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Glyoxal-induced modification enhances stability of hemoglobin and lowers iron-mediated oxidation reactions of the heme protein: An in vitro study

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ABSTRACT

Glyoxal, a reactive α-oxoaldehyde, increases in diabetic condition. It reacts with different proteins to form advanced glycation end products (AGEs) following Maillard-like reaction. Considering the significance of AGE-mediated protein modification by glyoxal, here we have investigated the in vitro effect of the reactive α-oxoaldehyde (10, 20 μM) on the heme protein hemoglobin (HbA_0) (100 μM) after incubation for one week at 25 °C. In comparison with HbA_0, glyoxal-treated HbA_0 exhibited decreased absorbance around 280 nm, reduced intrinsic fluorescence and lower surface hydrophobicity. Glyoxal treatment was found to increase the stability of HbA_0 without significant perturbation of the secondary structure of the heme protein. In addition, H_2O_2-mediated iron release and subsequent iron-mediated oxidative (Fenton) reactions were found to be lower in presence of glyoxal-treated HbA_0 compared to HbA_0. Mass spectrometric studies revealed modification of arginine residues of HbA_0 (Arg-31α, Arg-40β) to hydroimidazolone adducts. AGE-induced modifications thus appear to be associated with the observed changes of the heme protein. Considering the increased level of glyoxal in diabetes mellitus as well as its high reactivity, glyoxal-derived AGE adducts might thus be associated with modifications of the protein including physiological significance.

1. Introduction

Post-translational modifications of proteins play important roles in controlling their functions. Reducing sugars react with amino groups of proteins, a process known as non-enzymatic glycation (Maillard reaction) resulting in browning, fluorescence and cross-linking of proteins [1]. The reaction consists of several steps, including Schiff's base formation, Amadori rearrangement etc. finally leading to formation of advanced glycation end products (AGEs). Formation of AGEs in vivo contributes to pathophysiologies associated with aging and complications of diabetes [2], which may be significant due to increased levels of several active carbonyl compounds.

The reactive α-oxoaldehydes namely, glyoxal, methylglyoxal and 3-deoxyglucosone are known to initiate Maillard-like reactions and are more reactive than the parent hexose sugars with respect to their ability of protein modification and AGE formation [3,4]. Methylglyoxal is mainly derived from triose phosphates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate during glycolysis in eukaryotic cells and its blood level increases in both type 1 and type 2 diabetes mellitus [5–7]. It has been reported to react with several proteins namely, hemoglobin, cytochrome c, myoglobin, etc., resulting in either protein cross-linking and aggregation or formation of non-crosslinking AGE adducts (namely, hydroimidazolones, argpyrimidine, carboxyethyllysine, etc.) [8,9–12].

Like methylglyoxal, glyoxal is another reactive oxoaldehyde and its concentration increases from 215 to 230 nM in normal individuals to 350–470 nM in diabetic subjects [7]. It is a major product of glucose degradation under oxidative conditions [13] and has been reported to interact with several proteins, namely, α-crystallin [14], bovine serum albumin [15], myoglobin [16] and hemoglobin [17,18]. Glyoxal predominantly modifies lysine and arginine residues of proteins to form several products, such as carboxymethyllysine [19], carboxymethylarginine [20], dihydroxyimidazolidines and hydroimidazolones.

Several findings have reported interaction of methylglyoxal with hemoglobin leading to their modifications in vivo and in vitro [8,9,21,22]. In recent studies, glyoxal has been found to induce structural modifications of hemoglobin in vitro [17,18]. We have recently reported glyoxal-induced aggregation of hemoglobin

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in vivo [23]. However, in the above studies, only glyoxal-induced structural modifications of the heme protein have been reported and no effect of the reactive dicarbonyl compound on any functional properties of the hemoglobin has been reported. Considering this, in the present study we have characterized glyoxal-modified hemoglobin with respect to changes in structure, stability and function in connection to the above findings and correlated the same with the dicarbonyl-induced AGE modifications. Possible implications of the changes have been further discussed.

2. Materials and methods

2.1. Materials

Glyoxal, Sephadex G-100, 1-anilino-naphthaline-8-sulfonate (ANS), sequencing grade trypsin, α-cyano-hydroxycinnamic acid matrix (CHCA), Deoxyribose, Ferrozine, Hydroxylamine Hydrochloride, Thiobarbituric acid (TBA) were purchased from Sigma Chemical Company, USA. Biorex-70 resin (200–400 mesh) was obtained from Bio-Rad, India. All other reagents were AR grade and purchased locally.

2.2. Methods

2.2.1. Separation of non-glycated hemoglobin (HbA_0) from blood sample

Human blood samples were collected from healthy volunteers (non-smokers) aged 20–25 years following ethical principles formulated by the Institutional Ethics Committee. Total hemoglobin was isolated and purified from whole blood sample by using Sephadex G-100 size-exclusion column chromatography [24]. Different glycated hemoglobin species (HbA_{1a1}, HbA_{1a2}, HbA_{1b} and HbA_{1c}) and non-glycated hemoglobin (HbA_0) were separated from total hemoglobin following the method of Cohen and Wu [1]. The concentration of HbA_0 was determined from Soret absorbance using an extinction coefficient (ε_{415nm}) of 125 mM^{-1} cm^{-1} (heme basis) [25].

2.2.2. In vitro reaction of hemoglobin (HbA_0) with glyoxal

HbA_0 (100 μM) was incubated with two different glyoxal concentrations (10, 20 μM) under sterile conditions for 7 days at 25 °C. For control experiments, HbA_0 solution was incubated without glyoxal under identical conditions.

2.2.3. Spectrophotometric study

Absorption spectra of control and glyoxal-incubated samples (5 μM each) were recorded in the region 250–600 nm in a UV/VIS Spectrophotometer (Hitachi U 2000) using 1 ml quartz cuvette of path length 1 cm.

2.2.4. Spectrofluorimetric study

Intrinsic fluorescence emission of control and glyoxal-incubated samples (5 μM each) were monitored in a spectrofluorimeter (Hitachi F-3010) after excitation at 280 nm using 3 ml quartz cuvette of path length 1 cm.

For ANS binding study, the fluorescence emission spectra of the samples were recorded with excitation at 370 nm after incubating the samples (5 μM each) with ANS (20 μM) for 10 min at room temperature.

2.2.5. CD study

Far UV CD spectra (190–250 nm) of control and glyoxal-treated samples (3 μM each) were recorded in a spectropolarimeter (Jasco 600) using 1 mm path length cuvette. The α-helical contents of the proteins were determined according to the method of Chen et al. [26].

2.2.6. Stability studies

Conformational stability of samples was measured by fluorescence and CD studies. Chemical denaturant-induced unfolding was studied by recording fluorescence emission spectra (300–400 nm) of samples (5 μM each) with excitation at 280 nm after overnight incubation with guanidine hydrochloride (1 M). CD spectra of the samples (3 μM each) were also recorded and compared in the far UV region after overnight incubation with guanidine hydrochloride (1 M).

Thermal stability of protein samples was investigated by CD studies. CD spectral pattern of control and glyoxal-incubated samples (3 μM each) were recorded in the far UV region after thermal treatment at 70 °C, 75 °C, 80 °C and 85 °C for 10 min, respectively.

2.2.7. MALDI-TOF mass spectrometry

Control and glyoxal-treated HbA_0 were digested with sequencing-grade trypsin in solution at 37 °C for 16 h using enzyme: protein ratio 1:100 (w/w) and subjected to mass spectral analysis using the linear positive ion mode of MALDI-TOF MS. Digested samples (0.5 μl each) were loaded directly to the MALDI plate, mixed with 0.5 μl of saturated CHCA solution (prepared in 50% acetonitrile and 0.1% trifluoroacetic acid) and allowed to dry and crystallize. Mass spectra were subsequently recorded in a 4800 Proteomics Analyzer (MALDI-TOF/TOF mass spectrometer, Applied Biosystems) using the linear positive ion mode of MALDI-TOF MS at 20 kV acceleration voltage.

For identification of probable modified peptides and the specific glyoxal derived AGEs formed, a theoretical digestion of the heme protein was performed, considering up to two trypsin miscleavages (peptidemass, Expasy, http://www.expasy.ch/tools/peptide-mass.html) and peptide masses with specific mass increments due to AGE adducts were searched. The particular peptides of interest (i.e. having mass consistent with the mass increment due to AGE) were selected for MS/MS fragmentation by Collision Induced Dissociation (CID) to obtain sequence information using 1 kV collision energy. 1000 laser shots were collected for each MS/MS spectrum using a fixed laser intensity of 4500 V. Raw data was generated by using GPS Explorer Software. The identification of AGE modified peptides were done by manual interpretation of the MS/MS spectra.

2.2.8. Estimation of free iron

H_2O_2-induced iron release from HbA_0 and glyoxal-treated HbA_0 was estimated according to the method of Panter [27]. Protein samples (50 μM each) were incubated with H_2O_2 (250 μM) at 37 °C for 1 h. 250 μl cold TCA (20%) was added to 250 μl sample. 250 μl protein-free supernatant was treated with 2.5 ml iron buffer reagent (1.5% hydroxylamine hydrochloride in 0.2 M acetate buffer, pH 4.5) and 50 μl iron color reagent (0.85% ferrozine in iron buffer reagent), incubated at 37 °C for 30 min and absorbance was measured at 560 nm.

2.2.9. Deoxyribose degradation

Deoxyribose (0.67 mM) was incubated with HbA_0 or glyoxal-treated HbA_0 (10 μM) in presence of H_2O_2 (0.67 mM) for 1 h at 37 °C. TBA reactivity was developed by adding TBA (1%) and TCA (2.8%) and then heated for 10 mins in a boiling water bath. The resulting chromogen was extracted with *n*-butanol and fluorescence intensity was measured at 553 nm by exciting at 523 nm.

2.2.10. Oxidative DNA degradation experiment

For DNA degradation experiment, the reaction mixture (250 μl) containing plasmid (pGEM, 3 Kb) DNA (approximately 300 ng), HbA_0 or glyoxal-treated HbA_0 (10 μM) and H_2O_2 (250 μM) was