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In Vitro Nonenzymatic Glycation Enhances the Role of Myoglobin as a Source of Oxidative Stress

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Metmyoglobin (Mb) was glycated by glucose in a non-enzymatic *in vitro* reaction. Amount of iron release from the heme pocket of myoglobin was found to be directly related with the extent of glycation. After *in vitro* glycation, the unchanged Mb and glycated myoglobin (GMb) were separated by ion exchange (BioRex 70) chromatography, which eliminated free iron from the protein fractions. Separated fractions of Mb and GMb were converted to their oxy forms -MbO_2 and $GMbO_2$, respectively. H_2O_2-induced iron release was significantly higher from $GMbO_2$ than that from MbO_2. This free iron, acting as a Fenton reagent, might produce free radicals and degrade different cell constituents. To verify this possibility, degradation of different cell constituents catalyzed by these fractions in the presence of H_2O_2 was studied. $GMbO_2$ degraded arachidonic acid, deoxyribose and plasmid DNA more efficiently than MbO_2. Arachidonic acid peroxidation and deoxyribose degradation were significantly inhibited by desferrioxamine (DFO), mannitol and catalase. However, besides free iron-mediated free radical reactions, role of iron of higher oxidation states, formed during interaction of H_2O_2 with myoglobin might also be involved in oxidative degradation processes. Formation of carbonyl content, an index of oxidative stress, was higher by $GMbO_2$. Compared to MbO_2, $GMbO_2$ was rapidly auto-oxidized and co-oxidized with nitroblue tetrazolium, indicating increased rate of Mb and superoxide radical formation in $GMbO_2$. GMb exhibited more peroxidase activity than Mb, which was positively correlated with ferrylmyoglobin formation in the presence of H_2O_2. These findings correlate glycation-induced modification of myoglobin and a mechanism of increased formation of free radicals. 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, particularly in diabetic patients.

Keywords: Myoglobin; Nonenzymatic glycation; Free iron; Oxidative stress

INTRODUCTION

Blood glucose is known to cause slow chemical modification (glycation) of long-lived proteins namely, serum albumin,[1] α-crystallin,[2] collagen,[3] low-density lipoprotein,[4] hemoglobin,[5] etc. This modification may be significant with increased level of blood glucose over prolonged periods of time in diabetes mellitus. The key step in the modification of proteins by glucose is Schiff base formation, followed by the 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] The concentration of HbA_{1c} is proportionately increased with hyperglycemia in diabetic patients and is used to monitor the extent of the disease condition.[8] Many studies have been made on glycation-induced structural and functional modification of hemoglobin.[5,9–15] Findings from our laboratory indicate that HbA_{1c} may be a source of free radicals and oxidative stress.[14,15] 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 in heme pocket. This iron has been termed "mobile reactive iron",[16] which can catalyze Haber–Weiss reaction producing free

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radicals, particularly hydroxyl (OH) radicals, and in turn, may damage different cell constituents.[17] 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.[14] This iron may cause increased level of free radicals, which have been suggested to be involved in pathological complications of diabetes mellitus.[14,15] H_2O_2 is known to induce iron release from hemoglobin.[18] We have shown[15] that H_2O_2 promotes more iron release from HbA_{1c} than that from nonglycated hemoglobin (HbA_0). Iron-mediated free radical reactions are also more pronounced in HbA_{1c}, compared to HbA_0.

Like hemoglobin, the muscle protein myoglobin is another heme-containing protein involved in the storage and transfer of oxygen within muscle cells.[19] The α and β subunits of tetrameric hemoglobin ($\alpha_2\beta_2$) and monomeric myoglobin are alike in the sense that each of them has eight α-helical segments, same percent (75%) of α-helix and similar three-dimensional organization. Although glycation of hemoglobin is well known, there has been no study on glycation of myoglobin.

In contrast with red blood cells, muscle cells possess insulin-dependent glucose transport system and very low glucose concentration. Thus, glycation of myoglobin may not be important in these cells. However, vigorous repeated exercise produces muscle damage releasing myoglobin into the circulation,[20,21] where the protein comes in contact with high concentration of glucose. Free myoglobin in circulation is a potentially damaging molecule, which can, for example, lead to kidney failure.[22] Raised level of serum myoglobin associated with diabetic ketoacidosis has been reported.[23,24] Myoglobinuric renal failure in diabetic condition is also known.[24,25] One suggested mechanism for renal failure associated with hypermyoglobinemia is free radical damage involving "free" iron release.[20]

Considering these facts, we have undertaken this study to understand myoglobin-catalyzed iron-mediated free radical reactions. The findings provide evidence that myoglobin may be a source of catalytic iron and modification by glycation further enhances this functional behavior of the protein. However, glycation also potentiates formation of ferryl moiety of higher oxidation state of iron. Thus, free myoglobin in circulation, if becomes glycated, particularly in diabetic patients, may cause more oxidative stress causing pathophysiological complications.

MATERIALS AND METHODS

Materials

Sephadex G25, arachidonic acid, deoxyribose, ferrozine, *o*-dianisidine, nitroblue tetrazolium (NBT), horse heart myoglobin, bovine serum albumin (BSA), desferrioxamine (DFO) and thiobarbituric acid (TBA) were purchased from Sigma Chemical Company, USA. Biorex-70 resin (200–400 mesh) was purchased from Bio-Rad, India. All other reagents were AR grade and purchased locally.

Methods

In Vitro Glycation of Myoglobin

Metmyoglobin (Mb) was dissolved in 50 mM potassium phosphate buffer, pH 6.6 and its concentration was determined[26] using extinction coefficient $\varepsilon_{408\,nm} = 116\,mM^{-1}\,cm^{-1}$. *In vitro* glycation of Mb was performed essentially according to the method of hemoglobin glycation.[6] In this method Mb solution (5 mg/ml) in 50 mM potassium phosphate buffer, pH 6.6 was incubated with different amount of glucose (final volume, 1.5 ml). Mb and glucose solutions were sterile-filtered. The samples were incubated in stoppered glass vials for 6 days at 25°C. After incubation, the amount of free glucose left in the samples was estimated by glucose oxidase method,[27] from which the amount of glucose consumed was determined.

Estimation of Free Iron in In Vitro Glycated Myoglobin (GMb) Samples

Purchased Mb is mostly in the met form. After *in vitro* glycation, GMb and unchanged Mb present in the reaction mixtures were converted to their respective oxy-forms — $GMbO_2$ and MbO_2 according to the method of Dixon and McIntosh[28] and eluted through sephadex G25 column. Free iron in these mixtures containing MbO_2 and $GMbO_2$ was estimated according to the method of Panter.[16] A 250 μl cold TCA (20%) was added to 250 μl reaction mixture. After centrifugation, the supernatant (250 μl) was transferred to a test tube containing 250 μl distilled water, 2.5 ml iron buffer reagent (1.5% hydroxylamine hydrochloride in acetate buffer, pH 4.5) and 50 μl iron color reagent (0.85% ferrozine in iron buffer reagent). The color was developed for 30 min at 37°C and read at 560 nm. Results were expressed as μg of iron/g of protein.

Separation of GMb and Mb and Conversion to the Oxy Forms

After *in vitro* glycation, GMb and Mb were separated by ion exchange chromatography using Biorex 70 resin (7 × 1.0 cm), pre-equilibrated with 50 mM phosphate buffer, pH 6.0. For the separation of GMb and Mb fractions, elution buffers of different pH-6.6 and 7.0 were used, respectively. Glycation in GMb was detected by TBA reaction according to

the method developed by Flukinger and Winterhalter[29] for testing glycation in hemoglobin. The colored chromophore gave a characteristic absorption maxima around 443 nm. However, TBA test was found to be negative for Mb. Mb and GMb thus separated were converted to their oxy forms.[28]

Assay of Lipid Peroxidation and Deoxyribose Degradation

Free radical-induced lipid (arachidonic acid) peroxidation and deoxyribose degradation were measured in the presence of MbO_2 or $GMbO_2$ and H_2O_2 according to the method of Sadrzadeh *et al.*[30] and Gutteridge,[17] respectively. For lipid peroxidation, the reaction mixture (1 ml) containing MbO_2/$GMbO_2$ (6 μM), arachidonic acid (160 μM) and H_2O_2 (1 mM) was incubated at 37°C for 1 h. DFO (20 μM) and mannitol (5 mM) were used as required. The reaction was initiated by adding H_2O_2 and stopped with 20% TCA. After centrifugation, 0.5 ml each of 1% TBA and 50 mM citrate buffer, pH 3.0 was added to the supernatant and heated in a boiling water bath for 30 min. The absorbance was measured at 530 nm and the values were corrected for endogenous TBA reactive substances present in arachidonic acid solution. The results were expressed as MDA formation (nmol/h). For deoxyribose degradation assay, deoxyribose (0.67 mM) in 50 mM phosphate buffer, pH 6.6 was incubated for 1 h at 37°C with MbO_2 or $GMbO_2$ (8 μM), H_2O_2 (0.67 mM) and other additions (DFO or mannitol) as required. TBA reactivity was developed by adding 0.5 ml each of TBA (1%) and TCA (2.8%), and then heated for 10 min in a boiling waterbath. The resulting chromogen was extracted with *n*-butanol and fluorescence intensity was measured at 553 nm by exciting at 523 nm.

Assay of DNA Degradation

For the assay of H_2O_2-induced myoglobin-catalyzed DNA degradation, approximately 500 ng plasmid (pGEM) DNA (3 Kb) was incubated at 37°C for 1 h with 3 μM MbO_2 or $GMbO_2$ and 0.1% H_2O_2. The reaction was stopped with glycerol.[31] Different forms of DNA were separated by agarose (1%) gel electrophoresis and visualized by ethydium bromide staining.

Assay of Carbonyl Formation

MbO_2/$GMbO_2$-induced free radical-mediated carbonyl formation was estimated following the method of Levine *et al.*[32] The reaction mixture (1 ml) containing 7 μM myoglobin sample, 1 mg BSA and 2 mM H_2O_2 was incubated at 37°C for 1 h. A 10 mM DNPH (50 μl) was added and thoroughly mixed. After addition of 250 μl TCA (20%) and centrifugation, the pellet was collected and washed three times with ethanol:ethylacetate (1:1) mixture. The pellet was then dissolved in 0.3 ml 6 M guanidine solution and incubated at 37°C for 15 min. After centrifugation, the supernatant was collected and absorbance taken at 370 nm.

Auto-oxidation of Oxymyoglobins and their Co-oxidation with NBT

Auto-oxidation of MbO_2 and $GMbO_2$ as well as their co-oxidation with NBT were studied essentially according to the method of Winterbourn.[33] For auto-oxidation, the protein samples (8 μM) were stored at 4°C in sterile condition and met formation was estimated every 24 h interval for 8 days using the extinction coefficient, $\varepsilon_{630\,nm} = 4\,mM^{-1}\,cm^{-1}$. NBT facilitates oxidation of oxymyoglobin to its met form. For co-oxidation, NBT (50 μM) was added to the protein samples and incubated at 37°C for estimation of met formation.

Assay of Peroxidase Activities of Mb's

The peroxidase activities of Mb samples were estimated according to the method of Everse *et al.*[34] The reaction mixture (2 ml) contained 50 mM citrate buffer, pH 5.4, 1.5 μM Mb or GMb and 0.002% *o*-dianisidine. The reaction was initiated by adding 17.6 mM H_2O_2. The absorbance at 450 nm was taken for 3 min. The oxidation of Mb and GMb to their respective ferryl formation by H_2O_2 was estimated according to the method of Giulivi and Cadenas.[35] Addition of H_2O_2 to the metprotein is associated with visible absorption spectral changes, consisting of an increase in absorbance in 520–600 nm region, with major peaks at 548 and 582 nm and a decrease at 630 nm. This spectral change is ascribed to appearance of ferrylmyoglobin. For estimation of ferrylmyoglobin, Mb was measured at different time points of H_2O_2 addition. The fall in Mb content was used to measure the amount of ferrylmyoglobin.

RESULTS AND DISCUSSION

Iron Level in *In Vitro* Reaction Mixture (MbO_2 and $GMbO_2$)

In vitro glycation of Mb was done essentially according to the method of glycation of hemoglobin developed by Cohen and Wu.[6] The amount of glucose consumed was found to increase proportionately with the amount of glucose incubated in the reaction mixture (Table I) and it might be directly related with total glycation of Mb. After *in vitro* glycation, nonglycated and glycated heme protein

TABLE I Amount of iron released from myoglobin after *in vitro* glycation

Concentration of glucose (mg/100 ml)	Glucose consumed (mg/100 ml)	Free iron in Mb samples (μg/gm of protein)
0	0	37.5
65	11.1	75.0
130	25.0	150.0
196	40.4	180.0
260	51.0	212.0
330	60.6	250.0

Five milligrams of Mb was incubated in 0.05 M potassium phosphate buffer, pH 6.6, containing different amount of glucose (final volume, 1.5 ml) for 6 days at 25°C. The reaction mixture contained both Mb and GMb, which were converted to the oxy forms. Free iron in samples containing both MbO_2 and $GMbO_2$ was estimated by ferrozine reaction. The results are mean of four individual sets of experiments (SD < 10%).

present in mixtures were converted to the respective oxy forms -MbO_2 and $GMbO_2$[28] and free iron levels in these mixtures were estimated by ferrozine reaction.[16] Ferrozine reacts with free ferrous iron. The iron level was positively correlated with the extent of total *in vitro* glycation (Table I). Thus glycation of Mb leads to iron release. Free iron may be detrimental to the cell, because it may catalyze free radical reactions.[17,18]

Separation of Mb and GMb and their Conversion to the Respective Oxy Forms

For further characterization of the effect of glycation, experiments were done with nonglycated and glycated proteins, which were separated by cation exchange chromatography shown in Fig. 1. The separated myoglobin fractions were converted to their oxyforms — MbO_2 and $GMbO_2$. The absorption spectra of both MbO_2 and $GMbO_2$ were found to be identical (not shown). The absorption spectra of MbO_2 and $GMbO_2$ thus prepared showed three characteristic absorption peaks in the visible region (380–700 nm) — around 415, 540 and 577 nm. The concentration of these oxy-forms were determined[26] from extinction coefficient value $\varepsilon_{418\,nm} = 128\,mM^{-1}\,cm^{-1}$. The glucose adduct of the glycated fraction was confirmed by positive reaction with TBA.[29] The colored chromophore gave characteristic absorption maxima around 443 nm (not shown). The TBA test was found to be negative for nonglycated sample.

H_2O_2-induced Iron Release from MbO_2 and $GMbO_2$

H_2O_2-induced iron release from hemoglobin and its effect on free radical-mediated reactions are known.[14,15,17] Effects of H_2O_2 on myoglobin with respect to formation of compound I,[36] ferrylmyoglobin analogous to compound II,[35] protein radicals and heme-protein cross-links[37,38] have been reported. H_2O_2-induced iron release from oxyforms of myoglobin was studied by incubating MbO_2 and $GMbO_2$ with different concentrations (0, 125, 250, 500, 750 and 1000 μM) of H_2O_2 at 37°C for 1 h and the amount of iron released was estimated by ferrozine method.[16] H_2O_2 induced iron release from both MbO_2 and $GMbO_2$. However, more iron was released from $GMbO_2$ than that from MbO_2 (Fig. 2). Whether iron is released directly from the heme pocket of myoglobin or heme degradation is followed by iron release is not clear. Puppo and Halliwell[39] and Rice-Evans *et al.*[40] also reported H_2O_2-induced iron release from myoglobin. H_2O_2-mediated iron release from myoglobin may thus be a source of oxidative stress and cellular injuries, which may be more effective with glycated species. Therefore, if Mb or MbO_2 comes out of muscle cells and is glycated, particularly in uncontrolled diabetes, it may be a potential source of catalytic iron. Although there is

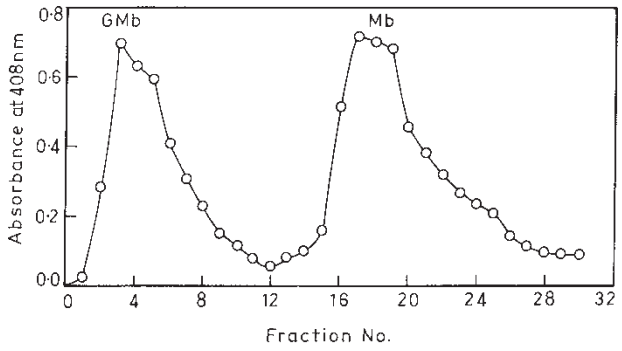

FIGURE 1 The elution profile of the glycated and nonglycated myoglobin fractions separated by ion exchange chromatography (BioRex 70). Fractions of GMb and Mb were separated by using different pH of 50 mM phosphate elution buffer, pH 6.6 and pH 7.0, respectively.

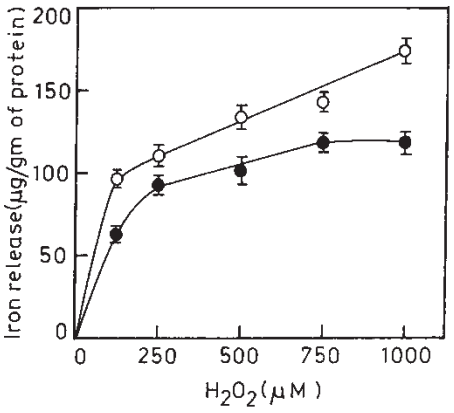

FIGURE 2 H_2O_2-induced iron release from MbO_2 (●) and $GMbO_2$ (○). Protein samples were incubated at 37°C for 1 h with varying concentrations of H_2O_2. After protein precipitation with TCA, the supernatant was used to estimate free iron by ferrozine reaction. The results are mean ± SEM of four observations.

no report on human subjects, Takasu *et al.*[41] reported stimulation of H_2O_2 generation in streptozotocin-induced diabetic rats.

To understand the effect of myoglobin glycation on free radical-mediated injuries of cell constituents, lipid peroxidation, deoxyribose degradation and DNA breakdown were measured in the presence of H_2O_2.

MbO_2 and $GMbO_2$-catalyzed Breakdown of Cell Constituents in the Presence of H_2O_2

Lipid Peroxidation and Deoxyribose Degradation

Aldehydes are produced when lipid hydroperoxides breakdown in biological systems. MDA is the most abundant aldehyde resulting from lipid peroxidation and its determination by TBA is used as an index of the extent of lipid peroxidation. Assay of H_2O_2-mediated arachidonic acid peroxidation by MbO_2 and $GMbO_2$ shows that the latter degraded the lipid more efficiently than the former. $GMbO_2$ formed approximately 1.35 nmole MDA/h, while MbO_2 formed approximately 0.75 nmole MDA/h (Table II).

OH radicals specifically attack the pentose sugar 2-deoxy-D-ribose to yield a mixture of products. On heating with TBA at low pH, some or all of these products react to form a pink chromogen that can be measured spectrophotometrically or spectrofluorimetrically. MbO_2 and $GMbO_2$-mediated deoxyribose breakdown in the presence of H_2O_2 was estimated. TBA reactivity (in arbitrary fluorescence emission intensity) of $GMbO_2$ was considerably higher (80 units), compared to that of nonglycated protein (61 units) under similar experimental conditions (Table II). Thus, like lipid peroxidation, $GMbO_2$-mediated deoxyribose breakdown is more efficient than MbO_2-mediated reaction. Both these processes were strongly inhibited by DFO, if added prior to the addition of H_2O_2 (Table II). DFO can act as an iron chelator as well as a suppressor of iron release from the heme protein.[40] Mannitol exhibited almost complete inhibition of lipid peroxidation and deoxyribose degradation by both MbO_2 and $GMbO_2$ in the presence of H_2O_2. Similar results were obtained with catalase (data not shown). Myoglobin-catalyzed lipid peroxidation in the presence of H_2O_2 has been mostly attributed to the formation of peroxyl radical.[42–44] Our study, however, suggests that iron liberated from heme of myoglobin by H_2O_2 acts as a Fenton reagent and forms OH radicals, which, in turn, initiate free radical-mediated chain reactions. When free iron is chelated or its release is suppressed by DFO, the free radical reactions are inhibited. Removal of H_2O_2 by catalase also inhibits the reactions. As H_2O_2-induced iron release is higher in $GMbO_2$ than in its nonglycated analog (Fig. 2), the former is more effective in causing oxidative damage (Table II). However, besides nonheme iron-mediated OH radicals, heme-mediated peroxyl radicals may also be effective in causing myoglobin associated oxidative damage.

TABLE II H_2O_2-mediated lipid peroxidation and deoxyribose degradation by nonglycated (MbO_2) and glycated myoglobin ($GMbO_2$)

Reaction mixture containing	Arachidonic acid peroxidation MDA formed (nmole/h)	Deoxyribose degradation TBA reactivity (Fluorescence Em intensity)
MbO_2	0.10	54
$MbO_2 + H_2O_2$	0.75	61
$MbO_2 + H_2O_2 +$ DFO	0.10	55
$MbO_2 + H_2O_2 +$ Mannitol	0.11	54
$GMbO_2$	0.11	59
$GMbO_2 + H_2O_2$	1.35	80
$GMbO_2 + H_2O_2 +$ DFO	0.21	58
$GMbO_2 + H_2O_2 +$ Mannitol	0.15	61

For lipid peroxidation, the reaction mixture (1 ml) contained $MbO_2/GMbO_2$ (6 μM) and arachidonic acid (160 μM). H_2O_2 (1 mM) and DFO (20 μM) or mannitol (5 mM) were added as indicated. MDA generated from arachidonic acid was quantitated by TBA reaction as described in "Materials and Methods" section. For deoxyribose degradation, the reaction mixture (1 ml) contained $MbO_2/GMbO_2$ (8 μM), deoxyribose (0.67 mM) in 50 mM phosphate buffer (pH 6.6). H_2O_2 (0.67 mM) and DFO (100 μM) or mannitol (5 mM) were added as indicated. TBA reactivity was developed, extracted and estimated from fluorescence emission at 553 nm by exciting at 523 nm. The results are mean of three observations for each experiment (SD < 10%).

DNA Breakdown

DNA damage is associated with oxidative stress. Radical-mediated DNA damage involving transition metal ion-induced reactions is in part initiated by OH 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-generating systems.[45,46] H_2O_2-induced myoglobin-catalyzed DNA (plasmid) breakdown was studied (Fig. 3). DNA was incubated with MbO_2 or $GMbO_2$ in the presence or absence of H_2O_2 and subjected to agarose gel electrophoresis. In the presence of H_2O_2 only, DNA was degraded to some extent (approximately 23%) converting form I to form II (lane 1: only DNA, lane 2: DNA + H_2O_2). Since H_2O_2 itself does not degrade DNA,[47] the degradation is probably due to metal ion contamination. MbO_2 (lane 4) or $GMbO_2$ (lane 6) alone could not degrade DNA. In the presence of both H_2O_2 and protein (lanes 3 and 5), DNA degradation was considerably higher than that of H_2O_2-induced effect (lane 2). However, $GMbO_2$ and H_2O_2 together (lane 5) exerted more effect than MbO_2 and H_2O_2 (lane 3). In the representative experiment shown in Fig. 3, DNA breakdown in DNA + MbO_2 + H_2O_2 system (lane 3) was found to

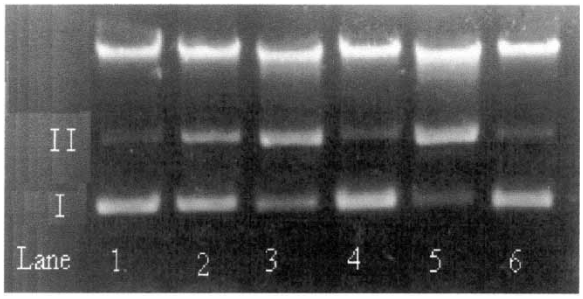

FIGURE 3 H_2O_2-mediated DNA (plasmid) breakdown by MbO_2 and $GMbO_2$. DNA was incubated with MbO_2/$GMbO_2$ and H_2O_2 at 37°C for 1 h, 5% glycerol (v/v) was added to the reaction mixture and then subjected to agarose gel electrophoresis for separation of different forms of DNA. Lane 1: Only DNA; Lane 2: DNA + H_2O_2; Lane 3: DNA + H_2O_2 + MbO_2; Lane 4: DNA + MbO_2; lane 5: DNA + H_2O_2 + $GMbO_2$; Lane 6: DNA + $GMbO_2$.

be approximately 65%, while that in DNA + $GMbO_2$ + H_2O_2 system (lane 5) was about 78%. This result further suggests that modification of myoglobin by glycation leads to increased formation of free radicals, which may cause breakdown of different cell constituents — lipids, carbohydrates, nucleic acids, etc.

Carbonyl Formation in BSA by H_2O_2-induced Free Iron from MbO_2 and $GMbO_2$

Metal-catalyzed oxidation may cause covalent modification of proteins by introducing carbonyl groups into amino acid residues of proteins.[48] A cation capable of redox cycling (Fe^{2+}/Fe^{3+}) binds to a divalent cation-binding site on the protein. Reaction 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 important in several physiological and pathological processes.[49,50] Experiments were done to detect the extent of carbonyl formation in BSA by H_2O_2-induced free iron from MbO_2 and $GMbO_2$. As shown in Table III, carbonyl content was found to be significantly higher in reaction mixture containing $GMbO_2$ and H_2O_2 (0.77 μmole/h) in comparison with that in MbO_2 and H_2O_2 (0.45 μmole/h), which indicates an increased level of oxidative modification by the glycated protein. In both cases, DFO and mannitol inhibited carbonyl formation, suggesting the process as an iron-dependent free radical-mediated process.

Oxidation of MbO_2 and $GMbO_2$

MbO_2 is oxidized easily to met form with generation of superoxide anion, which can be converted by the spontaneous dismutation into H_2O_2, this being also a potent oxidant of MbO_2.[51] Oxidation of MbO_2 and $GMbO_2$ was studied according to the method of Winterbourn.[33] The spectral analysis (450–700 nm) at different time intervals showed gradual elevation of absorbance at 630 nm indicating met formation (spectra not shown). Figure 4 shows that the met formation from $GMbO_2$ by auto-oxidation (Fig. 4a) as well as NBT-induced co-oxidation (Fig. 4b) was significantly higher than that from nonglycated protein. Thus glycation causes more oxidative stress in this respect also.

TABLE III H_2O_2-induced MbO_2 and $GMbO_2$-catalyzed carbonyl formation in the presence of BSA

Reaction mixture containing	Carbonyl formed (μmole/h)
BSA	0.23
BSA + MbO_2	0.27
BSA + MbO_2 + H_2O_2	0.45
BSA + MbO_2 + H_2O_2 + DFO	0.36
BSA + MbO_2 + H_2O_2 + Mannitol	0.27
BSA + $GMbO_2$	0.50
BSA + $GMbO_2$ + H_2O_2	0.77
BSA + $GMbO_2$ + H_2O_2 + DFO	0.41
BSA + $GMbO_2$ + H_2O_2 + Mannitol	0.45

Reaction mixture (1 ml) containing 1 mg BSA, 7 μM MbO_2/$GMbO_2$, 2 mM H_2O_2, 50 μM DFO and 5 mM mannitol, as indicated, was incubated at 37°C for 1 h. The carbonyl groups thus generated were quantitated by reaction with DNPH as described in "Materials and Methods" section. The results are mean of four individual sets of experiments in each case (SD < 15%).

Peroxidase Activities of Mb and GMb and H_2O_2-induced Ferryl Formation

Besides H_2O_2-mediated iron release from myoglobin, H_2O_2 also interacts with the heme protein to yield a potent oxidant (ferrylmyoglobin) capable of oxidizing a wide range of electron donors like phenol, aromatic amines and iodide.[52] This peroxidase-like activity of the heme protein (met form) was assayed. The reaction condition of this assay was different from that of lipid peroxidation assay, where

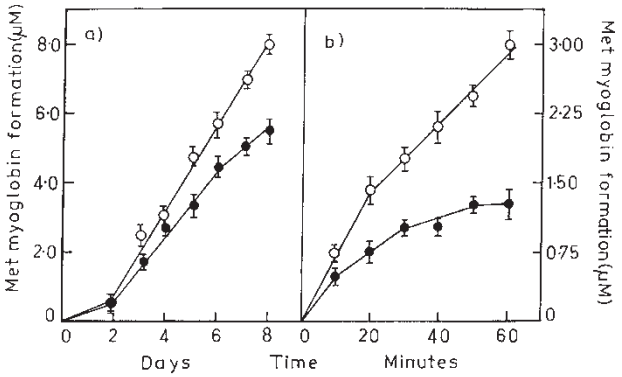

FIGURE 4 Time course of auto-oxidation (a) and NBT-induced co-oxidation (b) of MbO_2 (●) and $GMbO_2$ (○). For auto-oxidation, the protein sample alone was incubated at 4°C. For co-oxidation, protein sample was incubated with NBT at 37°C. Met formation was quantitated from the absorbance at 630 nm. The results are mean ± SEM of four experiments.

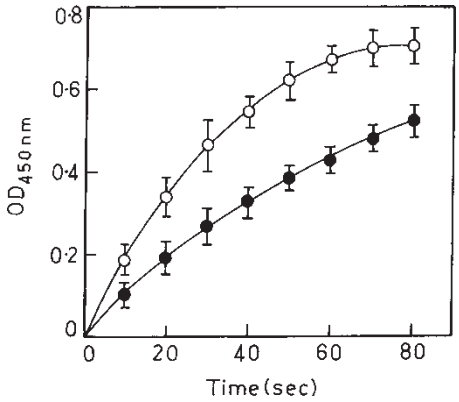

FIGURE 5 Peroxidase-like activity of Mb (●) and GMb (○) as a function of time. The reaction was initiated by adding H_2O_2 to the mixture containing citrate buffer, protein sample and *o*-dianisidine. The absorbance at 450 nm was monitored. The results are mean ± SEM of four experiments.

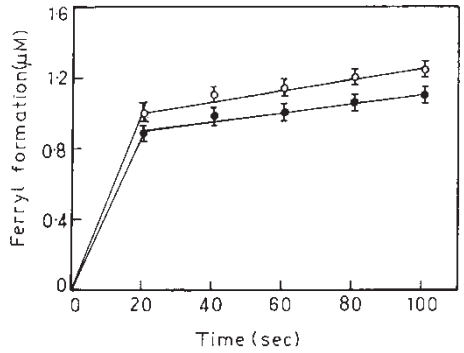

FIGURE 6 Time course of ferrylmyoglobin formation due to interaction of H_2O_2 with Mb (●) and GMb (○). H_2O_2: protein ratio of peroxidase assay was used for ferryl formation. The concentration of ferrylmyoglobin was calculated from the difference between initial metmyoglobin concentration and metmyoglobin concentration remained at different time points after addition of H_2O_2. The results are mean ± SEM of three experiments.

the reaction mixture was incubated at 37°C for 1 h to facilitate the iron release from the protein. For assay of peroxidase activity, the reaction rate was followed just after addition of H_2O_2. Compared to Mb, GMb exhibited more peroxidase-like activity (Fig. 5). This is in contrast with the finding on peroxidase activity of hemoglobin.[11,15] In comparison with HbA_0, HbA_{1c} possessed less peroxidase activity using *o*-dianisidine as the substrate.[15] A reduced peroxidase activity of glycated hemoglobin was also reported by Khoo *et al*.[11] using 5-aminosalicylic acid as the substrate. A modulation mechanism linked to structural change of the protein was suggested. Modification of Mb by glycation may also cause structural change in the protein, which may help the rate of entry of the substrate molecule *o*-dianisidine to heme pocket and consequently increase the peroxidase activity. Another possibility is the increased level of ferryl formation due to glycation, leading to higher peroxidase activity. To verify this possibility, ferryl formation was estimated from Mb and GMb using the same protein: H_2O_2 ratio of the peroxidase activity assay. Ferryl formation from GMb was found to be slightly higher than that from its nonglycated species (Fig. 6). However, even a small increase in ferryl formation may be effective in causing a considerable increment in peroxidase activity. The increased ferryl formation as well as increased peroxidase activity may be related to glycation-induced structural modification of the protein.

CONCLUSION

H_2O_2 interacts with both met and oxy form of myoglobin to produce ferrylmyoglobin and/or free iron. The preferred pathway probably depends on reaction conditions namely, temperature, pH, incubation time, concentration of H_2O_2, etc. Formation of either product is harmful and causes oxidative damage. In the presence of H_2O_2, both $GMbO_2$ and GMb induce more iron release from the proteins as well as enhance ferryl formation, leading to increased oxidative damage. Thus free myoglobin in circulation, if glycated, particularly in uncontrolled diabetes, may cause serious threat with respect to oxidative damage, which may, in turn, aggravate pathophysiological complications.

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