ANALYSIS OF PROTEIN POSTTRANSLATIONAL MODIFICATIONS BY MASS SPECTROMETRY: WITH SPECIAL REFERENCE TO HAEMOGLOBIN

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ABSTRACT

Mass spectrometry provides a convenient platform for the study of different protein post translational modifications from clinical specimen. Analysis of different post translational modifications of hemoglobin like glycation and glutathionylation can provide useful information on the disease progression and the possible outcome of therapies. In the present study, we have addressed post translational modifications of hemoglobin like glutathionylation and glycation in relation to diabetes and chronic renal failure. We found that both alpha and beta chains of human hemoglobin are glycosylated irrespective of the extent of glycemia as evidenced by a mass increment of 162 Da. The phenomenon of glutathionylation was observed with only the beta globin chain of hemoglobin probably due to the presence of an accessible cysteine residue indicated by a mass increment of 305 Da. Also, the extent of glutathionylation observed in the CRF patients could correlate with the severity of the oxidative stress owing to renal replacement therapies like dialysis and transplantation.

KEY WORDS

Glutathionylation, Glycation, Oxidative stress, End-stage renal disease, Renal replacement therapy.

INTRODUCTION

Most proteins undergo post translational modifications (PTMs), which are chemical modifications that alter the properties of a protein after its translation, either by proteolytic cleavage or by addition of a modifying group to one or more amino acids. Knowledge of these modifications are extremely important because they may alter physical and chemical properties, folding, conformation, distribution, stability, activity, and consequently, functions of the proteins. The most important and best studied PTMs include reversible phosphorylation, glycosylation, acetylation, methylation, sulphation, disulphide bond formation, deamidation and ubiquitination (1-3). Study of post translational modifications in relation to specific disease conditions are of importance in understanding the underlying disease processes and the possible outcome of specific

treatment regimen. This has prompted a great deal of research in clinical proteomics in the recent past in quest of new markers for diseases owing to developments in the field of mass spectrometry. Mass spectrometry (MS) has had a profound impact on addressing the structural problems involving many proteomes and other biopolymers (4). Much of the success of MS in biology has been largely due to the introduction of 'soft-ionization' techniques like electrospray ionization mass spectrometry (ESI-MS) (5,6) and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) (7,8), which can provide valuable information about the primary and higher orders of protein structures along with modifications without any disruption of covalent linkages (9).

MS analysis has been particularly useful in identifying different variants of hemoglobin in conditions like thalassemias and hemoglobinopathies, which are inheritable genetic disorders. Another most important isoform of hemoglobin studied in relation to disease has been glycosylated hemoglobin or HbA_{1c}, which serves as an indicator of the average blood glucose concentration in patients suffering from diabetes. In the past few years, a new species of hemoglobin adduct known as glutathionyl hemoglobin, belonging to the class of glutathionylated proteins has been considered in clinical

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research in relation to oxidative stress and has been proposed as a biochemical marker of oxidative stress in relation to diseases like cardiovascular, neurodegenerative, diabetes and atherosclerosis (10-12).

Mass spectrometry enables characterization of proteins and protein complexes including their modifications directly, without much purification. With macromolecular proteins like hemoglobin, one can study such modification from whole blood hemolysates without purification and separation of alpha/beta chains. MS is the candidate reference method for modification analysis, because PTMs lead to either mass increment or decrement with respect to the molecular weight expected on the basis of native protein sequence.

In the present study we have tried to characterize an irreversible (glycated hemoglobin) and a reversible (glutathionyl hemoglobin) hemoglobin PTM using MS platform.

Our main interest was to study the phenomenon of glycation in type-II diabetics and glutathionylation in Chronic Renal Failure (CRF) patients. While the utility of glycated hemoglobin in a clinical setting is well documented, that of glutathionylated Hb is still under investigation.

MATERIALS AND METHODS

The samples were obtained from Manipal Hospital, Bangalore for controls and patients suffering from type-II diabetics without any history of renal impairment and chronic renal failure patients. Mass spectrometric analyses were carried out at the molecular biophysics unit (MBU) housed at Indian Institute of Science, Bangalore.

Venous blood collected in EDTA tubes were the sample of choice. CRF patients consisted of subjects who had progressed to end stage renal disease (ESRD) and were on renal substitutive therapies like hemodialysis (HD), continuous ambulatory peritoneal dialysis (CAPD/PD) and renal allograft transplantation (Txp) for a minimum period of 6 months to 1 year. Prior consent was obtained from the hospital ethics committee and the patients. The control group consisted of master health check up individuals without any history of diabetes or CRF.

Glycated hemoglobin (HbA_{1c}): was estimated on whole blood by automated D-10 HPLC system (Bio Rad, USA) equipped with cation exchange column.

Sample preparation: Venous blood was centrifuged at 3500

rpm for 10 minutes to separate the plasma and the buffy coat. The red blood cells at the bottom of the tube was washed with three volumes of ice cold saline (0.9% NaCl), vortexed and centrifuged and the supernatant discarded. The procedure was repeated for two more times to remove the plasma proteins. The washed packed cells were then lysed by adding eight volumes of ice cold distilled water and centrifuged for 10 mins at 12000 rpm in a refrigerated centrifuge to sediment RBC membranes. The clear red hemoglobin rich supernatant was used for MS analysis.

Hemoglobin glutathionylation: Glutathionyl Hb was prepared *in vitro*, by incubating an aliquot of human hemoglobin (3.5%) from the previous step with large excess of oxidized glutathione (GSSG – 67 mM) in 0.5 M ammonium bicarbonate buffer (pH 8) for 12 hrs at 37°C. The reaction mixture was passed through a sephadex G-10 column to remove excess glutathione and buffer salts before MS analysis.

Liquid chromatography-Electrospray Ionization/Mass spectrometry: Electrospray ionization mass spectra were acquired in the positive ion mode using a single quadrupole mass spectrometer (Hewlett Packard: HP 1100 MSD series). 12 µl of diluted RBC hemolysates (800 fold dilution with distilled water) were injected through a C₁₈ reverse phase column (Zorbax, 4.6 mm x 150 mm, 5 µm) coupled to ESI. Hemoglobin was eluted using a linear gradient of acetonitrile containing 0.1% acetic acid (for protonation). Spectra were acquired over the mass range 200-300 m/z with fragmenter voltage kept at 110 V. Mass scale calibration was done using ES-tuning mixture of proteins supplied by the manufacturer. Molecular mass information was obtained after specifying the molecular mass range, abundance cutoff, and minimum peaks in a set for each of alpha and beta (α & β) globin chains and their molecular adducts of interest. Deconvolution of multiply charged species was performed after smoothing the spectra using HP Chemstation software.

RESULTS

ESI spectra typically yields multiply charged protonated species with Gaussian distribution for a macromolecular protein like hemoglobin, with charge acquisition ranging from +10 to +30. However, the most intense signals for molecular ions corresponding to alpha and beta subunits of Hb and their corresponding adducts were distributed predominantly in the range of +17 to +23 charges.

Figure 1 shows the ESI mass spectrum of normal adult hemoglobin. Integration of alpha and beta sub-unit peaks from

Fig No.1 SERUM NITRIC OXIDE LEVELS IN DIFFERENT STAGES OF LIVER TRANSPLANTATIONS

Figure 1: ESI mass spectrum of human adult hemoglobin. The top panel depicts the total ion chromatogram and deconvoluted peaks for alpha and beta subunits with masses of 15125 Da and 15866 Da respectively. The bottom panel depicts the mass spectra of hemoglobin along with the charge distribution for alpha and beta subunits of Hb.

Figure 2: ESI mass spectrum of human adult hemoglobin showing alpha and glycated alpha peaks of masses 15125 Da and 15287 Da in the upper panel. Lower panel shows beta, glycated beta and glutathionylated beta with masses 15865 Da, 16028 Da and 16171 Da, respectively

total ion chromatogram followed by deconvolution (transformed mass scale) yielded two prominent peaks with masses 15125 Da and 15866 Da corresponding to alpha and beta subunits of hemoglobin. Figure 2 shows ESI mass spectrum of normal adult hemoglobin with alpha and beta subunits along with their adducts yielded masses of 15125 Da and 15287 Da corresponding to alpha and glycated alpha, and masses 15865 Da and 16028 Da corresponding to beta and glycated beta chain, respectively and another mass peak corresponding to 16171 Da for glutathionylated beta chain.

With respect to patients from diabetic population, we found a mass increment of 162 Da corresponding to addition of glucose molecule, with both alpha and beta chains as with normoglycemic individuals, but with heightened intensities for 15287 Da and 16028 Da corresponding to mono-glycated adducts of hemoglobin. With increasing HbA_{1c} values, we observed corresponding increase in the peak intensities of both alpha and beta glycated peaks, with higher peak intensity for beta compared to alpha

Figure 3: ESI mass spectrum of human adult hemoglobin from a severe diabetic individual showing alpha and beta chains along with intense peaks for glycosylated alpha, glycosylated beta and glutathionylated beta chains

glycation. A representative mass spectrum from a severe diabetic patient with an HbA_{1c} value of 17.1% shows heightened peak intensities for glycosylated alpha (15286 Da) and glycosylated beta adduct (16028 Da) with along with an intense peak for glutathionylated beta (16170 Da) unit (Figure 3)

With respect to glutathionylation, a mass increment of 305 Da corresponding to addition of one glutathione molecule was observed with only beta chain of hemoglobin. No such mass increment was observed with alpha chain indicating absence of glutathionylation.

Figure 4: ESI mass spectrum of *in vitro* glutathionylation of human hemoglobin showing an intense peak of mass 16171 Da corresponding to glutathionylated beta compared to the beta peak of 15866 Da as shown in the inset.

In order to check the validity of the 16171 Da peak as glutathionyl beta, *in vitro* glutathionylation with oxidized glutathione (GSSG) yielded an intense peak of mass 16171 Da as shown in figure 4. To confirm the identity, an aliquot of glutathionyl Hb was incubated with 1, 4-Dithiothreitol (DTT) for two hours at 37°C and the resultant mixture was injected into the LC-ESI/MS, which showed complete absence of the 16171 Da peak.

The peak intensities observed with respect to different RRT groups showed a trend with glutathionylation. While the peak intensities observed with transplant group was almost similar to that of controls, dialysis group showed augmented intensities with most intense peaks observed in HD group. With respect to glycation, no much change was observed with transplant and the HD groups but PD group showed augmented peaks with beta glycation (Figure 5).

Figure 5: ESI mass spectrum of beta chain of human hemoglobin from non-diabetic ESRD patients. Panels (A) (B) and (C) show beta chain with its adducts of glycation and glutathionylation for Transplant, peritoneal dialysis and hemodialysis patient respectively

DISCUSSION

The study of different hemoglobin postranlational modifications has generated a lot of interest in clinical research, particularly in cases of chronic diseases like diabetes, uremia, and some neurodegenerative diseases. While in some cases the variations in the levels of a particular hemoglobin isoform has been correlated with the severity of the disease; as in case of glycated hemoglobin, in some like GS-Hb, they have been implicated in the pathogenesis and progression of the disease (13). In this regard GS-Hb has been recommended as a candidate molecule where oxidative stress has been implicated as a co-morbid factor (14).

Our study assumes importance as we have been able to successfully employ the mass spectrometric platform to address the structural modifications of hemoglobin directly from erythrocyte hemolysates without fractionation and separation of alpha/beta chains of Hb. The reason for selection of hemoglobin as a candidate molecule was because of its abundance, easy accessibility and a well characterized structure and functional relationship.

Hb-Glutathionylation: GSH, an important intracellular antioxidant, scavenges free radicals and prevents the oxidation of protein thiol groups, and gets itself oxidized to form GSSG in response to oxidative and nitrosative stresses (15, 16). GSSG reacts with thiol groups of proteins to form S-glutathionylated proteins (GSSPs). This reversible non-enzymatic modification could be an adaptive response to protect the critical thiol groups in proteins and may serve to transduce oxidative stimuli into a functional response at various levels of cellular signaling (17, 18). Thus in addition to the GSH/GSSG redox couple, the content of glutathionylated proteins (GSSPs) can indicate oxidative stress (19). Thus, it was of interest to analyze the glutathionylated adduct of Hb in ESRD patients on RRT, which is considered a stressful event, especially dialysis. This is reflected in GS-Hb peaks of dialysis patients; HD group in particular, where we found that the intensities were higher compared to transplant, which did not show any significant difference than that of controls.

A number of previous studies have demonstrated that uremia *per se* represents a pro-oxidant state (20, 21), which is further enhanced by substitutive therapies, especially dialysis where low values for cellular GSH have been reported (22-25). So it is plausible to consider that the concentration of GS-Hb would be higher in the dialysis group compared to transplants owing to factors like bioincompatible dialysis membranes, solutions, loss of valuable vitamins like ascorbate in dialysis, malnutrition, inflammation, iron therapies, etc., all of which could be responsible for an increased oxidative stress (26). In case of transplant patients, though persistence in oxidative stress has been documented, it seems to be more of a contribution from immunosuppressive regimen with drugs like cyclosporine (27, 28).

While it is clear that the mass increment of 305 Da with beta globin chain of Hb could be due to glutathionylation, absence of any such mass increment with alpha chain indicates that glutathionylation did not happen with alpha subunit. The primary structure of alpha chain has one cysteine residue at α -102 while beta has two cycteine residues at positions β -112 and β -93. Absence of glutathionylation with alpha subunit

indicates that the cysteine residue is not accessible for thiol exchange as supported by accessibility co-efficient ($0.0\text{\AA}^2$), whereas with beta chain presence of monogluthionylated peak indicates that one of the two cysteines got glutathionylated. Of the two cysteines in beta chain, Cys-93 is found to be liable for thiol exchange reactions owing to its surface accessibility ($10.9\text{\AA}^2$) compared to β Cys-112 ($2.4\text{\AA}^2$). This is in agreement with previous findings that only β Cys-93 is preferred for thiol exchange reactions, making it the reactive cysteine residue (29-31). Thus, glutathionylation should happen with cys-93 of beta globin chain of hemoglobin showing a mass of 16171 Da. Whether glutathionylation merely serves as an indicator of oxidative stress or a definitive role can be assigned to this PTM in the pathogenesis/progression of the disease needs to be further evaluated.

Hb-Glycation: The transformed mass spectra from normoglycemic and the diabetic patients principally showed peaks corresponding to moglycated adducts of alpha and beta chains. Traditionally the HbA_{1C} values used for clinical assessment of diabetes refers to attachment of a glucose moiety to the terminal valine residue of the beta globin chain of hemoglobin (as per IFCC) and does not include alpha glycation. But, when we are measuring total glycatd Hb, it should necessarily include both alpha and beta glycations as evidenced in our MS study. As per Variant and other commercially available HPLC based methodologies, including the equally popular boronate affinity chromatography, an equivalent of about one globin per alpa-beta dimer of the Hb carries glycation (32). So in all probability the focus is on beta glycation. But as per our observation, we found that signal intensities for both the alpha and beta glycations were significantly higher corresponding to higher HbA_{1C} values in diabetics, though the peak intensities for beta was comparatively higher, an observation similar to that of Roberts *et al* (33). Also, there have been some serious debate as to whether the A_{1C} species measured in automated HPLC method is truly HbA_{1C} or it also measures alpha glycation, especially in the diabetic range (>7% HbA_{1C}). What ever be the species (glycated alpha and beta, nonglycated alpha and glycated beta) that co-elutes along with HbA_{1C} is reported as HbA_{1C} in cation exchange HPLC (34). The same may be true with some of the certified boronate affinity chromatographic methods (32) as they are essentially calibrated using HPLC methods as the standard reference. With MS, the glycation identified with beta chain and quantified by some groups, either by ESI-MS or MALDI-MS, have pointed to the fact that HbA_{1C} measured by HPLC shows good correlation with beta glycation, which in true sense should be considered as 'chemical HbA_{1C}' (*sans* alpha glycation).

Mass spectrometry provides an accurate and unambiguous tool for characterization of different clinically relevant protein PTMs. Glutathionylation of hemoglobin happens with only beta chain Hb due to an accessible cysteine residue in the β subunit. GS-Hb can serve as a marker of oxidative stress in chronic diseases like uremia. Glycation of hemoglobin indicates that both alpha and beta globin chains are glycated, though the extent of alpha glycation is lesser compared to its beta counterpart. Clinically relevant HbA_{1C} and beta chain glycation could be the same entity.

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