deoxy and oxy forms of glycated and glutathionylated HbE and HbD Punjab: The deoxy forms of glycated and glutathionylated HbE and HbD Punjab were prepared by adding respective samples to 50 mM NH₄HCO₃/D₂O buffer, pH 7.4, containing 50 mM sodium dithionite. Nitrogen gas was bubbled to ensure deoxy state was maintained during the entire period of the experiment. The oxy forms of glycated and glutathionylated HbE and HbD Punjab were prepared by bubbling oxygen through respective samples in 50 mM NH₄HCO₃ buffer, pH 7.4, prior to the isotope exchange. Oxygen gas bubbling was continued throughout the experiment to ensure that haemoglobin was in oxy state.

Hydrogen/deuterium exchange (H/DX) experiment: H/DX experiment was done according to the protocol described previously. In brief, the isotope exchange kinetics was initiated by diluting the sample with 15-fold excess of 50 mM NH₄HCO₃/D₂O buffer (pH 7.4) at 25°C. Osmolarity of solution was maintained at 300 milliosmoles. At different time intervals, the exchange reaction was quenched by mixing of 10 μl of aliquot with 90 μl of ice-cold aqueous 0.1% TFA solution of pH 2.5. An aqueous pepsin solution (2 μl) was added immediately to the acidified protein solution maintaining molar ratio of enzyme : substrate as 1 : 10, and proteolytic digestion was performed for 5 min at 0°C. Equal volumes of digested sample and matrix solution (5 mg/ml CHCA) were mixed and 1 μl was spotted on a MALDI plate. The spot was dried rapidly using moderate vacuum in a desiccator and mass spectra were acquired immediately. The 0% and 100% control experiments were done as described previously.

Data acquisition and analysis: Mass analyses were performed on a SynaptTM HDMS MALDI mass spectrometer (Waters) in positive ion V mode, using 200 Hz solid state laser (λ = 355 nm). Mass spectrometer was calibrated using an external calibrant, PEG mix. Mass spectra were acquired by integrating an average of 50 shots/spots and analysed using MassLynx software ver. 4.1. The data analysis was performed as described elsewhere.
Results and discussion

In the present study, we analysed the conformational dynamics of HbA, HbE and HbD Punjab and compared it with their respective glycated and glutathionylated analogues. Using literature reports on these PTMs of HbA, we established the structure–function correlation of post-translationally modified HbA. Subsequently, we compared conformational dynamics of the signature peptides of modified HbE and HbD Punjab with that of HbA and assessed the functional disorder that might be associated with glycation and glutathionylation of HbE and HbD Punjab. We predicted that both glycation and glutathionylation of HbE might result in stronger oxygen binding, whereas, in case of HbD Punjab, glycation might result in weakening in the oxygen binding but glutathionylation might cause stronger oxygen binding.

Glycation of proteins is a non-enzymatic process called Maillard reaction, where primary amine groups of a protein are modified with aldehyde group of glucose, thereby forming a labile aldime called Schiff’s base which slowly isomerizes to a stable ketoamine by Amadori rearrangement. It was reported that for HbA, glycation primarily occurs at the N-terminal valine residue of β-globin chain in vivo. Half-life of human red blood cells is 120 days. Once modified, the glucose moiety becomes a part of haemoglobin molecule. Thus glycated haemoglobin (HbAco) serves as a potential biomarker of glycemic index for the past 120 days in diabetes mellitus. Upon glycation, the molecular mass of β-chain increases by 162 Da.

2.3-Diphosphoglycerate (2,3-DPG), an allosteric regulator, is present in the erythrocytes in equimolar concentrations with haemoglobin. In the deoxy state, it is bound non-covalently between two β-globin chains of a tetrameric haemoglobin molecule via ionic interactions with β-Val1, β-His2, β-Lys82 and β-His143 residues of both β-globin chains. Upon oxygenation of haemoglobin, 2,3-DPG dissociates from the tetramer, making these interacting residues free. Using H/DX-MS, we reported the change in conformational dynamics upon oxygenation of peptic peptide with m/z 1494.9 which comprised of N-terminal β-globin chain spanning residues 1–14 (refs 13, 14 and 17). Upon glycation of HbA, the N-terminal fragment appeared with m/z 1656.9. H/DX kinetics data showed that glycated normal haemoglobin (GHbA) had an increase in flexibility following the deoxy to oxy transition (ΔΣkiPi = 41.13). This increase was 5.62-folds less than that of HbA (ΔΣkiPi = 231.21) (Table 1). Figure 1a illustrates the best-fit curve of H/DX kinetics for this peptide of GHbE and GHbD Punjab. In the deoxy to oxy transition of GHbA, the flexibility of signature peptide after oxygenation was relatively less than that of HbA indicating that the required conformation of the oxy state of GHbA had been partly attained in the deoxy state itself. The presence of glucose moiety at N-terminus of β-globin chain causes reduction in the affinity of T-state of GHbA for 2,3-DPG by 2.6 times compared to T-state of HbA11. It has been reported that glycated haemoglobin has a higher affinity for oxygen compared to HbA11,18–20, thereby shifting its oxygen dissociation curve (ODC) to the left with a decreased P50 value, the partial pressure of oxygen required to saturate haemoglobin by 50%. Our observed results indicated that increased oxygen affinity might be attributed to the reduced affinity of modified haemoglobin towards 2,3-DPG.

Haemoglobin variants are also susceptible to glycation as this modification is functional group specific. We studied the conformational dynamics of glycated haemoglobin from samples of diabetic patients with haemoglobinopathies. Due to limited accessibility of diabetic variant sample, we conducted H/DX experiments in a mixed population of both native haemoglobin variants HbE and HbD Punjab, and their respective glycated forms, GHbE and GHbD Punjab in different sets. Using H/DX of peptide backbone amide hydrogens, we investigated the structural transition associated with oxygenation of both HbE and HbD Punjab and measured P50 values. Due to presence of both native and glycated haemoglobin variants in the experimental sample, the comparative structural analysis was restricted with signature peptide of glycated haemoglobin, N-terminal β-globin fragment. Figure 1b and c illustrates the best-fit curves of H/DX kinetics for this peptide of GHbE and GHbD Punjab in deoxy and oxy states respectively. Comparing kinetics data of HbE and GHbE, we observed a similar trend in the change in the rate of isotope exchange of peptide with m/z 1494.9 upon glycation of HbE, as observed for HbA (Table 1). The rate of isotope exchange of this peptide in deoxy GHbE was much higher (ΣkiPi = 324.08) than deoxy HbE (ΣkiPi = 24.54). Similarly, the rate of isotope exchange of deoxy GHbD Punjab (ΣkiPi = 312.13) was higher than deoxy HbD Punjab (ΣkiPi = 147.27). The increase in the flexibility of signature peptide after oxygenation of HbE was observed to be 2.14-folds, whereas for GHbE, it was 1.68-folds with a large increase in the flexibility of glycated fragment in its deoxy state. This indicated that compared to HbE, the oxygen affinity of GHbE is expected to be higher. Thus, there might be a left shift in the ODC for GHbE compared to HbE with a concomitant decrease in P50 value as observed during glycation of HbA. However, change in the flexibility of signature peptide after oxygenation of GHbD Punjab was found to be in the reverse direction.
Figure 1. H/DX kinetics of the peptide with m/z 1656.9 of GHbA, GHbE and GHbD Punjab in their deoxy (blue diamond) and oxy (red triangle) states. Panels a–c represent the best-fit curves of the kinetics data for this peptide of GHbA, GHbE and GHbD Punjab respectively. Y-axis is labelled with the number of deuterium incorporated and X-axis with the corresponding exchange time. m/z, Globin subunit, and residues of the peptide are indicated in each panel.

Table 1. H/DX kinetic parameters of peptic peptides of deoxy and oxy states of HbA, HbE and HbD Punjab and GHbA, GHbE and GHbD Punjab

| Peptide | Residues | Molecule       | \(\sum k_i P_{\text{oxy}}\) | \(\sum k_i P_{\text{deoxy}}\) | \(\Delta \sum k_i P_i = \sum k_i P_{\text{oxy}} - \sum k_i P_{\text{deoxy}}\) | Fold diff. = \(\frac{\sum k_i P_{\text{oxy}}}{\sum k_i P_{\text{deoxy}}}\) |
|---------|----------|----------------|-----------------------------|-------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------|
| 1494.9  | \(\beta^{1-14}\) | HbA            | 298.25                      | 67.04                         | 231.21                                                                          | 4.45                                                          |
| 1656.9  | \(\beta^{1-14}\) | GHbA           | 348.61                      | 307.47                        | 41.13                                                                          | 1.13                                                          |
| 1494.9  | \(\beta^{1-14}\) | HbE            | 52.39                       | 24.54                         | 27.85                                                                          | 2.14                                                          |
| 1656.9  | \(\beta^{1-14}\) | GHbE           | 543.71                      | 324.08                        | 219.63                                                                          | 1.68                                                          |
| 1494.9  | \(\beta^{1-14}\) | HbD Punjab     | 332.59                      | 147.27                        | 185.32                                                                          | 2.26                                                          |
| 1656.9  | \(\beta^{1-14}\) | GHbD Punjab    | 188.72                      | 312.13                        | -123.40                                                                         | 0.60                                                          |

compared to unmodified HbD Punjab. Upon oxygenation, the flexibility was increased by 2.26-folds in HbD Punjab whereas there was a decrease in flexibility (0.6-folds) of the signature peptide after oxygenation of GHbD Punjab (Table 1). This indicated that there might be a decrease in the oxygen affinity leading to right shift in the ODC of GHbD Punjab with concomitant increase in \(P_{50}\) compared to the native HbD Punjab.

Glutathionylation is another non-enzymatic PTM occurring under conditions of oxidative stress in a cell. Oxidative stress occurs when there is an imbalance between production of reactive oxygen species (ROS) and levels of antioxidants in the system. Under the condition of oxidative stress, the increased ROS is destroyed by the natural antioxidant, GSH present in the cell, which in turn gets oxidized to GSSG. In addition to the reduction of GSSG by glutathione reductase, excess GSSG might undergo thiol disulfide exchange with free accessible cysteine residues of proteins thereby forming glutathionylated proteins. Glutathionylated haemoglobin appears to serve as a potential biomarker of oxidative stress under several disease conditions. It has been shown that in HbA, only \(\beta\)-Cys93 is the preferred site for glutathionylation. Upon glutathionylation, the molecular mass of \(\beta\)-globin chain is increased by 305 Da. Previously, the structural perturbation induced on HbA upon glutathionylation was monitored using H/DX-MS. That data was reprocessed and reanalysed to compare the conformational transition of glutathionylated haemoglobin variants of HbE and HbD Punjab with that of GSHbA.

Sequence assignment of the peptic peptide with m/z 1921.0 confirmed it as a \(\beta\)-globin fragment consisting of residues 86–102 including the site of glutathionylation, \(\beta\)-Cys93. After glutathionylation, the same fragment appeared with m/z 2226.1. Figure 2a illustrates the best-fit curve of H/DX kinetics data for this peptide of GSHbA in deoxy and oxy states. On the basis of NMR data, Craescu et al. showed that the characteristic intra-subunit salt bridge \(\beta\)-Asp94–\(\beta\)-His146 and inter-subunit hydrogen bond at the \(\alpha\beta\) interface, \(\beta\)-Asp99–\(\alpha\)-Tyr42 in
Figure 2. H/DX kinetics of the peptide with m/z 2226.0 of GSHbA, GSHbE and GSHbD Punjab in their deoxy (blue diamond) and oxy (red triangle) states. Panels a–c represent the best-fit curves of the kinetics data for this peptide originated from GSHbA, GSHbE and GSHbD Punjab respectively. Y-axis is labelled with the number of deuterium incorporated and X-axis with the corresponding exchange time. m/z, Globin subunit, and residues of the peptide are indicated in each panel.

Table 2. H/DX kinetic parameters of peptic peptides of deoxy and oxy states of HbA, HbE and HbD Punjab and GSHbA, GSHbE and GSHbD Punjab

| Peptide | Residues | Molecule         | $\sum k_i P_{oxy}$ | $\sum k_i P_{deoxy}$ | $\Delta \sum k_i P_i$ | Fold diff. = $\sum k_i P_{oxy}/\sum k_i P_{deoxy}$ |
|---------|----------|------------------|-------------------|----------------------|-----------------------|-----------------------------------------------|
| 1921.0  | $\beta$86-102 | HbA              | 328.42            | 54.15                | 274.27                | 6.07                                           |
| 2226.0  | $\beta$86-102 | GSHbA            | 208.64            | 104.28               | 104.36                | 2.00                                           |
| 1921.0  | $\beta$86-102 | HbE              | 212.00            | 120.66               | 91.34                 | 1.76                                           |
| 2226.0  | $\beta$86-102 | GSHbE            | 510.18            | 353.84               | 156.34                | 1.44                                           |
| 1921.0  | $\beta$86-102 | HbD Punjab       | 331.75            | 294.03               | 37.73                 | 1.13                                           |
| 2226.0  | $\beta$86-102 | GSHbD Punjab     | 346.68            | 265.88               | 80.80                 | 1.30                                           |

the deoxy form of HbA, were perturbed significantly on glutathionylation. H/DX kinetics data of the peptide 2226.1 m/z showed an increase in the rate of exchange upon oxygenation of GSHbA ($\Delta \sum k_i P_i = 104.36$), which is 2.62-folds lower than that of unmodified HbA ($\Delta \sum k_i P_i = 274.27$) (Table 2). This decrease might be due to the increase in the conformational dynamics of deoxy form of GSHbA ($\sum k_i P_i = 104.28$) by 1.93-folds compared to the respective unmodified deoxy HbA ($\sum k_i P_i = 54.15$). It was concluded that glutathionylation induced conformational constraints such that GSHbA adopts an oxyhaemoglobin-like conformation in its deoxy state. GSHbA was reported to have high oxygen affinity with a significant left shift of ODC with lower $P_{50}$ value compared to HbA.$^{17}$

As glutathionylation is a functional group specific reaction, haemoglobin variants are also susceptible to this PTM under oxidative stress conditions. In this study, the conformational dynamics of in vitro synthesized glutathionylated haemoglobin variants was investigated. GSHbE and GSHbD Punjab yielded the molecular abundance of ~60%. As a result, the reaction mixture for H/DX experiments was a mixed population of unmodified and glutathionylated molecules. The structure–function correlation of native HbE and HbD Punjab upon oxygenation was investigated using H/DX and ODC of the respective variant haemoglobin. The presence of both native and glutathionylated haemoglobin molecules in the reaction set restricted our analysis with signature peptide 2226.1 m/z containing glutathione moiety attached to $\beta$-Cys93 in the respective variant haemoglobin molecules. Figure 2b and c shows the best-fit curves of H/DX kinetics data for this peptide of GSHbE and GSHbD Punjab in deoxy and oxy states, respectively. Comparing the rate of isotope exchange of 2226.1 m/z in GSHbE with that of 1921 m/z in HbE during deoxy to oxy transition, we observed a similar increase in the flexibility ($\Delta \sum k_i P_i = 156.34$) as observed for HbA ($\Delta \sum k_i P_i = 104.36$). The rate of isotope exchange of this peptide in deoxy GSHbE was
much higher ($\Sigma kP = 353.84$) than deoxy HbE ($\Sigma kP = 120.66$). The increase in the flexibility of signature peptide upon oxygenation of HbE was observed to be 1.76-folds whereas the same for GSHbE was 1.44-folds (Table 2). This indicated that the oxygen affinity of HbE after glutationation might be increased with a left shift in the ODC and lower $P_{50}$ value. In case of HbD Punjab, the rate of isotope exchange of signature peptide in deoxy GSHbD Punjab ($\Sigma kP = 265.88$) was close to that of deoxy HbD Punjab ($\Sigma kP = 294.03$). The increase in flexibility of the peptide 1921.0 m/z upon oxygenation of HbD Punjab was observed to be 1.13-folds, however, the increase in flexibility for the signature peptide 2226.1 m/z for GSHbD Punjab was observed to be slightly higher, 1.3-folds. This indicated that there might be an increase in the affinity of oxygen for GSHbD Punjab compared to its native haemoglobin with a lower $P_{50}$ value.

Based on the structure–function correlation of two PTMs of HbA, the conformational dynamics of glycated and glutationylated forms of HbE and HbD Punjab were used to predict the probable changes in the oxygen affinity of post-translationally modified HbE and HbD Punjab in comparison with their respective parent variant haemoglobin. It was expected that glycation and glutationylation of HbE might result in tighter oxygen binding compared to HbE. In case of HbD Punjab, we predicted that glycation might result in weaker oxygen binding whereas glutationylation was expected with tighter oxygen binding compared to native HbD Punjab.

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