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D-Ribose induced glycoxidative insult to hemoglobin protein: An approach to spot its structural perturbations

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ABSTRACT

Glycation of biological macromolecule leads to the establishment of advanced glycation end products (AGEs) having implications in metabolic disorders. D ribose appears to be the most reactive among the naturally occurring sugars and contribute significantly to the glycation reactions *in vivo*, however, no report have been published yet to discuss D ribose induced glycation of hemoglobin (Hb). Therefore, the present study was designed to investigate D ribose induced glycoxidative damage to Hb protein. Briefly, the commercially available Hb was glycated with D ribose for varying time intervals. The structural perturbation induced in glycated Hb (GHb) was confirmed by biophysical techniques *viz*., UV–visible, fluorescence spectroscopy, circular dichroism, Fourier transform infrared spectroscopy, dynamic light scattering, MALDIthermal denaturation by UV–visible spectrophotometer and DSC. Biophysical techniques confirm the secondary and tertiary structural perturbation in GHb as compared to native Hb. The values of carbonyl content, hydroxy methyl furfural, thiobarbituric acid reactive substance and nitro blue tetrazolium were found to be increased and free lysine and free arginine content were decreased in the GHb due to structural change. Thus, results of this study have established that glycation with D ribose lead to the structural changes in the native Hb which might play an important role in pathophysiology metabolic diseases.

1. Introduction

Glycation (Maillard Reaction) of protein begins with the complex series of sequential non-enzymatic reactions between the carbonyl group of reducing sugars and nucleophilic free amine groups of the biomolecules, which is the driving force for the formation of many products including 'Schiff's base' [1]. These bases rearrange through acid-base catalysis reaction to produce stable products known as ketoamine or 'Amadori products [2]. After the Amadori product formation, the reaction becomes more diverse and complicated and consequently forms fluorescent cross linking adducts called advanced glycation end products (AGEs) *via* dehydration, cyclization and oxidation [3]. AGE formation is a slow and continuous process under normal physiological conditions, but due to the persistent hyperglycemic condition, this process is enhanced 5–6 fold [4]. Glycation causes structural perturbations in the secondary and tertiary level of protein structure and ultimately interfere with the stability and normal functioning of the protein [5]. Glycation rate basically depends upon monosaccharides concentration, anomerization rate and is inversely proportional to the number of carbon atoms present in the reducing monosaccharide [6].

Previous studies strongly suggest the accumulation of AGEs in human and animal tissues during aging. Association of AGEs with V-domain of RAGE, increase the pathophysiology of various diseases, including diabetes, diabetic microangiopathy, nephropathy, atherosclerosis [7], neurodegenerative related disease such as Alzheimer's disease, Parkinson's disease [8] and also cystic fibrosis [9].

Hb is a tetrameric, heme containing globular protein having four globin chains *i.e.* two α (141 amino acids) and two β (146 amino acids), which are attached with each other by non-covalent interactions. The molecular weight of Hb is 64.45 kDa and its concentration in the red blood cell is 330 mg/ml [10]. Hb contains six tryptophan residues present near the heme moiety which are exposed to the quaternary confirmation of the molecule [11]. The *in vivo* key function of Hb is to bind and deliver oxygen to various tissues of the body parts. Glycated human hemoglobin (HbA1c) is primarily detected *in vivo* among all the glycated proteins [12]. HbA1c level is constantly increased in diabetic patients

Abbreviations: AGEs, advanced glycation end products; Hb, Hemoglobin; FTIR, Fourier transform infra-red spectroscopy; CD, circular dichroism; DLS, dynamic light scattering; DSC, differential scanning calorimetry; MALDI, matrix assisted laser desorption/ionization; NBT, nitro blue tetrazolium; HMF, hydroxymethyl furfural; CC, carbonyl content; TBARS, thiobarbituric acid reducing substance; FI, fluorescence intensity.

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with continuous hyperglycemic stage. The measurement of HbA1c level helps in the detection and management of diabetes mellitus [13]. Under normal physiological condition, level of HbA1c is approximately 5–6% of normal HbA but this value can be increased to 15% or more in diabetic condition. This elevation of HbA1c level is responsible for long term insulin resistance [14].

Glycation of Hb is not only restricted to N-terminal valine residue of β subunit, but also possible at other positions on the α and β subunits in which glucose moiety is directly linked to an ε-amino group of one or more lysine residues (Lys 17, Lys 59, Lys 61, Lys 65, Lys 66, Lys 82, Lys 95, Lys 120, Lys 132 and Lys 144) [15]. Sometimes arginine containing guanidinium group are also involved in the glycation of Hb. Therefore, glycation of Hb causes biological, physiological, conformational and structural perturbation, which depends on the degree of glycation. Meanwhile, studies revealed that GHb causes iron release from Hb molecule that involved in Fenton reaction. Fenton reaction enhances the oxidative stress and damage the host cell [11].

As we know, glucose is the most abundant sugar in the every living organism and the glycation of protein with glucose are very well documented [16,17]. According to current research, it is also revealed that not only glucose, but also other reducing monosaccharides can be good candidates for the glycation process because of their extreme reactivity compared to glucose. In this regard, ribose is very reactive among the naturally occurring monosaccharides [18]. D ribose is a basic component of riboflavin, ribonucleic acids and adenosine tri-phosphate (ATP). It has been shown that D ribose has the capacity to react with proteins and produce various glycated derivatives. The level of D ribose in the human body is about ~100 μM [19], but some study also reported the concentration of free ribose in the human blood plasma is 7 μM (0–17 μM) [20].

To the best of our knowledge, so far no study has been performed on D-ribose induced glycation of Hb; even though D ribose is a very reactive sugar. However haemophilia, haemolytic anaemia, diabetes mellitus and other types of diseases are associated with the alteration in Hb molecule [21]. Therefore, we have carried out a detailed study of D ribose induced glycoxidative deformation and perturbation of the Hb macromolecule. The conformational and structural changes induced in the Hb macromolecule were studied by spectroscopic and biophysical techniques such as UV–visible spectroscopy, Fourier transform infrared spectroscopy(FTIR), dynamic light scattering (DLS), circular dichroism (CD) and matrix assisted laser desorption/ionization(MALDI) as well as we have also determined the thermal denaturation of Hb before and after glycation by using reducing sugar D ribose by UV–visible spectrophotometer and differential scanning calorimetry (DSC). The level of ketoamine content was also estimated with reference to the non-glycated Hb by nitro blue tetrazolium (NBT) reduction assay. Moreover, generation of hydroxymethyl furfural (HMF), thiobarbituric acid reactive substance (TBARS) and carbonyl content (CC) were also estimated to confirm the generation of free radicals. Detection of side chain modification was done by free lysine and free arginine residue estimation. Characterization of Hb specifically by MALDI has been the subject of some degree of sample purification that was most often required. For instance, MALDI was used to examine the extent of glycation of Hb as a possible indicator for diabetes [22]. Delayed extraction, combined with reflecting time-of-flight (TOF) mass analysis, provides high resolution and allows accurate mass measurements in the ppm range for sample components of fairly complex mixtures [22]. MALDI-TOF MS has therefore been used by many research groups as a convenient and rapid means for the characterization of proteins and peptides derived from biological samples.

2. Material and methods

2.1. Materials and chemicals

Hemoglobin (Hb) was obtained from MP Biologicals, sodium carbonate, sodium bicarbonate, sodium di hydrogen phosphate, di sodium hydrogen phosphate, sodium chloride, D ribose, nitro blue tetrazolium (NBT), oxalic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), sodium hydroxide, sodium chloride and phosphotungstic acid were obtained from Hi-Media. Sodium azide and ethanol were obtained from Merck and 2, 4, 6 trinitrobenzene 1 sulphonic acid (TNBS) was purchased from G-Biosciences. Phenanthrenequinone and triphenylphosphine were purchased from Sigma Aldrich. Ethylacetate and dinitrophenylhydrazine (DNPH) were purchased from Rankem. Guanidium hydrochloride was purchased from S. D. Fine-chem limited. Hydrochloric acid (HCl) was purchased from Fisher Scientific. All other chemicals used in this study were of analytical grade. All the solutions were filter sterilized using Puradisc TM 0.2 mM syringe filter (Whatman, GE Healthcare UK limited, UK) and were dispensed into culture tubes, under aseptic conditions.

2.2. Methods

2.2.1. Glycation of hemoglobin (Hb)

In the present study, commercially available Hb (50 μg/ml) was modified with varying concentration of D ribose (1, 2, 5 and 10 mM) at 100 mM phosphate buffer saline (PBS), pH (7.4) under sterile conditions. The reaction mixture was incubated at 37 °C for varying time periods (1–21 days) followed by the extensive dialysis against PBS to remove unbound constituents. Native Hb was served as control. The reaction mixture was stored at −20 °C for further use.

2.2.2. Absorbance spectroscopy

The spectral profiles were done as per previous published literature [23]. The absorption spectra of the native and glycated Hb (GHb) samples, incubated for varying time periods (1–21 days), were recorded on Biospectrum-Kinetics spectrophotometer (Eppendorf) in the wavelength range of 220–400 nm by using a quartz cuvette of 1 cm path length. Individual aliquots of the reaction mixtures were analyzed for the absorbance at 280 nm. Hyperchromicity can be calculated by using following formula

$$\%\ \text{Hyperchromicity at 280 nm} = \left(\frac{\text{OD of Glycated Hb} - \text{OD of Native Hb}}{\text{OD of Glycated Hb}}\right) \times 100 \quad \text{(i)}$$

2.2.3. Nitro Blue Tetrazolium (NBT) reduction assay

Amadori products (fructosamine) in the glycated protein samples were determined by NBT reduction test [24]. The native and glycated protein sample (20 μl) was mixed with 180 μl of 100 mM sodium carbonate-bicarbonate buffer (pH 10.8) containing 0.25 mM NBT and incubated at 37 °C for 25 min. The final absorbance was recorded at 525 nm against 100 mM sodium carbonate – bicarbonate buffer of pH (10.8). Absorbance values were recorded each day by removing aliquots from the reaction mixture to determine the presence of ketoamine moieties. Finally, the content of amadori products (nM/ml) was determined by using an extinction coefficient of 12,640 M^{-1} cm^{-1} [25].

2.2.4. Hydroxymethyl furfural (HMF) content estimation

HMF content in the native and GHb samples were quantitatively estimated as described previously by thiobarbituric acid assay (TBA) [26]. HMF is an intermediary product of the Maillard reaction and was investigated in the native and glycated sample of the protein. HMF content in the native and glycated protein samples were estimated by mixing 1 ml sample with 1 M of oxalic acid. The mixture was kept in a water bath for 1 h, and 40% of trichloroacetic acid (TCA) was mixed in the solution after cooling at room temperature. TBA was mixed after removing precipitate by filtration. This mixture was incubated for half an hour. The colour was developed and the amount of HMF (nmol/ml) was calculated using a molar extinction coefficient value of 4×10^4 M^{-1} cm^{-1} at 443 nm.