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Effect of nonenzymatic glycation on functional and structural properties of hemoglobin

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Abstract

HbA_{1c}, the major glycated hemoglobin increases proportionately with blood glucose concentration in diabetes mellitus. H_2O_2 promotes more iron release from HbA_{1c} than that from nonglycated hemoglobin, HbA_0. This free iron, acting as a Fenton reagent, might produce free radicals and degrade cell constituents. Here we demonstrate that in the presence of H_2O_2, HbA_{1c} degrades DNA and protein more efficiently than HbA_0. Formation of carbonyl content, an index of oxidative stress, is higher by HbA_{1c}. Compared to HbA_0, HbA_{1c} is more rapidly autooxidized. Besides these functional changes, glycation also causes structural modifications of hemoglobin. This is demonstrated by reduced α-helix content, more surface accessible hydrophobic tryptophan residues, increased thermolability and weaker heme-globin linkage in HbA_{1c} than in its nonglycated analog. The glycation-induced structural modification of hemoglobin may be associated with its functional modification leading to oxidative stress in diabetic patients.

Keywords: Hemoglobin; Non-enzymatic glycation; Diabetes mellitus; Oxidative stress; Free iron

1. Introduction

The central identifying feature of diabetes mellitus is chronic and substantial elevation of the circulating glucose concentration. The increased blood glucose stimulates nonenzymatic glycation of proteins namely, serum albumin [1], α-crystallin [2], collagen [3], low-density lipoprotein [4], hemoglobin [5] etc. The key step in the modification of proteins by glucose is Schiff base formation, followed by Amadori rearrangement [6]. The Amadori product can then undergo oxidative cleavage, resulting in the formation of advanced glycation end products (AGEs) [7]. The first indication that a very simple chemical reaction between glucose and free amino groups on protein can lead to irreversible modification came with the characterization of hemoglobin A_{1c} (HbA_{1c}), which has the N-terminus of the β chain (valine) linked to glucose [6]. HbA_{1c} concentration is proportionately increased in diabetic patients with ambient hyperglycemia and reflects the extent as well as management of diabetic condition [8]. Several reports have been made on glycation-induced structural and functional modification of hemoglobin [5,9–16]. From computer modeling and electron paramagnetic resonance spectral studies, allosteric structure of glycated hemoglobin was speculated to be fixed in 'T' or Tough state [9,10]. Using ESR spectroscopic study, Watala et al. [10] reported the decreased mobility of the lysine residue in glycated hemoglobin and suggested a change in the conformation of the molecule. Compared to HbA_0, a reduced peroxidase activity of HbA_{1c} was reported by both Khoo et al. [12] and Kar and Chakraborti [13]. A modulation mechanism linked to structural change of the protein was suggested. Compared to HbA_0, HbA_{1c} was reported to exhibit moderately high oxygen affinity [14]. According to Inouye et al. [15], glycation of hemoglobin via chronic hyperglycemia was significantly correlated with cholesterol peroxidation in erythrocytes. Hemoglobin glycation was suggested to induce oxygen-derived free radicals causing oxidative damage to endogenous molecules, including cholesterol.

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Although free radicals and associated oxidative stress have been implicated in eliciting diabetes mellitus [17,18], the mechanism of increased formation of free radicals in diabetes is not yet clear. Findings from our laboratory indicate that HbA_{1c} may be a source of free radicals and oxidative stress [13,16]. Ferrous iron with six coordination states is bound in heme pocket of hemoglobin. Under specific circumstances, iron can be liberated from the heme and ligated to another moiety, probably distal histidine (E7) near heme pocket. This iron has been termed 'mobile reactive iron' [19], which can catalyze Haber–Weiss reaction producing free radicals, particularly hydoxyl ($OH^{\cdot}$) radicals, and in turn, may damage different cell constituents [20]. We have shown that free reactive iron level in purified hemoglobin, isolated from blood of diabetic patients is proportionately increased with increased level of blood glucose [16]. This iron may cause increased level of free radicals, which have been suggested to be involved in pathological complications of diabetes mellitus [13,16]. In a recent study, Cussimanio et al. [21] have demonstrated that hemoglobin and myoglobin are extremely susceptible to damage by glucose in vitro through a process that leads to complete destruction of heme group. The iron released from heme destruction enhances AGE formation.

H_2O_2 is known to induce iron release from hemoglobin [22]. We have shown that H_2O_2 promotes more iron release from HbA_{1c} than that from HbA_0 and iron-mediated free radical reactions namely, lipid peroxidation and deoxyribose degradation are more pronounced with HbA_{1c} in comparison with HbA_0 (13). These results are also in agreement with our recent findings, which correlate glycation-induced modification of myoglobin and a mechanism of iron-mediated increased formation of free radicals [23]. Although myoglobin glycation is not significant within muscle cells, free myoglobin in circulation, if becomes glycated, may pose a serious threat by eliciting oxidative stress in diabetic patients. The modified functional properties of glycated heme proteins—hemoglobin and myoglobin may thus be correlated with increased free radical reactions.

To verify the correlation further, we have undertaken the present study to understand hemoglobin-catalyzed iron-mediated free radical reactions with respect to DNA breakdown, protein degradation and carbonyl formation. The findings confirm the role of hemoglobin as a source of catalytic iron and its modification by glycation enhances further this functional property of the protein. Glycation also stimulates oxidation of hemoglobin, which may further complicate oxidative reactions in diabetic condition. Besides functional modification, we have also studied glycation-induced structural modification of hemoglobin with respect to conformations, surface accessible tryptophan residues, thermal stability and stability of heme-globin linkage. The findings in this communication suggest that glycation causes both structural and functional modifications, which may be associated with pathophysiological complications of diabetes mellitus.

2. Experimental

2.1. Materials

Sephadex G-25, sephadex G-100, human serum albumin (HSA), bovine serum albumin (BSA), agarose, ethidium bromide, alanine dehydrogenase, nicotinamide adenine dinucleotide (NAD^+), hydrazine hydrate, dinitrophenylhydrazine (DNPH), acrylamide, desferrioxamine (DFO), mannitol, thiobarbituric acid (TBA) and hemin chloride were purchased from Sigma Chemical, USA. BioRex-70 resin (200–400 mesh) was purchased from BioRad, India. Other chemicals were of analytical grade and purchased locally.

2.2. Preparation of hemoglobin, separation of HbA_{1c} and HbA_0 from hemoglobin and their characterization

Human blood samples from patients of age group 40–50 years with noninsulin-dependent diabetes mellitus were collected in heparinised condition. Hemoglobin was isolated and purified from red blood cells (RBC) using Sephadex G-100 column chromatography [24]. Hemoglobin was used to separate the fractions of HbA_{1c} and HbA_0 by an ion exchange chromatography [6]. Hemoglobin solution was applied to cation exchange column containing BioRex-70 resin (20×1.5 cm) preequilibrated with 50 mM phosphate buffer, pH 6.6. Fractions (0.5 ml) of glycated and nonglycated hemoglobins were separated by stepwise increase of NaCl concentration in elution buffer according to the method of Cohen and Wu [6]. Fractions HbA_{1a1} and $HbA_{1a2,}$ containing β-*N*-fructose 1,6-diphosphate and β-*N*-glucose 6-phosphate, respectively were eluted without any added NaCl (Buffer A). HbA_{1b} having β-*N*-carbohydrate was collected in the fraction containing 0.05 M NaCl in the elution buffer (Buffer B). HbA_{1c} was separated at salt concentration of 0.1 M (Buffer C) and finally, HbA_0 or nonglycated major human adult hemoglobin was eluted at 1.0 M NaCl concentration (Buffer D). A representative elution profile of the separated fractions is shown in Fig. 1, in which absorbances of different hemoglobin fractions at 415, 540 and 670 nm have been plotted against fraction numbers. The first two small peaks in the plot are for HbA_{1a1} and HbA_{1a2}, the third one for HbA_{1b}, fourth represents HbA_{1c} and the final peak is for HbA_0. HbA_{1c} and HbA_0 peak fractions were collected for this study. HbA_0 fraction was diluted with phosphate buffer immediately after separation to 0.1 M NaCl concentration. This was done to reconstitute back to tetrameric hemoglobin from dissociated

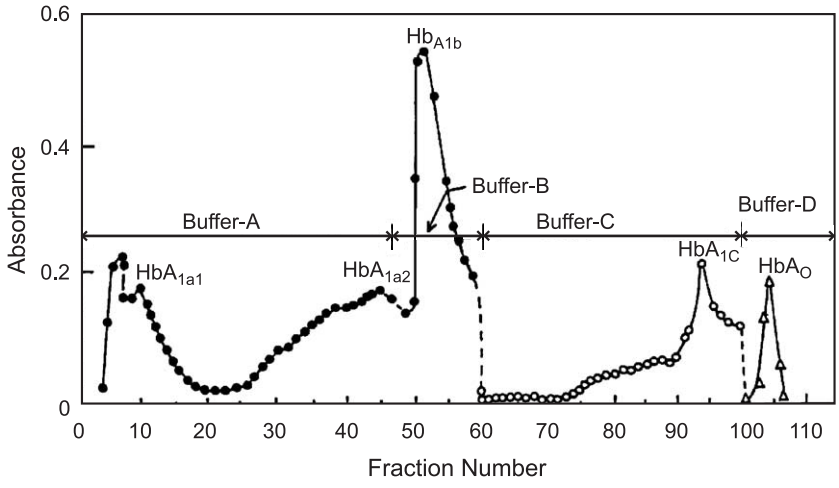

Fig. 1. The elution profile of the separated fractions of glycated and nonglycated hemoglobin species on ion-exchange chromatography (BioRex-70). Fractions of hemoglobin were separated by stepwise increase of NaCl concentrations (0–1.0 M) in 50 mM phosphate buffer, pH 6.6. Buffer A: no NaCl, Buffer B: 0.05 M NaCl, Buffer C: 0.1 M NaCl, Buffer D: 1.0 M NaCl. Absorbances of isolated fractions were taken at 415 nm (●), 540 nm (○) and 670 nm (△) in order to get readings for drawing in the same scale.

subunits formed, if any, at high concentration of NaCl [24]. Both HbA_{1c} and HbA_0 in 50 mM phosphate buffer, pH 6.6 showed a single elution peak corresponding to tetrameric hemoglobin molecular weight 66,800, when applied to a calibrated sephadex G-100 column. Both fractions showed three absorption peaks around 415, 540 and 577 nm. Glycation in HbA_{1c} was detected by TBA reaction according to the method of Flukinger and Winterhalter [25] (data not shown). However, TBA test was negative for HbA_0. The extents of oxygenation in HbA_{1c} and HbA_0 estimated from their respective absorption spectra following Huang and Redfields' [26] equation were found to be nearly 100%.

$$\text{Oxygen content}(\%) = 12.73 - 9.92(A_{560}/A_{540}) \times 100/3.77 + 3.88(A_{560}/A_{540}),$$

where A_{560} and A_{540} indicate absorbances at 560 and 540 nm, respectively.

HbA_{1c} and HbA_0 concentrations were measured from their Soret absorbances with extinction coefficient, ε_{415nm} as 125 mM^{-1} cm^{-1} (monomer basis) [27].

2.3. H_2O_2-mediated HbA_0 and HbA_{1c}-catalyzed DNA degradation

For the assay of H_2O_2-induced hemoglobin-catalyzed DNA degradation, approximately 450 ng plasmid (pGEM) DNA (3 kb) was incubated at 37 °C for 1 h with 25 μM HbA_0 or HbA_{1c} and 0.3% H_2O_2. The reaction was stopped with 10% (v/v) glycerol [28]. Different forms of DNA were separated by agarose (1%) gel electrophoresis and visualized by ethidium bromide staining.

2.4. H_2O_2-mediated protein degradation by HbA_0 and HbA_{1c}

Protein degradation assays were performed essentially according to the method of Pacifici and Davies [29] by using RBC. Purified human RBC (3.6×10^8 cells) was incubated with HbA_0 or HbA_{1c} (20 μM) and H_2O_2 (2 mM) for 2 h at 37 °C. Proper controls were prepared. DFO (50 μM) or mannitol (10 mM) was added to the reaction mixture as indicated. After incubation, 200 μl of perchloric acid (0.1 M) was added for lysis of RBC. The reaction tubes were left on ice water for 10 min followed by centrifugation at $3000 \times g$ for 15 min. Five hundred microliters of supernatant was taken and its pH was adjusted to 9.0 by addition of 400 μl of 2 M KOH and 700 μl of 0.5 M Tris–HCl buffer, pH 9.0, kept on ice for 1 h, during which perchlorate crystallizes out of the solution and alanine formation was measured in the supernatant. To 500 μl supernatant, 500 μl Tris–EDTA (0.5 M Tris, pH 9.0 + 40 mM EDTA), 500 μl of 6.6% hydrazine hydrate, 100 μl of NAD^+ (200 ng/ml) and 100 μl of alanine dehydrogenase (0.2 mg/ml, 30 U/mg) were added. The mixture was incubated at 37 °C for 1 h. NADH was estimated spectrofluorimetrically with 340 nm excitation and 450 nm emission.

2.5. Assay of carbonyl formation

HbA_0 or HbA_{1c}-induced free radical-mediated carbonyl formation was estimated following the method of Levine et al. [30]. The reaction mixture (1 ml) containing 47 μM hemoglobin sample, 2.0 mg BSA and 2.0 mM H_2O_2 was incubated at 37 °C for 1 h. Five hundred microliters DNPH (10 mM) was added and thoroughly mixed. After addition of 250 μl TCA (20%) and centrifugation, the pellet was collected and washed three times with 1 ml ethanol:ethyl acetate (1:1) mixture. The pellet was then dissolved in 0.6

ml 6 M guanidine solution and incubated at 37 °C for 15 min. After centrifugation, the supernatant was collected and carbonyl content was estimated from the absorbance at 370 nm using a molar absorption coefficient 22,000 M^{-1} cm^{-1}.

2.6. Autooxidation of HbA_0 and HbA_{1c}

Sterile-filtered HbA_0 or HbA_{1c} (40 μM) in 50 mM PBS, pH 6.6 was taken in sterile tubes and incubated at 4 °C for 15 days. The aliquots were taken out aseptically every 24-h interval and the absorbances at 577 and 630 nm were measured. The extent of oxidation, as represented by methemoglobin formation, was estimated from the relation [31]:

$$[\text{MetHb}], \mu\text{M} = 279A_{630\text{nm}} - 3A_{577\text{nm}}.$$

2.7. Circular dichroism studies

Circular dichroic measurements (200–600 nm) of HbA_0 and HbA_{1c} were done in Jasco 600 spectropolarimeter. Molar ellipticity [θ] values were obtained using the relation [32]:

$$[\theta] = [M_rW]\theta/10.l.c,$$

where c is the concentration of hemoglobin (HbA_0 or HbA_{1c}) in gm/ml, θ (obtained directly from the CD chart) is the observed rotation in degrees (mdeg), l is the path length in cm and [M_rW], the mean residual molecular weight of the protein is 110. The α-helical contents of HbA_0 and HbA_{1c} were determined according to the relation [32]:

$$\text{Fraction of } \alpha - \text{helix} = [\theta]_{222} + 2340/ - 30,300,$$

where $[\theta]_{222}$ is the molar specific ellipticity at 222 nm.

2.8. Spectrofluorimetric tryptophan quenching titration using acrylamide as a neutral quencher

Surface accessibility of tryptophan residues in HbA_0 and HbA_{1c} was estimated from the dynamic quenching of its fluorescence by acrylamide acting as a neutral quencher. When excited at 285 nm, emission maxima of HbA_0 and HbA_{1c} was at 330 nm. Quenching of tryptophan fluorescence intensity of HbA_0 or HbA_{1c} (6.5 μM) in the presence of the added acrylamide was measured from the change of the respective intensity at 330 nm. Excitation of acrylamide at 285 nm does not contribute significantly to the fluorescence emission around 330 nm. Fraction accessibility of tryptophan residues was estimated from Lehrer plot of $F_0/\Delta F$ versus $1/L_t$ following the relation [34]:

$$F_0/\Delta F = 1/f + 1/f \cdot K \cdot 1/L_t,$$

where $\Delta F = F_0\text{-}F$; F_0 and F represent fluorescence emission intensities of HbA_0 or HbA_{1c} in the absence and presence of total acrylamide concentration (L_t), respectively, f is the fraction of tryptophan quenched by acrylamide.

2.9. Thermal denaturation of HbA_0 and HbA_{1c}

The precipitation test of hemoglobin by thermal denaturation was done essentially according to the method of Olsen [35]. For this experiment, HbA_0 and HbA_{1c} in 50 mM phosphate buffer saline, pH 6.6 were further subjected to sephadex G-100 column equilibrated with 100 mM phosphate buffer, pH 7.4 and single eluted fraction corresponding to tetrameric protein of molecular weight 66,800 was collected. The sample of HbA_0 or HbA_{1c} (20 μM) in 100 mM phosphate buffer, pH 7.4 was incubated at 62 °C in a water bath. At different time intervals, samples were withdrawn and chilled on ice immediately and centrifuged to remove denatured protein precipitates. The absorbances of the supernatants at 523 nm were measured. The percentage of denaturation of protein was estimated according to the relation [35]:

$$\text{Percentage of denaturation} = (A_0 - A_t) \times 100/A_0,$$

where A_0 and A_t are absorbances without incubation and with incubation for a particular time, t, respectively.

2.10. Stability of heme-globin linkage in HbA_0 and HbA_{1c}

The heme transfer from Hb^+ (Hb^+A_0 or Hb^+A_{1c}) to human serum albumin (HSA) forming methemalbumin (MHA) was determined spectrophotometrically according to the method of Benesch [36]. HbA_0 or HbA_{1c} in 50 mM phosphate buffer, pH 6.6 were oxidized to ferric form by adding 1.2 equivalent potassium ferricyanide followed by passing through sephadex G-25 column (20 × 1.5 cm) preequilibrated with 50 mM Tris–HCl buffer, pH 7.5 to remove ferro and ferricyanide. The concentration of Hb^+ was determined from absorbance at 540 nm ($\varepsilon_{540\text{nm}} = 11.0 \times 10^3$ $M^{-1}cm^{-1}$). The reaction was started by adding HSA to a temperature-equilibrated cuvette (25 °C) containing 1.0 ml each of 0.5 M Tris–HCl, pH 9.05 and Hb^+ in 0.05 M Tris–HCl, pH 7.5. The final pH was 9.0. The concentrations of Hb^+A_0 or Hb^+A_{1c} and HSA were 40 μM each in final volume. The change in absorbances at 510 and 580 nm were recorded at different time intervals. The concentration of MHA formed in the reaction mixture was calculated from a 0.25 mM MHA standard treated in identical condition. To prepare MHA standard, equal amounts of 1 mM hemin chloride in 0.5 M Tris–HCl, pH 9.05 and 1 mM HSA in 0.05 M Tris–HCl, pH 7.5 were mixed and diluted to 20-folds to get 0.25 mM MHA standard solution. In the first phase of the heme transfer reaction, the heme from β chains of hemoglobin is released to form MHA. During this phase, the sum of [Hb^+] and [MHA] should remain constant and equal to initial Hb^+

concentration. The percentage of [MHA] formed at different time intervals was calculated according to the relation [36]:

$$\text{Percentage of } [\text{MHA}] = [\text{MHA}] \times 100/[\text{Hb}^{+}] + [\text{MHA}] = [\text{MHA}] \times 100/\text{Hb}^{+}_{\text{initial}}$$

3. Results

3.1. Glycation-induced functional modification of hemoglobin

3.1.1. H_2O_2-induced HbA_0 and HbA_{1c}-catalyzed DNA breakdown

H_2O_2-induced hemoglobin-catalyzed DNA breakdown was studied (Fig. 2). DNA was incubated with HbA_0 or HbA_{1c} in the presence or absence of H_2O_2 and subjected to gel electrophoresis. In the presence of H_2O_2 only, DNA was not degraded (Lane 1: only DNA, Lane 2: DNA + H_2O_2). HbA_0 (Lane 3) or HbA_{1c} (Lane 5) alone could not degrade DNA. However, DNA degradation was clearly evident with conversion of form I to form II in the presence of both H_2O_2 and protein (Lanes 4 and 6). HbA_{1c} and H_2O_2 together (Lane 6) exerted more effect than the combined effect of HbA_0 and H_2O_2 (Lane 4). In the representative experiment shown in Fig. 2, DNA breakdown in DNA + HbA_0 + H_2O_2 system (Lane 6) was found to be approximately 15% higher than in DNA + HbA_0 + H_2O_2 system (Lane 4).

3.1.2. H_2O_2-induced HbA_0 and HbA_{1c}-catalyzed protein degradation

RBC are unable to synthesize alanine de novo or by metabolic interconversion. Alanine appearance is, therefore, an indication of protein degradation. H_2O_2-induced HbA_0 and HbA_{1c}-mediated protein degradation in perchloric acid extracts of RBC were studied. Alanine residues released from RBC were estimated spectrofluorimetrically by alanine

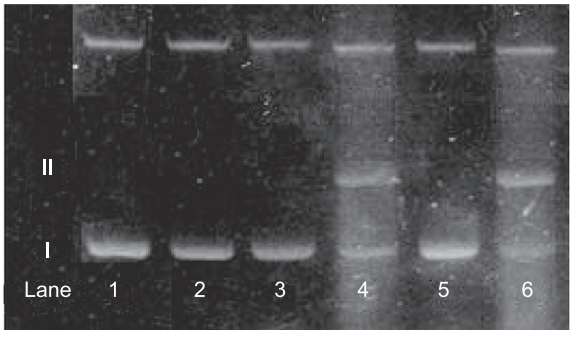

Fig. 2. H_2O_2-mediated DNA (plasmid) breakdown by HbA_0 and HbA_{1c}. DNA was incubated with HbA_0 or HbA_{1c} and H_2O_2 at 37 °C for 1 h, 5% glycerol (v/v) was added to the reaction mixture and than subjected to agarose gel electrophoresis for separation of different forms of DNA. Lane 1: only DNA; Lane 2: DNA + H_2O_2; Lane 3: DNA + HbA_0; Lane 4: DNA + HbA_0 + H_2O_2; Lane 5: DNA + HbA_{1c}; Lane 6: DNA + HbA_{1c} + H_2O_2.

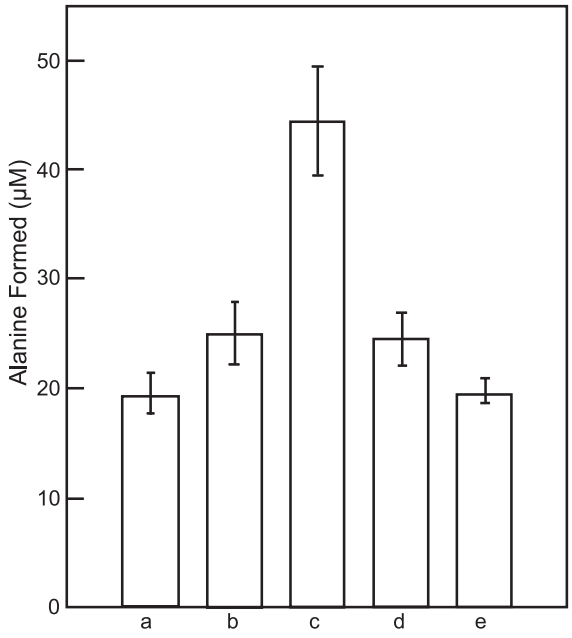

Fig. 3. Protein degradation assay on normal RBC induced by HbA_0 or HbA_{1c} in the presence of H_2O_2. RBC was incubated with (a) H_2O_2, (b) HbA_0 and H_2O_2, (c) HbA_{1c} and H_2O_2, (d) HbA_{1c}, H_2O_2 and DFO and (e) HbA_{1c}, H_2O_2 and mannitol for 2 h at 37 °C, after which the cells were lysed with perchloric acid. Alanine residues released from the proteins were estimated spectrofluorimetrically. The results are mean ± S.D. of three experiments.

dehydrogenase-linked NADH oxidation and the results have been presented in Fig. 3. As shown in the figure, HbA_{1c}-mediated protein breakdown in the presence of H_2O_2 was significantly higher than HbA_0-mediated process. In the absence of H_2O_2, either HbA_0 or HbA_{1c} alone did have very little effect on protein degradation (data not shown). Both DFO, which chelates free iron or suppresses its release and mannitol, a hydroxyl radical quencher inhibited the protein degradation reactions. The results of inhibition in HbA_{1c}-mediated process have only been shown.

3.1.3. Carbonyl formation as an indication of oxidative stress

Oxidation-induced carbonyl formation in HbA_0 and HbA_{1c} has been presented in Table 1. In the presence of H_2O_2, carbonyl formation in HbA_{1c} was higher (30 μM) than in HbA_0 (23 μM). HbA_{1c}-mediated carbonyl formation was also higher in the presence of an exogenous protein BSA. DFO and mannitol inhibited carbonyl formation catalyzed by both HbA_0 and HbA_{1c}, suggesting the reactions as iron-dependent free radical-mediated processes.

3.1.4. Oxidation of HbA_0 and HbA_{1c}

Oxidation of HbA_0 and HbA_{1c} was studied according to the method of Winterbourn [31]. The spectral analysis (450–700 nm) at different time (days) intervals showed gradual elevation of absorbance at 630 nm indicating met formation (spectra not shown). The rate of methemoglobin

Table 1
H_2O_2-induced carbonyl formation in HbA_0 and HbA_{1c} in the absence or presence of BSA

Reaction mixture containing	Carbonyl content (μM)
HbA_0	17.3 ± 0.8
$HbA_0 + H_2O_2$	23.8 ± 1.5
$HbA_0 + BSA + H_2O_2$	26.8 ± 1.4
$HbA_0 + BSA + H_2O_2 + DFO$	15.3 ± 0.4
$HbA_0 + BSA + H_2O_2 +$ mannitol	15.0 ± 0.5
HbA_{1c}	19.2 ± 1.2
$HbA_{1c} + H_2O_2$	30.0 ± 2.0
$HbA_{1c} + BSA + H_2O_2$	36.2 ± 2.2
$HbA_{1c} + BSA + H_2O_2 + DFO$	27.3 ± 1.0
$HbA_{1c} + BSA + H_2O_2 +$ mannitol	27.0 ± 1.3

Reaction mixture (1 ml) containing different components, as indicated, was incubated at 37 °C for 1 h. The carbonyl groups thus generated were quantitated by reaction with DNPH. The results are mean ± S.D. of four individual sets of experiments in each case.

formation was estimated from the absorbances at 577 and 630 nm and plotted in Fig. 4. Met formation by autooxidation was significantly higher from HbA_{1c} than that from nonglycated protein HbA_0.

3.2. Glycation-induced structural modification of hemoglobin

3.2.1. Circular dichroic spectral analysis of HbA_0 and HbA_{1c}

Glycation-induced conformation change of hemoglobin was studied by circular dichroic (CD) spectral analysis. Fig. 5 exhibits CD spectra of HbA_0 and HbA_{1c} at far UV region (200–250 nm). Compared to HbA_0, HbA_{1c} showed a slight decrease in negative ellipticity in the region 210–225 nm. The α-helix contents of HbA_0 and HbA_{1c} were measured according to the relation of Chen et al. [33] and were found to be approximately 72% and 65%, respectively. The CD spectra of HbA_0 and HbA_{1c} in near UV region (250–300 nm) and visible region (400–600 nm) were almost identical (spectra not shown).

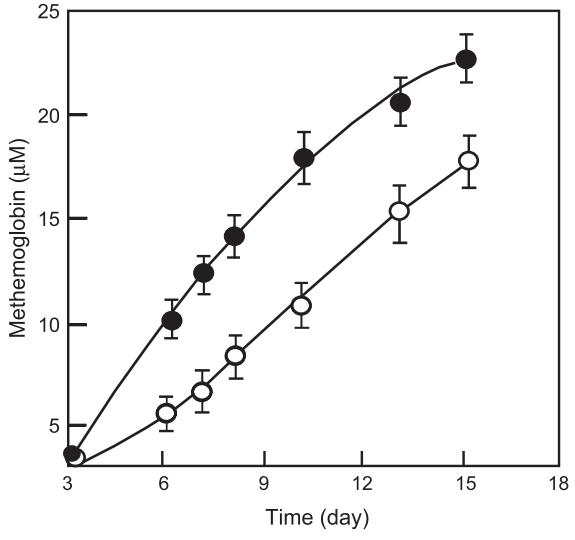

Fig. 4. Time course of autooxidation of HbA_0 (○) or HbA_{1c} (●). The protein samples were incubated at 4 °C and met formation was quantitated at different time (days) intervals. The results are mean ± S.D. of five experiments.

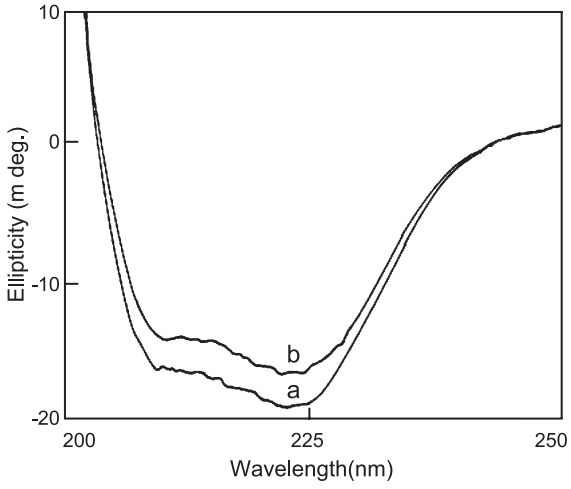

Fig. 5. Circular dichroic spectra of HbA_0 (a) and HbA_{1c} (b) at far UV region (200–250 nm).

3.2.2. Estimation of surface accessible tryptophans in HbA_0 and HbA_{1c}

Tryptophan fluorescence is a useful monitor of ligand binding in a variety of systems. To measure the surface accessibility of tryptophan residues in HbA_0 and HbA_{1c}, tryptophan fluorescence quenching was studied with addition of increasing concentration of acrylamide. Fig. 6 is a representative Lehrer [34] plot of $F_0/\Delta F = 1/f + 1/fK(1/L_t)$; f is the fraction of tryptophan quenched as estimated from the intersection of above linear plot with $F_0/\Delta F$ axis. Accessible

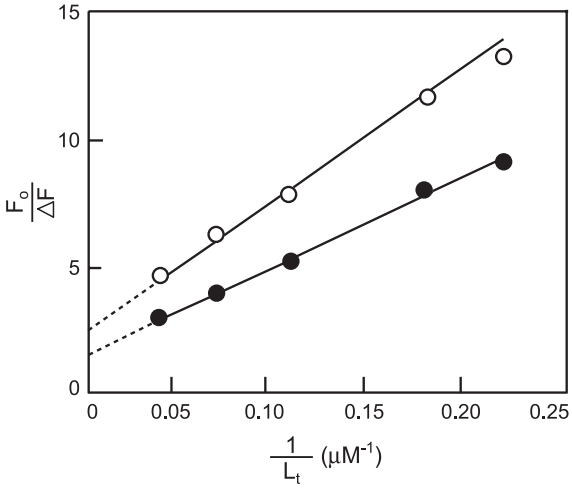

Fig. 6. A representative Lehrer plot $F_0/\Delta F$ versus $1/L_t$ of HbA_0 (○) and HbA_{1c} (●) for the determination of fraction of tryptophan molecules, accessible to acrylamide for quenching interaction. $\Delta F = F_0\text{-}F$, where F_0 is the fluorescence emission of protein at 330 nm in the absence of acrylamide and F is the quenched fluorescence emission after successive addition of acrylamide (L_t).

fraction of tryptophan residues in HbA_{1c} appeared to be 80%, compared to 50% in HbA_0.

3.2.3. Thermal denaturation of HbA_0 and HbA_{1c}

Thermal denaturation at 62 °C of HbA_0 and HbA_{1c} was studied and percentage of denaturation as a function of time has been plotted (Fig. 7). At each time point, the extent of denaturation of HbA_{1c} was found to be greater than that of HbA_0, depicting higher thermolabile nature of HbA_{1c} than its nonglycated analog, HbA_0.

3.2.4. Stability of heme-globin linkage in HbA_0 and HbA_{1c}

The affinity between heme and globin moiety of hemoglobin can be estimated by measuring the rate of heme exchange between hemoglobin and HSA [36]. However, this affinity in oxygenated hemoglobin is too strong to measure this exchange. Therefore, the heme exchange between methemoglobin (Hb^+) and HSA is measured by estimating the product MHA. Although the spectra of these two proteins Hb^+ and HSA are quite similar in neutral or acid solution, they show a difference at pH 9.0. Transfer of heme from Hb^+ to HSA can thus be measured at that pH by taking absorbances at two wavelengths (510 and 580 nm) simultaneously at different time points for analysis of two colored compounds in a mixture. Based on this principle, the rate of heme exchange from Hb^+A_0 or Hb^+A_{1c} to HSA was measured at pH 9.0, where the absorption spectrum of the product MHA was distinctly different from Hb^+A_0 or Hb^+A_{1c} spectrum. A representative plot of heme transfer experiment from equimolar concentration of Hb^+A_0 or Hb^+A_{1c} to HSA has been shown in Fig. 8. Percentage of MHA formation was plotted against time. The percentage of MHA formed from interaction of HSA with oxidized heme

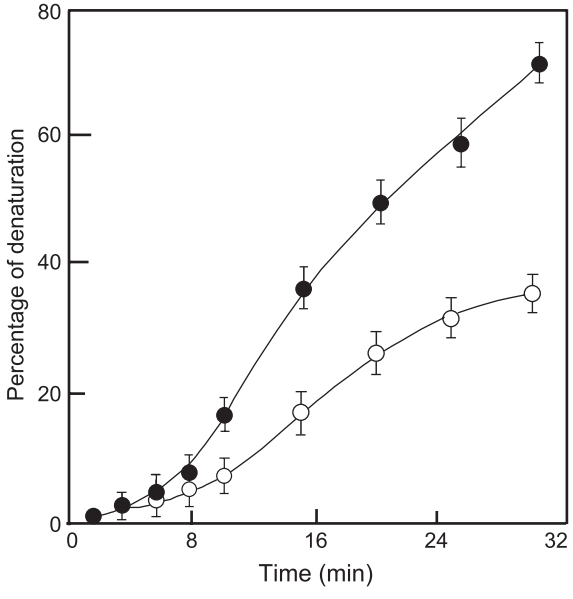

Fig. 7. Time course of thermal denaturation of HbA_0 (○) and HbA_{1c} (●) at 62 °C. The results are mean ± S.D. of four observations.

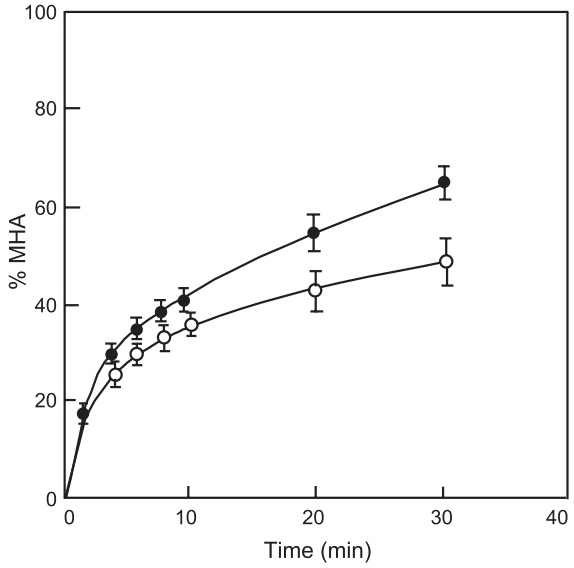

Fig. 8. Time course of heme transfer from Hb^+A_0 (○) and Hb^+A_{1c} (●) to HSA forming MHA. % MHA=[MHA] × 100/[Hb^+]+[MHA]. The results are mean ± S.D. of four observations.

in hemoglobin was significantly higher in case of Hb^+A_{1c} than in Hb^+A_0.

4. Discussion

In hemoglobin, ferrous iron is completely domesticated and tamed within protoporphyrin cage. Under certain conditions, this iron is liberated and may be detected by reaction with ferrozine [19]. We have shown earlier that free iron level in purified hemoglobin isolated from blood of diabetic patients is proportionately increased with increased level of blood glucose or extent of the disease condition [16]. We have also found that ferrozine-detected iron level is increased proportionately in in vitro-glycated hemoglobin with extent of the glycation. These findings suggest that glycation induces iron release from heme pocket of hemoglobin. Hemoglobin glycation is quite significant in diabetes mellitus. Of different glycated hemoglobin species, HbA_{1c} is most important and is used as a marker of the disease condition [8]. H_2O_2 induces iron release from hemoglobin [22]. We have shown that H_2O_2 promotes more iron release from HbA_{1c} than that from its nonglycated analog, HbA_0 [13]. Free iron may be a source of free radicals. Takasu et al. [17] reported stimulation of H_2O_2 generation in streptozotocin-induced diabetic rats. Gutteridge [20] measured iron released from hemoglobin by H_2O_2 and other hydroperoxides and suggested a possible source of $OH^{\cdot}$ radicals through iron-dependent Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+}OH^- + OH^{\cdot}$. Cussimanio et al. [21] also proposed that heme degradation in in vitro-glycated hemoglobin and myoglobin might be initiated by H_2O_2 formation, followed by reaction with superoxides generated or hydroxyl radicals or with both. H_2O_2-mediated increased level of iron release from HbA_{1c}

[13] may, thus, be a source of oxidative stress and cellular injuries, which may be caused by Fenton reaction in uncontrolled diabetes mellitus, where HbA_{1c} level is significantly elevated. We have shown that iron-mediated free radical reactions namely, lipid peroxidation and deoxyribose degradation are more pronounced in HbA_{1c} than in HbA_0 [13]. These findings are particularly important, because although oxidative stress has been suggested to be associated with diabetes mellitus, the source of the stress is not yet clear. A recent study from our laboratory also shows that H_2O_2-induced iron release is significantly higher from glycated myoglobin than that from its nonglycated analog and the free reactive iron, in turn, causes free radical reactions [23]. The present study is an attempt to understand the role of glycated hemoglobin in eliciting oxidative stress in diabetes mellitus. The structural properties of HbA_0 and HbA_{1c} have also been studied to find the role of glycation on structural modification of hemoglobin. There are several reports on glycation-induced structural changes of hemoglobin [9–11]. In these studies, computer modeling [9], ESR spectroscopy [10] and electrospray ionization mass spectrometry [11] have been used. In the present study, we have used CD spectroscopy, measurement of surface accessible tryptophan residues, thermal denaturation and stability of heme-globin linkage.

H_2O_2-induced HbA_0 and HbA_{1c}-catalyzed DNA (plasmid) breakdown has been studied (Fig. 2). H_2O_2 alone cannot degrade DNA [37]. Similarly, HbA_0 or HbA_{1c} alone cannot degrade DNA. However, in the presence of H_2O_2, HbA_0 or HbA_{1c}-catalyzed DNA breakdown is clearly evident. HbA_{1c}-mediated DNA breakdown is more effective than HbA_0-mediated process. DNA damage is associated with oxidative stress. Radical-mediated DNA damage involving transition metal ion-induced reactions is, in part, initiated by $OH^{\cdot}$ radical attack on DNA constituents. These reactions modify the chemical structure of DNA subunits (nucleobases and deoxyribose moieties), and they mark the onset of subsequent biochemical and biological effects in $OH^{\cdot}$-generating systems [38,39].

Protein degradation experiment as shown in Fig. 3 also indicates that H_2O_2-induced HbA_{1c}-mediated protein degradation is more effective than HbA_0-mediated process. Inhibition of the reactions by DFO and mannitol further indicates that protein degradation is an iron-mediated hydroxyl radical-catalyzed reaction. These results are in agreement with our earlier reports on lipid peroxidation and deoxyribose degradation by glycated hemoglobin as well as glycated myoglobin [13,23]. Modification of heme proteins—hemoglobin or myoglobin by glycation thus leads to increased formation of free radicals, which may cause breakdown of different cell constituents.

In the presence of H_2O_2, carbonyl formation in HbA_{1c} is higher than that in HbA_0. Similarly, the extent of carbonyl formation in BSA by H_2O_2-induced free iron from HbA_{1c} is significantly higher than that from HbA_0 (Table 1). Metal-catalyzed oxidation may cause covalent modification of proteins by introducing carbonyl groups into amino acid residues of proteins [40]. A cation capable of redox cycling (Fe^{2+}/Fe^{3+}) binds to a divalent cation-binding site on the protein. Reaction of hemoglobin with H_2O_2 generates a free radical, which oxidizes amino acid residues at or near that cation-binding site introducing carbonyl groups, which provide a moiety for quantification with DNPH. Such oxidative modification is an index of oxidative stress and may be significant in several physiological and pathological processes [41,42]. Our findings (Table 1) thus suggest an increased level of oxidative modification by the glycated protein HbA_{1c}, which is in conformity with the findings on DNA breakdown (Fig. 2) and protein degradation (Fig. 3). The inhibition of carbonyl formation by DFO and mannitol indicates that inhibition in HbA_0-containing system is considerably higher than in HbA_{1c}-containing system (Table 1). Since H_2O_2 releases more iron from HbA_{1c} than that from HbA_0 [13], the degree of inhibition by same amount of DFO or same amount of mannitol may be less in case of oxidation-induced carbonyl formation in HbA_{1c}-containing system. Higher DFO concentration is required to chelate iron released from HbA_{1c}. Similarly, higher concentration of mannitol is needed to quench $OH^{\cdot}$ radicals formed in HbA_{1c}-containing system.

In the aqueous media and in the absence of methemoglobin reductase system, the ferrous iron of the heme group of oxyhemoglobin is continuously autooxidized to methemoglobin and superoxide radicals ($O_2^{\cdot-}$). The rate of autooxidation of HbA_{1c} appears to be significantly higher than that of HbA_0 (Fig. 4). Similar results were obtained with NBT-induced cooxidation of HbA_0 and HbA_{1c} [13] as well as nonglycated and glycated myoglobin [23]. In normal physiological condition, it is estimated that approximately 3% of hemoglobin is autooxidized in 24 h [43]. Equilibrium is generally maintained between the rates of formation of methemoglobin (Hb^+A_0) and its reduction to oxyhemoglobin (HbA_0). If HbA_{1c} induces the increased rate of autooxidation, the diabetic patients with increased level of HbA_{1c} might form more methemoglobin within erythrocytes. Again, increased formation of methemoglobin may promote hemichrome and finally Heinz body formation, which damage irreversibly the erythrocyte membranes [31,44]. Superoxide radicals, which are produced from autooxidation of hemoglobin, may further react with water to form H_2O_2 increasing reactive oxygen species (ROS) level within the cell. Thus, increased level of HbA_{1c} and its susceptibility to oxidation may be another possible source of increased level of ROS and cellular degradation in diabetes mellitus.

Besides functional modification, glycation also induces structural modifications of hemoglobin. CD spectral analysis of HbA_0 and HbA_{1c} exhibits change at far UV region (Fig. 5). The reduced level of α-helix content of HbA_{1c} (about 7%) represents possibly an increased volume of the tertiary structure of hemoglobin due to glycation. Such a change may represent an unfolding of tetrameric structure of the protein. This change may also alter the spatial arrange-

ment of inter and intrachain amino acid residues and may manifest upon functional activities of hemoglobin as a whole. Estimation of surface accessible tryptophans in HbA_0 and HbA_{1c} (Fig. 6) also suggests a conformational change of hemoglobin due to glycation. Hemoglobin molecule has six tryptophan residues, one each at 14th position of α chains and two each at 15th and 37th position of β chains. These hydrophobic amino acid residues are partially exposed in quaternary conformation of hemoglobin molecule. Our findings indicate that glycation induces conformational change in hemoglobin molecule in such a fashion that more tryptophan moieties are exposed.

Thermal stability of a protein is an important index for its structural integrity as well as its functional property. Thermal denaturation experiment thus provides a direct measurement of its stability. The fact that HbA_{1c} is more thermolabile than HbA_0 (Fig. 7) strengthens the view of structural modification of hemoglobin due to glycation. It has been reported that cytochrome *P-450*, a heme containing thermolabile protein is degraded under certain pathological conditions and releases its heme in the system to create oxidative stress [44]. Since HbA_{1c} is also relatively more thermolabile than HbA_0, experiments have been done to test the stability of heme-globin linkage in both HbA_{1c} and HbA_0 (Fig. 8). The results suggest that heme-globin linkage is weaker in HbA_{1c} than in HbA_0. This finding is important with respect to structural aspect of HbA_{1c}, as it indicates that glycation at the N-terminal sites of β chains in hemoglobin alters the steric pattern of the molecule and influences the stability of the heme-globin linkage. Weaker heme-globin linkage in HbA_{1c} may have an implication that quicker heme loss from glycated hemoglobin under certain conditions may cause an oxidative stress. The weak heme-globin linkage may be a preferential target of attack by H_2O_2 leading to heme degradation and iron release. Our findings also get support from the study of Cussimanio et al. [21] according to which, hemoglobin and myoglobin are sensitive to destruction by nonenzymatic glycation through a process that leads to complete destruction of the essential heme group. It has been suggested that the reaction of heme proteins with H_2O_2 can cause heme degradation and the iron is released via ferryl intermediate [22]. The peroxide complex of the ferryl heme readily autooxidizes to form the highly reactive superoxide radicals [43,45]. Therefore, besides nonheme iron-mediated $OH^{\cdot}$ radicals, heme-mediated ferryl radicals and other degradation products may also be effective in causing hemoglobin associated oxidative damage. Glycation of heme proteins may enhance the degradation of heme and subsequent processes. We have shown that H_2O_2 promotes more iron release as well as ferryl myoglobin formation from glycated myoglobin than that from its nonglycated analog [23].

The present findings thus explain, at least in part, the facts that H_2O_2-induced iron release from HbA_{1c} is more pronounced than that from HbA_0 [13] and free iron level in hemoglobin samples isolated from diabetic patients is progressively higher with extent of the disease condition [16]. Glycation-induced structural and functional modifications of hemoglobin may thus have deleterious effects of the protein leading to oxidative stress and pathological complications of diabetes mellitus. However, further studies are necessary to elucidate how glycation-induced structural changes of hemoglobin are related to the functional changes of the protein.

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