Fluorescence and Biochemical Characterization of Glycated Hemoglobin

Nadanathangam Vigneshwaran, Gopalakrishnapillai Bijukumar[a], *Nivedita Karmakar*[*], *Sneh Anand, Anoop Misra*[b]

Centre for Biomedical Engineering, Indian Institute of Technology Delhi, New Delhi – 110016, India. [a]Biomedical Engineering Unit, [b]Department of Medicine, All India Institute of Medical Sciences, New Delhi – 110029, India.

SUMMARY: Isoelectric focusing (IEF) of glycated hemoglobin (GHb) was carried out in ultra-thin polyacrylamide gels to separate the hemoglobin-advanced glycation endproducts (Hb-AGEs) from the hemoglobin-A_{1C} (HbA_{1C}) fraction. Precast polyacrylamide gels (Ampholine® PAGplate) were used in Pharmacia LKB Multiphor II for this purpose. The separated bands for Hb-AGE and HbA_{1C} based on their isoelectric point (pI), were confirmed with the purifed fractions obtained from the cation exchange chromatographic technique. From the calibration curve, the pI values were found to be 6.748 and 6.495 for HbA_{1C} and Hb-AGE, respectively. The lowering of pI values for glycated hemoglobin, when compared to unglycated hemoglobin (pI = 6.852), can be attributed to the glycation at the amino terminals of the peptide chains. Increased reduction in pI value for Hb-AGE can be attributed to the effect of glycation of amino groups at various sites on the peptide chains, apart from the terminal amino groups. Fluorescence analysis was carried out for the purified fraction of Hb-AGE which showed the formation of a new fluorophor adduct having the excitation and emission maxima at 308 nm and 345 nm, respectively. Time-dependent formation of Hb-AGE under *in vitro* conditions was monitored by fluorescence (308/345 nm) over a period of 120 days, which showed its formation only after 3 weeks of incubation.

Introduction

The adult human erythrocyte has, in addition to the major hemoglobin (HbA), several minor hemoglobins (HbA_{1a}, HbA_{1b} and HbA_{1C}). In hyperglycaemic conditions, the carbonyl group of the open chain structure of glucose reacts with free aminogroups of Hb, via nucleophilic addition to form an aldimine. The labile Schiff base formed undergoes Amadori rearrangement, resulting in a stable non-reversible ketoamine adduct. The formation of GHb is dependent upon the surrounding blood glucose

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concentration and the life span of the erythrocytes. Correspondingly, GHb disappears according to the half-life of the erythrocytes. GHb has been widely accepted as an objective and quantitative index of long-term blood-glucose levels and is today in routine use in monitoring diabetes[1]. But the long-term assessment of glucose control by Hb-AGE measurement was demonstrated recently by Wolffenbuttel *et al.*[2]. Turk *et al.*[3] compared the Hb-AGE and HbA_{1C} for the assessment of diabetic control, showing good correlation between these two parameters in diabetic subjects. The level of Hb-AGE has been reported to be a better index for monitoring the long-term complication of diabetes for the past 60 days (half life of red cells)[2]. The Hb-AGE accounts for 0.42% of the circulating hemoglobin in normal human beings whereas in diabetic patients it has been reported to rise to 0.75%[4]. The immunological assay of Hb-AGE is done by the competitive AGE-ELISA[5] method, which is tedious and requires expensive reagents.

Quantitative determination of HbA_{1C} by thin layer IEF was demonstrated by Mortensen[6]. Polymorphism in hemoglobin found in Podolian cattle was characterized by Scaloni et al.[7], using IEF. By IEF, they were able to separate the hemoglobin variants having the difference of 0.03 in pI value. IEF is an electrophoretic method for the separation of proteins, according to their pI values, in a stabilized pH gradient. IEF in immobilized pH gradients (IPG) represent the latest development in focusing methods, i.e., electrophoretic techniques able to create and maintain a pH gradient in an electric field throughout the duration of the separation process until the attainment of steady-state conditions. The specific advantages of focusing technique includes the excellent resolution of 0.01 pH units and independent over the total protein load, mode of sample preparation and the time of operation[8]. Comparison between the pI of a parent molecule and that of one to several of its derivatives will be made easier by this method; hence, it will be an effective method for the separation of HbA_{1C} and Hb-AGE.

A combination of chromatrographic and mass spectrometric techniques was also used to evaluate the extent and distribution of glycation within the glycated hemoglobin molecule. Detailed structural analysis of the phenylboronate affinity chromatgraphy/ion-exchange (IE) HPLC-separated sub populations of GHb was explored by Zhang et al.[9].

The fluorescence property of the AGEs may form the basis of a significant technique for

both the qualitative and quantitative identification of the same. An age-related increase in fluorescence and brown pigment formation was observed *in vivo* with long-lived proteins, such as lens crystallin, collagen and myelin[10]. Collagen AGE-specific fluorescence determination was performed by measuring emission at 440 nm upon excitation at 370 nm. The intensity is known to increase with an advance in diabetes[5]. Fluorescence of pentosidine, an intermediate adduct formed during non-enzymatic browning of ribonuclease and lysozyme by glucose has been characterized and observed to accumulate in tissue proteins with age and in diabetes[11]. The emission maxima for pentosidine has been observed at 385 nm when excited at 335 nm. Spectroscopy-based fluorescence determination is more sensitive than the absorbance spectroscopy[12].

Materials and Methods

In vitro analysis of Hb-AGE formation

Bovine serum hemoglobin (purchased from Sigma Co. St.Louis, USA), D(+)-glucose, 0.4 M sodium phosphate buffer (pH 7.4) were used for the *in vitro* experiment. All solutions were made in deionised water. Two different hemoglobin samples were prepared, with 5.0 mM and 20.0 mM glucose to mimic the normoglycemic and hyperglycemic conditions, respectively, while the control was maintained without glucose. Hemoglobin at the concentration of 50.0 mg/ml [5] was dissolved in 0.4 M sodium phosphate buffer (pH 7.4) and incubated in a rotary shaking water bath at 37°C for 120 days under sterile and dark conditions. Intermittent sampling was done to check for contamination by microorganisms. At weekly intervals, a sample was withdrawn and diluted with 0.4 M sodium phosphate buffer (pH 7.4) to obtain the final concentration of 1.0mg/ml[13] [diluted samples were used to avoid the inner filter effect]. The diluted hemoglobin samples were used for fluorescence measurement, using a Luminescence Spectrofluorimeter (Perkin Elmer, LS 50 B UK). Samples taken after 3 months of incubation were used for purification and characterization of Hb-AGE.

Isolation of Glycated Hemoglobin

Total GHb was isolated using the phenyl boronate affinity chromatography kit [Sigma Co. St.Louis, USA] which uses an affinity resin in a disposable column that has an

affinity for cis-diols, e.g, glucose molecules attached to hemoglobin. The eluted fraction which contains the GHb was used for further analysis.

Separation of HbA_{1C} and Hb-AGE

The GHb (eluted fraction of affinity chromatography) was further separated on a cation-exchange column (CM-Cellulose) using a flow rate of 0.8 ml/min. The fractions were detected by absorbance at 415nm. A binary buffer system was used: buffers A and B consisted of 20 mM Bis-Tris-1 mM KCN (pH 6.7) and 20 mM Bis-Tris-1 mM KCN - 0.1 M NaCl (pH 6.5), respectively. The desired pHs of the buffers were obtained by titration with HCl. The column was equilibrated with buffer A, and step elution was carried out with the buffer B. The fractions manually collected were monitored as mentioned above.

Isoelectric Focusing

In vitro glycated hemoglobin samples, after 90 days of incubation, were gel filtered (column packed with Sephadex G-25) to remove unreacted glucose. Using the absorbance value at 280 nm the final concentration was adjusted to be 5 mg/ml.

The Multiphor II™ electrophoresis system (Pharmacia LKB Biotechnology, Sweden) was used for isoelectric focusing. Precasted Ampholine® PAG plate (pH: 3.5 to 9.5) of size 245 × 110 × 1 mm made of polyacrylamide gel, having the ampholine concentration of 2.2% [w/v], was used. The anolyte and catholyte used were 1.0 M phosphoric acid and 1.0 M NaOH, respectively. The temperature was maintained at 10°C by using the MultiTemp II™ thermostatic circulator. IEF applicator strips (size : 1.0 × 0.5 cm) were used for the purpose of sample application. Samples were applied at the concentration of 5 mg/ml and 20µl was loaded per strip. An isoelectric focusing calibration kit (Pharmacia) having the pI markers was used to generate the calibration curve. The voltage applied in sequence was in the order of: 500 V for 10 min, 1000 V for 10 min and 1500 V for 2 h. A MultiDrive™ XL power supply was used. While running, after 1 h, application strips were removed. At the end of the run, the gel was washed in fixing solution for 20 min and stained by Coomassie Blue R 250, to observe the bands. A standard curve was derived by plotting pI values against the relative

mobility of the protein bands. The bands for HbA_{1C} and Hb-AGE were distinguished by comparing with the lanes having the purified fractions obtained from cation exchange chromatography. By comparing with the standard curve, the pI values of HbA_{1C} and Hb-AGE were calculated.

Fluorescence Analysis

The purified fraction of Hb-AGE was subjected to fluorescence analysis in a LS 50B PERKIN ELMER Spectrofluorimeter. Based on the prescan values, an emission scan was done. At weekly intervals, a sample was withdrawn from the *in vitro* glycated hemoglobin and diluted with 0.4 M sodium phosphate buffer (pH 7.4) to get the final concentration of 1.0 mg/ml [to avoid the inner filter effect]. Prescanning was carried out at the wavelength ranging from 200 to 800 nm. Based on the prescan results, the excitation wavelength was strandadized to be 308 nm and the emission scan was done in the range of 330 to 400 nm for emission wavelength.

To remove these scattering effects, an emission spectrum of blank solution was recorded and then substracted from the emission spectrum of the sample solution. Since Rayleigh scattering is largely a random effect, the scatter peaks in the two spectra may not be identical. Hence, the spectra were normalized before performing the background substraction. After normalization, the normalized blank spectrum was substracted from the normalized sample spectrum. The resultant spectra, after removing the Rayleigh and Raman scattering, were used for peak analysis. Major peaks were identified and their values were used for analysis.

Results

Figure 1 shows the cation-chromatrographic separation of Hb-AGE and HbA_{1C} by a stepwise elution method. The total GHb fraction isolated from the phenyl boronate affinity chromatography kit was loaded into the cation-exchange column for further separation of HbA_{1C} and Hb-AGE. The fraction eluted by the buffer of pH 6.7 corresponds to HbA_{1C} while that fraction eluted by the buffer of pH 6.5 corresponds to Hb-AGE. The fractions collected corresponding to the peaks were quantified, based on the absorbance values at 280 nm and used for IEF.

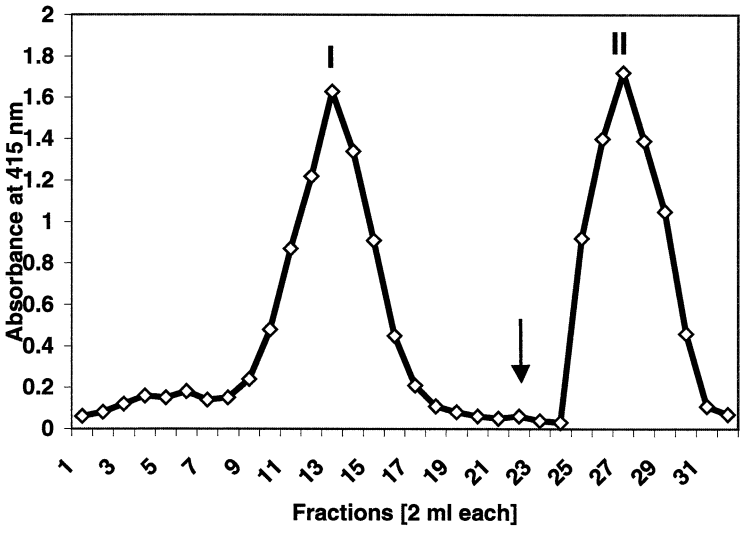

Fig. 1 Cation-exchange chromatographic separation of HbA_{1C} and Hb-AGE. First eluted fraction (I) represents HbA_{1C} whereas the second eluted fraction (II) represents Hb-AGE. Arrow indicates the addition of buffer B .

For IEF, the calibration curve was obtained by plotting the relative mobility of bands for the pI markers against their pI values. The pure hemoglobin was found to have a single band of pI value 6.852, as determined from the calibration curve. In the lanes having *in vitro* glycated hemoglobin as such we observed three bands, while the lanes having purified fractions of HbA_{1C} and Hb-AGE contained only one band each. These two bands corresponding to HbA_{1C} and Hb-AGE were in the anodic side (see Fig. 2). The relative mobility of these two bands were calculated and compared with the calibration curve. The following values were obtained: 6.748 and 6.495 for HbA_{1C} and Hb-AGE, respectively.

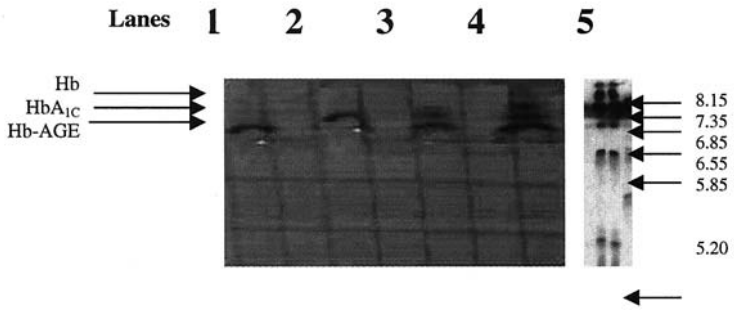

Fig. 2 Typical IEF of *in vitro* glycated hemoglobin. Lanes include purified Hb-AGE (1), purified HbA_{1C} (2), GHb (3), *in vitro* glycated hemoglobin (4) and marker (5) with pI values shown

Fluorescence studies demonstrated a typical excitation peak at 308 nm and an emission peak at 345 nm for the purifed Hb-AGE, whereas no such peak was observed for both the HbA_{1C} and pure Hb. *In vitro* studies over a period of 120 days showed an increase in the fluorescence at 308/345 nm after 3 weeks for samples incubated with glucose, while no such fluorescence was observed for the control samples (see Fig. 3).

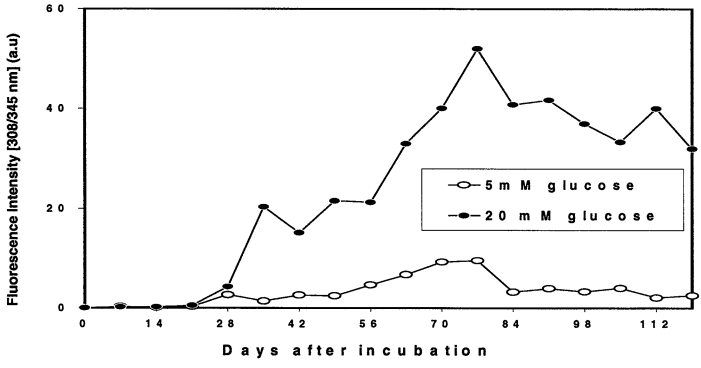

Fig. 3 Kinetics of formation of Hb-AGE under i*n vitro* conditions

Discussion

Modification, such as glycation, of the amino terminus of the peptide chains imparts enough negative charge to the hemoglobin molecule to allow separation of the respective hemoglobins by charge-dependent techniques. Stevens *et al.*[14] reported that the isoelectric points and column chromatographic properties of all the glycolytic intermediate-hemoglobin adducts were identical with those of the natural product HbA_{1b}. The IEF pattern of hemoglobin from diabetic patients have been reported to form two bands, corresponding to Hb and HbA_{1C}[6]. In contrast, our experiments showed one more band, which might be due to the formation of Hb-AGE. To confirm the identity, purified fractions of HbA_{1C} and Hb-AGE were also used in IEF. The *in vitro* sample produced three bands in IEF, corresponding to Hb, HbA_{1C} and Hb-AGE. The characteristic fluorescence nature of the Hb-AGE can be attributed to the formation of new aromatic glycated endproducts formed from HbA_{1C}, which differs from HbA_{1C} in its non-reversible nature. The significant increase in the fluorescence at 308/345 nm after the period of three weeks of incubation of Hb with glucose can be attributed to the formation of a new fluorophor adduct which will serve as a good monitoring index for diabetes mellitus.

Conclusion

The data presented here are a qualitative overview of the IEF pattern for the Hb-AGE. This proves the difference between the HbA_{1C} and Hb-AGE. The lowering of pI value for Hb-AGE compared to that of HbA_{1C} demonstrated the advanced stage of non-enzymative glycosylation of hemoglobin. Further research is going on in our laboratory to elucidate the structural differences between HbA_{1C} and Hb-AGE and the possibility of using Hb-AGE as a biosensing element for diabetes mellitus, using its intrinsic fluorescence nature.

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