26. Copeland, R. A. *et al.*, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 11202–11206.
27. Swinney, D. C., Mak, A. Y., Barnett, J. and Ramesha, C. S., *J. Biol. Chem.*, 1997, **272**, 12393–12398.
28. So On-Yee., Scarafia, E. E., Mak, A. Y., Callan, O. H. and Swinney, D. C., *J. Biol. Chem.*, 1998, **273**, 5801–5807.
29. Kothekar, V., Sahi, S. and Srinivasan, M., *J. Biomol. Struct. Dyn.*, 1999, **16**, 901–915.
30. Sahi, S., Srinivasan, M. and Kothekar, V., *J. Mol. Struct. (Theochem)*, 2000, **498**, 133–148.
31. Du Pont, L., *Acta Cryst.*, 1995, **C51**, 507–509.
32. Stewart, J. J. P., MOPAC 7.0 QCPE Bloomington, IN. USA, 1993.
33. Kothekar, V., Sahi, S. and Mishra, J., *Indian J. Biochem. Biophys.*, 1999, **35**, 273–283.
34. Protein Data Bank, Chemistry Department Building, 555 Brookhaven National Laboratory, P.O. Box 5000 USA.
35. Koradi, R. MOLMOL – A molecule analysis and display program, Institut fur Molekular Biology und Biophysik, ETH, Zurich, Spectrospin AG, Fallenden, Switzerland, 1997.
36. Kothekar, V. and Mrigank, *Phys. Educ.*, 1988, **5**, 169–176.
37. Case, D. A. *et al.*, AMBER 5.0: Assisted Model Building with Energy Refinement – A Computer Simulation Software developed by the University of California, USA, 1997.
38. Fletcher, R. and Reeves, C. M., *Comput. J.*, 1964, **7**, 149–154.
39. Sayle, R. A., RASMOL – Molecular Visualization Program Glaxo Research and Development, Greenfield, Middlesex, UK, 1994.
40. Verlett, L., *Phys. Rev.*, 1967, **130**, 98–103.
41. Hockney, R. W. and Eastwood, J. W., in *Computer Simulations using Particles*, McGraw Hill, New York, 1981.
42. Jorgenson, W. L., Chandrasekhar, J., Medura, J. D., Impey, R. W. and Klein, M. L., *J. Chem. Phys.*, 1983, **79**, 926–935.
43. Kollman, P., *Pharm. Res.*, 1998, **15**, 368–370.

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RESEARCH COMMUNICATIONS

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

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HbA_{1c}, the major glycosylated haemoglobin increases proportionately with blood glucose level in diabetes mellitus. Here we demonstrate that H_2O_2-induced iron release is more from HbA_{1c} than that from non-glycosylated haemoglobin (HbA_0). In the presence of H_2O_2, HbA_{1c} degrades arachidonic acid and deoxyribose more efficiently than HbA_0, which suggests that iron release is more with HbA_{1c} compared to HbA_0. Increased rate of oxidation of HbA_{1c} in the presence of nitrobluetetrazolium is indicated by an increase in methaemoglobin formation. HbA_{1c} exhibits less peroxidase activity than HbA_0. 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 peroxide[1–3]. ROS is responsible for tissue damaging effect, leading to pathophysiological complications[4,5]. 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 reductase[6,7] 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 (HbA_{1a}, HbA_{1b}, HbA_{1c}) in minor amounts in normal human blood. Of these species, HbA_{1c}, 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 sugar[12]. 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 reaction[13].

Recently, we have reported[14] 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 (HbA_0) and glycosylated haemoglobin (HbA_{1c}) from blood samples of diabetic

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patients and investigate their differential functional behaviour with respect to iron release and free radical-mediated reactions.

Sephadex G-100, thiobarbituric acid, arachidonic acid, nitrobluetetrazolium, 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[15]. 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 HbA_{1c} and HbA_0 were separated by increase of NaCl concentration in elution buffer according to the method of McDonald *et al.*[16] The concentrations of HbA_0 and HbA_{1c} were measured from their soret absorbances with extinction coefficient$_{415\ nm}$ as 125 $mM^{-1}cm^{-1}$ (monomer basis)[17]. Glycosylation in HbA_{1c} was detected according to the method of Flukinger and Winterhaulter[18].

Free iron levels in haemoglobin samples isolated from blood of diabetic patients are significantly higher than those from normal individuals[14]. Since concentration of HbA_{1c} 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, HbA_0 and HbA_{1c} were isolated from haemoglobin samples of diabetic patients. However, ferrozine-detected free iron could not be detected in either HbA_0 or HbA_{1c}. It was probably eliminated during purification by ion exchange chromatography. H_2O_2 induces iron release from haemoglobin[19]. We, therefore, studied the release of iron from HbA_0 and HbA_{1c} in the presence of increasing concentrations of H_2O_2 (0–1.25 mM), according to the method of Panter[13]. Figure 1 shows that HbA_{1c} releases significantly more ferrozine-detected free iron than HbA_0, with increasing concentrations of H_2O_2. Takasu *et al.*[3] reported stimulation of H_2O_2 generation in induced diabetes. Gutteridge[19] measured iron released from total haemoglobin by H_2O_2 and other hydroperoxides and suggested a possible source of $^{\cdot}OH$ radical through iron-dependent Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH$. The iron released from HbA_{1c} and HbA_0 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[20]. Such complication is often encountered in diabetes.

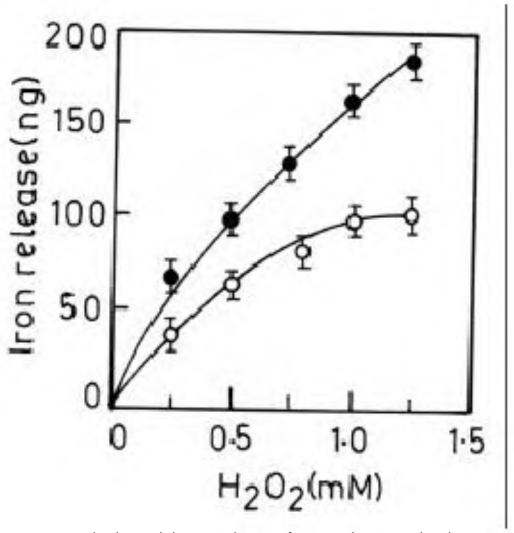

Figure 1. H_2O_2-induced iron release from HbA_0 and HbA_{1c}. Haemoglobin sample (50 µM) was incubated at 37°C for 1 h with varying concentrations of H_2O_2 (0–1.25mM). Protein was precipitated with 250 µl 20% TCA. 250 µl protein-free supernatant was treated with 2.5 ml iron buffer reagent (1.5% hydroxylamine hydrochloride in 0.2 M acetate buffer, pH 4.5) and 50 µl iron colour reagent (0.85% ferrozine in iron buffer reagent), incubated at 37°C for 30 min and read at 560 nm. Iron released from HbA_0 (○) and HbA_{1c} (●) was calculated from the standard curve using standard solution of iron buffer reagent. The results are mean ± SEM of three observations.

Table 1. H_2O_2-mediated lipid peroxidation by HbA_0 or HbA_{1c}.

Addition to the reaction mixture	MDA (nmole/h) formed from HbA_0	MDA (nmole/h) formed from HbA_{1c}
–	2.25	5.45
+ Catalase (35 units)	0	0
+ DFO (20 µM)	0.40	4.60
+ DFO (40 µM)	0	4.00
+ DFO (60 µM)	0	3.50
+ DFO (100 µM)	0	2.25

The reaction mixture (1 ml) containing HbA_0 or HbA_{1c} (40 µM), arachidonic acid (160 µM) and H_2O_2 (1 mM) was incubated at 37°C for 1 h. DFO or catalase was added as indicated. The reaction was initiated by adding H_2O_2 and stopped by adding 20% TCA. 0.5 ml each of 1% TBA and 50 mM citrate buffer, pH 3.0 were added. The supernatant was heated in a boiling waterbath 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 are mean of three experiments in each case (SD < 10%).

To understand the free radical insult, lipid (arachidonic acid) peroxidation and deoxyribose degradation were measured in the presence of HbA_0 or HbA_{1c} and H_2O_2 essentially according to the methods of Sadrzadeh *et al.*[5] and Gutteridge[19], respectively. Table 1 shows that HbA_{1c} degrades arachidonic acid more efficiently than HbA_0. As more iron is released from HbA_{1c} than HbA_0 (Figure 1), glycosylated haemoglobin is more efficient in degrading arachidonic acid than the nonglycosylated form as demonstrated in Table 1. OH radicals

Table 2. H_2O_2-mediated deoxyribose degradation by HbA_0 and HbA_{1c}.

Addition to the reaction mixture	TBA reactivity (fluorescence emission intensity, arbitrary units)	
	HbA_0	HbA_{1c}
–	4.1	4.2
+ H_2O_2 (0.67 mM)	45.6	69.2
+ H_2O_2 (0.67 mM) + DFO(135 µM)	7.9	8.7

The reaction mixture (1 ml) contained HbA_0 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 waterbath. The resulting chromogen was extracted with *n*-butanol. 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%).

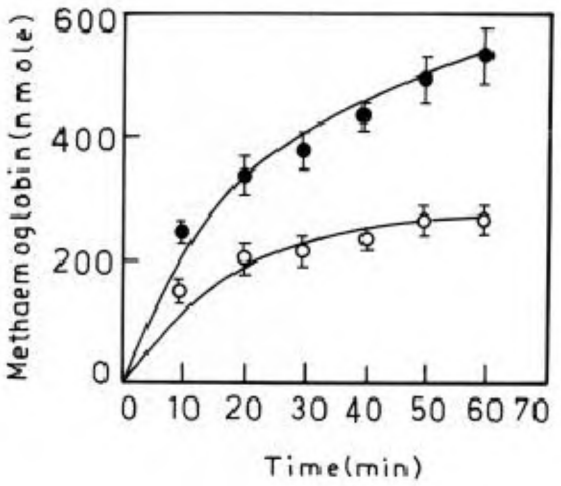

Figure 2. NBT-induced methaemoglobin formation from HbA_0 and HbA_{1c}. 40 µM HbA_0 (○) or HbA_{1c} (●) and 240 µM NBT were used. Methaemoglobin formed was estimated from absorbances at 577 and 630 nm at different time intervals using the relation[21]: Methaemoglobin (µM) = 279 $A_{630\,nm}$ – 3.0 $A_{577\,nm}$. The results are mean ± SEM of three experiments.

degrade deoxyribose. HbA_{1c}-mediated deoxyribose breakdown is more efficient than HbA_0-mediated breakdown (Table 2). These results suggest that increased oxidative stress in diabetes mellitus may be due to increased formation of HbA_{1c}. In the presence of desferrioxamine (DFO), an iron chelator, deoxyribose degradation and lipid peroxidation by HbA_0 or HbA_{1c} were significantly inhibited (Tables 1 and 2). This suggests that these processes are mediated by H_2O_2-induced free iron released from haemoglobin after interaction with H_2O_2. The effect of different concentrations of DFO on HbA_0 or HbA_{1c}-mediated arachidonic acid breakdown as shown in Table 1 indicates that although 40 µM DFO completely inhibited HbA_0-mediated degradation, 100 µM DFO could inhibit only 50% of the HbA_{1c}-mediated breakdown. This result suggests that since H_2O_2 releases more iron from HbA_{1c} than from HbA_0, 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 ferritine 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_0 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_0. 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_0 (data not shown).

Besides H_2O_2-mediated iron release from haemoglobin, H_2O_2 has another effect on the protein. Haemoglobin possesses peroxidase-like activity[24]. It interacts with H_2O_2 to yield a potent oxidant (ferryl haemoglobin) capable of oxidizing a wide range of electron donors

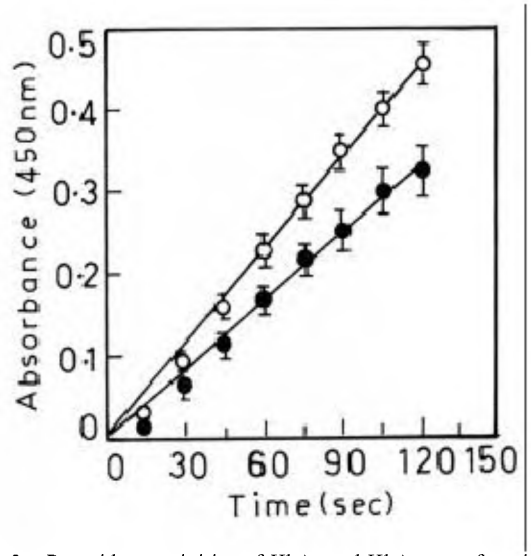

Figure 3. Peroxidase activities of HbA_0 and HbA_{1c} as a function of time. The reaction mixture (2 ml) contained 50 mM citrate buffer pH 5.4, 1.5 µM HbA_0 (○) or HbA_{1c} (●), 002% o-dianisidine and the reaction was initiated by adding 17.6 mM H_2O_2. The absorbance at 450 nm was monitored. The results are mean ± SEM of four experiments.

like phenol, aromatic amines and iodide[25]. We measured peroxidase activities of HbA_{1c} and HbA_0 using o-dianisidine as a substrate. Compared to HbA_{1c}, HbA_0 exhibited more peroxidase activity (Figure 3). Presence of iron in heme moiety is obligatory for peroxidase-like activity. Since H_2O_2 releases more iron from HbA_{1c} than HbA_0, availability of active form of HbA_{1c} required for peroxidase activity may be less in comparison with that of HbA_0, which may explain reduced peroxidase activity of HbA_{1c}. However, the difference in peroxidase activities between HbA_0 and HbA_{1c} may also be related to their structural changes. In HbA_{1c} 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.*[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.*[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_2O_2-mediated iron release, spontaneous or NBT-induced oxidation, lipid and deoxyribose degradation and peroxidase activity as demonstrated in this study.

1. Vijaylingam, S., Parthiban, A., Shanmugasundaram, K. P. and Mohan, V., *Diabetic Med.*, 1996, **13**, 715–719.
2. Heikkila, R. E., Winson, B., Cohen, G. and Barden, H., *Biochem. Pharmacol.*, 1976, **25**, 1085–1092.
3. Takasu, N., Komiya, I., Asawa, T. and Nagasawa, Y., *Diabetes*, 1991, **40**, 1141–1145.
4. Aruma, O. I., *Methods Enzymol.*, 1994, **233**, 57–66.
5. Sadrzadeh, S. M., Graf, E., Panter, S. S., Hallaway, P. E. and Eaton, J. W., *J. Biol. Chem.*, 1984, **259**, 14354–14356.
6. Adachi, T., Ohta, H., Hirano, K., Hayshi, K. and Marklund, S. L., *Biochem. J.*, 1991, **279**, 263–267.
7. Blakynty, R. and Harding, J. J., *Biochem. J.*, 1992, **288**, 303–307.
8. Urano, S., Hoshi-hashizume, M. and Tochigi, N., *Lipids*, 1991, **26**, 58–61.
9. Ceriello, A., Giugliano, D. and Quatraro, A., *Diabetes Care*, 1991, **14**, 68–72.
10. Som, S. *et al.*, *Metabolism*, 1981, **30**, 572–578.
11. Allen, D. W., Schroeder, W. A. and Balog, J., *J. Am. Chem. Soc.*, 1958, **80**, 1628–1634.
12. Goldstein, D. E., Parker, K. M. and England, J. D., *Diabetes*, 1982, **31**, 70–78.
13. Panter, S. S., *Methods Enzymol.*, 1994, **231**, 502–514.
14. Kar, M. and Chakraborti, A. S., *Indian J. Exp. Biol.*, 1999, **37**, 190–192.
15. Bhattacharyya, M., Chaudhuri, U. and Poddar, R. K., *Int. J. Biol. Macromol.*, 1990, **12**, 297–301.
16. McDonald, M. J., Shapiro, R., Bleichman, M., Solway, J. and Bunn, H. F., *J. Biol. Chem.*, 1978, **253**, 2327–2331.
17. Bhattacharyya, J., Bhattacharyya, M., Chakraborti, A. S., Chaudhuri, U. and Poddar, R. K., *Biochem. Pharmacol.*, 1994, **47**, 2049–2053.
18. Flukinger, R. and Winterhaulter, K. H., *FEBS Lett.*, 1976, **71**, 356–360.
19. Gutteridge, J. M. C., *FEBS Lett.*, 1986, **201**, 291–295.
20. Kluger, M. J. and Bullen, J. J., in *Iron and Infection* (eds Bullen, J. J. and Giffiths, E.) Wiley, New York, 1987, pp. 243–250.
21. Cutler, P., *Diabetes*, 1989, **38**, 1207–1209.
22. Winterbourn, C. C., *Methods Enzymol.*, 1990, **186**, 265–272.
23. Mal, A. and Chatterjee, I. B., *J. Biosci.*, 1991, **16**, 55–70.
24. Everse, J., Johnson, M. C. and Marini, M. A., *Methods Enzymol.*, 1994, **231**, 547–561.
25. Grisham, M. B. and Everse, J., in *Peroxidase in Chemistry and Biology* (eds Everse, J., Everse, K. E. and Grishman, M. B.), CRC Press, Boca Raton, 1991, vol. 1, pp. 335–344.
26. Khoo, U. Y., Newman, D. J., Miller, W. K. and Price, C. P., *Eur. J. Clin. Chem. Clin. Biochem.*, 1994, **32**, 435–440.
27. Watla, C., Golanski, J., Witas, H., Gurbiel, R., Gvowzdzinsky, K. and Trojanowski, Z., *Int. J. Biochem. Cell Biol.*, 1996, **28**, 1393–1403.

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