RESEARCH COMMUNICATIONS

Effect of glycosylation on iron-mediated free radical reactions of haemoglobin

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HbA1c, the major glycosylated haemoglobin increases proportionately with blood glucose level in diabetes mellitus. Here we demonstrate that H2O2-induced iron release is more from HbA1c than that from nonglycosylated haemoglobin (HbA0). In the presence of H2O2, HbA1c degrades arachidonic acid and deoxyribose more efficiently than HbA0, which suggests that iron release is more with HbA1c compared to HbA0. Increased rate of oxidation of HbA1c in the presence of nitroblue tetrazolium is indicated by an increase in methaemoglobin formation. HbA1c exhibits less peroxidase activity than HbA0. These findings on glycosylation-induced functional properties of haemoglobin suggest a mechanism of increased formation of free radicals and oxidative stress in diabetes mellitus.

In diabetes mellitus, oxidative stress is associated with increased production of reactive oxygen species (ROS) like superoxide radical, hydroxyl radical or hydrogen peroxide1–3. ROS is responsible for tissue damaging effect, leading to pathophysiological complications4,5. The mechanism of increased formation of free radicals in diabetes mellitus is still not clear, but prevailing theory suggests that a reduced level of scavenging enzymes like superoxide dismutase, glutathione reductase6,7 and deficiencies of antioxidants like vitamins E and C (refs 8–10) stimulate free radical formation in this pathological condition.

Allen et al.11 in 1958 first reported the existence of several glycated haemoglobin species (HbA1a, HbA1b, HbA1c) in minor amounts in normal human blood. Of these species, HbA1c, in which glucose is linked to N-terminal valine residues of β chains, is of utmost importance as its formation is increased in diabetic patients with ambient hyperglycemia and is used to monitor clinically for long-term control of blood sugar12. In normal physiological state, iron is tightly bound within protoporphyrin ring of heme pocket. Under specific circumstances, iron is released from heme and ligated to another moiety, perhaps the distal histidine in the heme pocket. This iron termed ‘free reactive iron’ can be detected by ferrozine reaction13.

Recently, we have reported14 that free reactive iron level in purified haemoglobin (total) isolated from blood of diabetic patients is proportionately increased with increased level of blood glucose. Since iron may be a source of free radicals, it may explain increased formation of free radicals and oxidative stress in diabetes mellitus. However, there has been no study on glycosylated haemoglobin-induced iron release and free radical-mediated biochemical reactions. This has led us to isolate nonglycosylated (HbA0) and glycosylated haemoglobin (HbA1c) from blood samples of diabetic
patients and investigate their differential functional behaviour with respect to iron release and free radical-mediated reactions.

Sephadex G-100, thiobarbituric acid, arachidonic acid, nitroblue tetrazolium, ferrozine, catalase and o-dianisidine were obtained from Sigma Chemical Company, USA and Biorex-70 resin (200–400 mesh) was purchased from Bio-Rad, India. All other reagents were AR grade and purchased locally.

Haemoglobin (total) was isolated and purified from heparinized blood samples donated by non-insulin-dependent diabetes mellitus volunteers belonging to the age group 40–55 years, by a method described elsewhere. This haemoglobin was applied to cation exchange column containing Biorex-70 resin (20 × 1.5 cm) pre-equilibrated with 50 mM phosphate buffer, pH 6.6. Fractions of HbA1c and HbA0 were separated by increase of NaCl concentration in elution buffer according to the method of McDonald et al. The concentrations of HbA0 and HbA1c were measured from their sorbed absorbances with extinction coefficient of 125 mM−1 cm−1 (monomer basis). Glycosylation in HbA1c was detected according to the method of Flukinger and Winterhalter.

Free iron levels in haemoglobin samples isolated from blood of diabetic patients are significantly higher than those from normal individuals. Since concentration of HbA1c is proportionately increased with hyperglycemia, this glycosylated haemoglobin species may be responsible for increased free iron concentrations in total haemoglobin samples isolated from diabetic patients. To understand the glycosylation-induced iron release from haemoglobin, HbA0 and HbA1c were isolated from haemoglobin samples of diabetic patients. However, ferrozine-detected free iron could not be detected in either HbA0 or HbA1c. It was probably eliminated during purification by ion exchange chromatography. H2O2 induces iron release from haemoglobin. We, therefore, studied the release of iron from HbA0 and HbA1c in the presence of increasing concentrations of H2O2 (0–1.25 mM), according to the method of Panter. Figure 1 shows that HbA1c releases significantly more ferrozine-detected free iron than HbA0, with increasing concentrations of H2O2. Takasu et al. reported stimulation of H2O2 generation in induced diabetes. Gutteridge measured iron released from total haemoglobin by H2O2 and other hydroperoxides and suggested a possible source of OH radical through iron-dependent Fenton reaction: Fe2+ + H2O2 → Fe3+ + OH · OH. The iron released from HbA1c and HbA0 found in this study may, thus, be associated with free radical-mediated cellular injury. Besides this, one pathological state that can result from increased concentration of free iron in blood is bacterial infection. Such complication is often encountered in diabetes.

To understand the free radical insult, lipid (arachidonic acid) peroxidation and deoxyribose degradation were measured in the presence of HbA0 or HbA1c and H2O2 essentially according to the methods of Sadrazadeh et al. and Gutteridge, respectively. Table 1 shows that HbA1c degrades arachidonic acid more efficiently than HbA0. As more iron is released from HbA1c than HbA0 (Figure 1), glycosylated haemoglobin is more efficient in degrading arachidonic acid than the nonglycosylated form as demonstrated in Table 1.
Table 2. H$_2$O$_2$-mediated deoxyribose degradation by HbA$_9$ and HbA$_{1c}$.

<table>
<thead>
<tr>
<th>Addition to the reaction mixture</th>
<th>TBA reactivity (fluorescence emission intensity, arbitrary units)</th>
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<tbody>
<tr>
<td></td>
<td>HbA$_9$</td>
</tr>
<tr>
<td>-</td>
<td>4.1</td>
</tr>
<tr>
<td>+ H$_2$O$_2$ (0.67 mM)</td>
<td>45.6</td>
</tr>
<tr>
<td>+ H$_2$O$_2$ (0.67 mM) + DFO(135 μM)</td>
<td>7.9</td>
</tr>
</tbody>
</table>

The reaction mixture (1 ml) contained HbA$_9$ or HbA$_{1c}$ (4 μM) and 0.67 mM deoxyribose in 50 mM phosphate buffer, pH 6.6. Different additions were made and incubated at 37°C for 1 h. TBA reactivity was developed by adding 0.5 ml each of TBA (1%) and TCA (2.8%), then heated for 15 min in a boiling water bath. The resulting chromogen was extracted with n-hexane. The product was estimated from fluorescence emission at 553 nm by exciting at 523 nm. The results are mean of three observations for each experiment (SD < 10%).

![figure](image)

**Figure 2.** NBT-induced methaemoglobin formation from HbA$_9$ and HbA$_{1c}$. 40 μM HbA$_9$ (Q) or HbA$_{1c}$ (○) and 240 μM NBT were used. Methaemoglobin formed was estimated from absorbances at 577 and 630 nm at different time intervals using the relation$^{22}$: Methaemoglobin (μM) = 279 A$_{577}$ nm – 3.0 A$_{630}$ nm. The results are mean ± SEM of three experiments.

breakdown as shown in Table 1 indicates that although 40 μM DFO completely inhibited HbA$_9$-mediated degradation, 100 μM DFO could inhibit only 50% of the HbA$_{1c}$-mediated breakdown. This result suggests that since H$_2$O$_2$ releases more iron from HbA$_{1c}$ than from HbA$_9$, more DFO is required to chelate the iron released from HbA$_{1c}$. Cutler$^{21}$ reported that DFO (10 mg DFO per kg body weight administered i.v. for six weeks) could improve pathophysiological complications in high ferritin diabetic patients. It was not clear how DFO worked. From the present study it seems that DFO chelates iron released from HbA$_{1c}$ and prevents oxidative stress. HbA$_{1c}$-mediated free radical insult may, thus, be associated with pathophysiological complications in diabetes mellitus.

Co-oxidation of HbA$_9$ and HbA$_{1c}$ with NBT was studied according to the method of Winterbourn$^{22}$. The spectral analysis (450–700 nm) at different time intervals showed gradual elevation of absorbance at 630 nm indicating methaemoglobin formation (spectra not shown). Figure 2 shows that the rate of methaemoglobin formation from HbA$_{1c}$ is significantly higher than that from HbA$_9$. The methaemoglobin formation can promote Heinz body and superoxide radical formation, which subsequently can damage erythrocyte membrane$^{23}$. Autooxidation of HbA$_{1c}$ was also found to be significantly higher than that of HbA$_9$ (data not shown).

Besides H$_2$O$_2$-mediated iron release from haemoglobin, H$_2$O$_2$ has another effect on the protein. Haemoglobin possesses peroxidase-like activity$^{24}$. It interacts with H$_2$O$_2$ to yield a potent oxidant (ferryl haemoglobin) capable of oxidizing a wide range of electron donors.

![figure](image)

**Figure 3.** Peroxidase activities of HbA$_9$ and HbA$_{1c}$ as a function of time. The reaction mixture (2 ml) contained 50 mM citrate buffer, pH 5.4, 1.5 μM HbA$_9$ (Q) or HbA$_{1c}$ (○), 0.02% di-2-ethylhexyl diisocyanate and the reaction was initiated by adding 17.6 mM H$_2$O$_2$. The absorbance at 450 nm was monitored. The results are mean ± SEM of four experiments.
like phenol, aromatic amines and iodide\textsuperscript{25}. We measured peroxidase activities of HbA\textsubscript{4c} and HbA\textsubscript{2} using o-dianisidine as a substrate. Compared to HbA\textsubscript{4c}, HbA\textsubscript{2} exhibited more peroxidase activity (Figure 3). Presence of iron in heme moiety is obligatory for peroxidase-like activity. Since H\textsubscript{2}O\textsubscript{2} releases more iron from HbA\textsubscript{4c} than HbA\textsubscript{2}, availability of active form of HbA\textsubscript{4c} required for peroxidase activity may be less in comparison with that of HbA\textsubscript{2}, which may explain reduced peroxidase activity of HbA\textsubscript{4c}. However, the difference in peroxidase activities between HbA\textsubscript{2} and HbA\textsubscript{4c} may also be related to their structural changes. In HbA\textsubscript{4c} the N-terminal valine of β chain is covalently blocked with ketoamine linkage due to nonenzymatic glycosylation. Change in conformation due to this chemical modification may alter the rate of entry of the substrate molecule o-dianisidine to heme pocket and consequently change the peroxidase activity. A reduced peroxidase activity of glycosylated haemoglobin was also reported by Khoo et al.\textsuperscript{26}, using 5-aminosalicylic acid as substrate. They suggested a modulation mechanism linked to structural change of the protein. From ESR spectroscopic study, Watla et al.\textsuperscript{27} reported the decreased mobility of the lysine residue adjacent to cysteine residue in glycosylated haemoglobin and suggested a change in conformation of the molecule. However, further studies are necessary on glycosylation-induced structural modification of haemoglobin to relate the consequential change in the functional activities of haemoglobin, namely H\textsubscript{2}O\textsubscript{2}-mediated iron release, spontaneous or NBT-induced oxidation, lipid and deoxyribose degradation and peroxidase activity as demonstrated in this study.