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Structural alterations of hemoglobin and myoglobin by glyoxal: A comparative study

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ABSTRACT

Glyoxal, a highly reactive oxoaldehyde, increases in diabetic condition. It reacts with different proteins to form advanced glycation end products (AGEs). Here we have studied the structural alterations as well as the sites and nature of amino acid modifications of two heme proteins, hemoglobin and myoglobin on incubation with glyoxal for seven days at 25 °C. In comparison with normal hemoglobin (HbA_0), glyoxal-treated hemoglobin ($GHbA_0$) exhibits decreased absorbance around 280 nm, reduced intrinsic fluorescence and lower surface hydrophobicity. However, glyoxal-treated myoglobin (GMb) exhibits the opposite effects in these respects when compared to normal myoglobin (Mb). Glyoxal increases the thermal stability of hemoglobin, while it decreases the stability of myoglobin. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)–mass spectrometry reveals modifications of Arg-31α, Arg-40β and Arg-104β of hemoglobin by glyoxal to hydroimidazolone adducts. On the other hand, glyoxal modifies Lys-133 and Lys-145 to carboxymethyllysine and Arg-31 to hydroimidazolone adducts in myoglobin. Thus the same oxoaldehyde exerts different effects on hemoglobin and myoglobin and may be associated with different structural properties of the proteins.

1. Introduction

Reducing sugars react with amino groups of proteins, a process known as non-enzymatic glycation (Maillard reaction) resulting in browning, fluorescence and crosslinking 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].

The α-oxoaldehydes namely, glyoxal, methylglyoxal and 3-deoxyglucosone are produced in different pathways including glycation reactions [3]. These compounds are highly reactive and known to cause protein modification and AGE formation more effectively than the parent hexose sugars [4,5]. Methylglyoxal has been reported to react with several proteins namely, cytochrome c, ceruloplasmin, myoglobin, hemoglobin, etc., resulting in either protein cross-linking and aggregation or formation of non cross-linking AGE adducts [6–10]. Like methylglyoxal, glyoxal is another reactive oxoaldehyde, and its concentration increases from 215–230 nM in normal individuals to 350–470 nM in diabetic subjects [11]. Glyoxal is a major product of glucose degradation under oxidative conditions [3]. Fructose, arabinose and ascorbate may also degrade to glyoxal, possibly through intermediate adducts to proteins. Glyoxal is formed directly during oxidative degradation of polyunsaturated fatty acids [12] and myeloperoxidase-mediated degradation of serine at sites of inflammation [13]. It has been reported to interact with several proteins, namely, α-crystallin [14], bovine serum albumin [15], α-synuclein [16] and hemoglobin [17]. Glyoxal modifies predominantly lysine and arginine residues of proteins to form several products, such as carboxymethyllysine (CML) [18], carboxymethylarginine (CMA) [19], dihydroxyimidazolidines (G-DH1 and G-DH2) and hydroimidazolones (G-H1, G-H2 and G-H3). It enters into red blood cells (RBC) and reduces their deformability probably by interacting with the cellular proteins [20].

Recent findings from our laboratory have shown that methylglyoxal interacts with the heme proteins myoglobin and hemoglobin leading to their modifications [8,10]. However, methylglyoxal-induced modifications differ with respect to structural changes, site of modifications and nature of AGEs formed in hemoglobin and myoglobin. The findings have prompted us to undertake the present study. Myoglobin, a simple monomeric protein, associated with storage and transfer of oxygen, exhibits higher percentage of α helicity and greater thermal stability compared to hemoglobin, a tetrameric protein associated with transport of oxygen. Considering increased concentration of glyoxal in diabetic

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condition as well as its high reactivity, we have studied structural alterations of hemoglobin and myoglobin, after in vitro reaction with glyoxal under identical conditions. In a recent study [17], hemoglobin–glyoxal interaction has been reported to promote aggregation and AGE cross-linking with quite high concentrations of glyoxal (20–90%, v/v). In the present study, we have used much low concentration of glyoxal (50 μM) to find how it affects the structure and stability of hemoglobin and myoglobin together with the sites and nature of adducts formed on the modified proteins.

2. Materials and methods

2.1. Materials

Horse heart myoglobin, glyoxal, Sephadex G-100, acrylamide, 1-anilino-naphthaline-8-sulfonate (ANS), Coomassie R250, sequencing grade trypsin and α-cyano-hydroxy cinnamic acid matrix (CHCA) 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. Separation of non-glycated hemoglobin (HbA_0) from blood sample

Blood samples were obtained from healthy non-smoking human subjects aged 25–30 years. Total hemoglobin was isolated and purified from RBC by using Sephadex G-100 column chromatography in 50 mM phosphate buffer (PB), pH 6.6. Non-glycated hemoglobin (HbA_0) was separated from total hemoglobin by cation exchange chromatography using Biorex-70 resin in elution buffer PB [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) [21].

2.3. In vitro reaction of HbA_0 and myoglobin (Mb) with glyoxal

Mb was dissolved in PB and its concentration was determined using ε_{408nm} = 116 mM^{-1} cm^{-1} [21]. 100 μM each of HbA_0 and Mb were separately incubated with different concentrations of glyoxal (5, 10, 20 and 50 μM) under sterile conditions for 7 days at 25 °C. For control experiments, HbA_0 or Mb solution was incubated in the absence of glyoxal under identical conditions.

2.4. Polyacrylamide gel electrophoresis (PAGE)

The incubated samples were subjected to native PAGE (10%) for 3 h at constant voltage (60 V). Control Mb and 50 μM glyoxal-treated Mb sample were also applied to SDS-PAGE (10%). For electrophoresis, 15 μl of each protein sample (15 μM) was loaded, followed by staining with Coomassie R250.

2.5. Spectrophotometric study

The absorption spectra of control and glyoxal-incubated samples (6 μM each) were recorded in the region 250–650 nm in a UV/VIS Spectrophotometer (Hitachi U 2000) using 1 ml quartz cuvette of path length 1 cm.

2.6. Spectrofluorimetric study

Fluorescence emission spectra of control and glyoxal-incubated samples (6 μM each) were monitored in the region 300–400 nm in a spectrofluorimeter (Hitachi F-3010) with excitation at 280 nm using 3 ml quartz cuvette of path length 1 cm.

For ANS binding study, the samples (8 μM each) were incubated with 20 μM ANS for 10 min at room temperature and the fluorescence emission spectra (450–600 nm) were recorded with excitation at 370 nm.

2.7. Circular dichroic (CD) study

CD spectra of control and glyoxal-treated samples (3 μM each) were recorded in a spectropolarimeter (Jasco 600) using 1 mm path length cuvette in the far UV region (190–250 nm). The α-helical contents of the proteins were determined according to the method of Chen et al [22].

2.8. Thermal stability study

In a differential scanning calorimetric (DSC) study, the melting profiles of 100 μM (1 ml) each of glyoxal (50 μM)-treated HbA_0 and Mb samples (termed as $GHbA_0$ and GMb, respectively) and the control samples were obtained in a VP-DSC Microcalorimeter by heating the samples (1 °C/minute) over a definite temperature range. Before introduction into the calorimetric cells, the protein samples were thoroughly degassed.

Thermal stability of the protein samples was also studied by PAGE. After heating HbA_0 and glyoxal-incubated HbA_0 samples (15 μM each) at 75 °C for 10 min and Mb and glyoxal-incubated Mb samples (15 μM each) at 80 °C for 10 min, the samples were subjected to native PAGE (10%), followed by staining with Coomassie R250. The temperatures of heating were kept around the melting temperatures of the heme proteins.

2.9. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)–mass spectrometric study

The samples (50 μM glyoxal treated proteins $GHbA_0$ and GMb and respective controls) were digested with sequencing-grade trypsin in solution at 37 °C for 16 h using enzyme:protein ratio 1:100 (w/w). For GMb, in-gel trypsin digestion was also performed after excising the lower band from the native gel. The 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 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. To identify probable modified peptides and the specific glyoxal derived AGEs formed, theoretical digestion of the heme proteins 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) using 1 kV collision energy. 1,000 laser shots were collected for each MS/MS spectrum using a fixed laser intensity of 4,500 V. Raw data were generated by using GPS Explorer Software. The identification of AGE modified peptides were done by manual interpretation of the MS/MS spectra.

3. Results and discussion

Glycation of proteins and their subsequent modifications have been ascribed to play significant roles in different pathological complications. Here we have studied the structural alterations of HbA_0 and Mb on reaction with glyoxal under identical conditions. The highest concentration of glyoxal used in the study was 50 μM. The concentration used was much higher than the plasma concentration of the dicarbonyl found in vivo [23]. However, in