Glycosylated Minor Components of Human Adult Hemoglobin

PURIFICATION, IDENTIFICATION, AND PARTIAL STRUCTURAL ANALYSIS*

Melisenda J. McDonald, Robert Shapiro, Margaret Bleichman, Julian Solway, and H. Franklin Bunn†

From the Division of Hematology, Department of Medicine, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Massachusetts 02115

Human hemolysate contains several minor components designated Hb A₁ₐ, Hb A₁ₘ, Hb A₁ₜ, which are post-translational modifications of the major hemoglobin component A_g. Individuals with diabetes mellitus have elevated levels of Hb A₁ₜ, a hemoglobin modified with a glucose moiety at the NH₂ terminus of each β chain. A new chromatographic technique using Bio-Rex 70 is described which not only allows complete separation of Hb A₁ₐ from Hb A₁ₚ but also resolution of Hb A₁ₜ into two components, designated Hb A₁ₚ and Hb A₁ₛ. Carbohydrate determinations with the thiobarbituric acid procedure revealed that Hb A₁ₚ, Hb A₁ₚ, and Hb A₁ₚ, as well as Hb A₁ₚ were glycosylated. Total phosphate analysis revealed 2.06 and 1.01 mol of phosphate/α/β dimer for Hb A₁ₚ and Hb A₁ₚ, respectively; Hb A₁ₚ and Hb A₁ₚ contained no detectable phosphate. Hemoglobin incubated with [14C]glucose-6-P co-chromatographs precisely with Hb A₁ₚ, strongly suggesting that Hb A₁ₚ is glucose-6-P hemoglobin. Levels of Hb A₁ₚ and Hb A₁ₚ are normal in individuals with diabetes mellitus. Furthermore, diabetic red cells contain normal levels of glucose-6-P. Therefore, glucose-6-P hemoglobin does not serve as a significant precursor to Hb A₁ₚ. Instead Hb A₁ₚ is formed by the direct reaction of hemoglobin with glucose. This suggests that hemoglobin can serve as a model system for nonenzymatic glycosylation of protein.

Hemoglobins A₁ₚ, A₁ₚ, and A₁ₚ are negatively charged minor components found in normal human red cells (1). Structural analysis of Hb A₁ₚ, the most abundant minor component, has shown that the NH₂ terminus of the β chain forms a Schiff base linkage with glucose and then undergoes an Amadori rearrangement to a stable ketoamine linkage (2-4). In contrast to Hb A₁ₚ, there is virtually no information to date on the structure of Hb A₁ₚ and Hb A₁ₚ.

* This work was supported by Grant AM1823 from the National Institutes of Health and the Nehemias Gorin Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Investigator, Howard Hughes Medical Institute.

In this report unmodified Hb A₁ₚ will be designated Hb A₁ₚ, in keeping with the nomenclature of Huisman and Meyering (6). Allen et al. (1) used the designation Hb A₁ₚ.
RESULTS AND DISCUSSION

Purification of Minor Hemoglobin Components - The elution profile of normal adult hemolysate on Bio-Rex 70 is shown in Fig. 1. This new column procedure not only allows complete separation of the minor hemoglobin components Hb A\(_{1p}\), Hb A\(_{2p}\), and Hb A\(_{4p}\), but also the separation of "Hb A\(_{1s}\)" into two components which we have designated Hb A\(_{1ps}\) and Hb A\(_{1ps}\). The non-hemoglobin peak, previously described as the "non-heme protein(s)," contains a variety of proteins including heme proteins that are not bound to the resin under the conditions employed. The two A\(_{1s}\) hemoglobins, which are retained somewhat by the column, can be separated from the non-hemoglobin protein peak as well as from each other. Hemoglobin A\(_{1s}\) is eluted as a well defined peak. Finally, hemoglobins A\(_{2s}\) and A\(_{4s}\) are eluted with 0.1 M NaCl and 1.0 M NaCl, respectively, in the starting buffer.

Rechromatography of Minor Components - The modified chromatographic procedure of Schnek and Schroeder (10) shown in Fig. 2A did succeed in separating Hb A\(_{sa}\) from the major hemoglobin peak and from minor components Hbs A\(_{1p}\) and A\(_{2p}\), and the non-hemoglobin peak. Note that the separation of Hb A\(_{sa}\) and A\(_{1ps}\) is not satisfactory. In order to interpret the chromatographic pattern shown in Fig. 1, we had to identify the minor hemoglobin peaks in terms of the elution pattern described by Allen et al. (1). This comparison is necessary since hemoglobins are not always eluted from Bio-Rex 70 in a single peak. The relative elution mobilities of the peaks of Hbs A\(_{1ps}\), A\(_{1ps}\), and A\(_{4ps}\) from the chromatogram shown in Fig. 2A were pooled separately, concentrated by ultrafiltration and rechromatographed on individual Bio-Rex 70 columns according to the new procedure described above. The elution profiles of these columns are shown as a composite in Fig. 2B. It is clear that pool 42 to 47, representing Hb A\(_{1ps}\), is resolved into two components which we have designated Hb A\(_{1ps}\) and Hb A\(_{1ps}\). Pool 49-55 is clearly Hb A\(_{4ps}\) and pool 65-85 is clearly Hb A\(_{4ps}\).

Alkaline Denaturation Studies - Allen et al. (1) found that under their conditions Hb F co-chromatographed with A\(_{1k}\) and acetylated Hb F co-chromatographed with A\(_{1k}\). Since it was possible that either Hb A\(_{1ps}\), A\(_{1ps}\), or A\(_{4ps}\) is or contains fetal hemoglobin, these components were subjected to alkaline denaturation according to the method of Ikehns et al. (12). Hb F is resistant to alkaline denaturation, whereas other types of human hemoglobin are rapidly denatured by the addition of alkali. As shown in Fig. 3A, all of these minor components isolated on Bio-Rex 70 (Fig. 1) resembles Hb A in their rapid denaturation by alkali, in contrast to Hb F in cord hemolysate. These results indicate that neither Hb A\(_{1ps}\), A\(_{1ps}\), A\(_{4ps}\), nor A\(_{4ps}\) contains a significant amount of fetal hemoglobin.

Identification of Modified Subunits - Analysis of hybrid hemoglobins formed from mixtures of the pured hemoglobin components with canine hemoglobin revealed that Hbs A\(_{1ps}\), A\(_{1ps}\), A\(_{1sp}\), and A\(_{4sp}\) all owe their low isoelectric points to modifications on the \(\beta\) chain only. These results are depicted in Table I.

Presence of Carbohydrate - Analysis of the minor hemoglobin components by the colorimetric assay of Fluckiger and Winterhalter (9) is shown in Fig. 4A. All the minor components assayed showed absorbance spectra typical of the complex that 5-hydroxyethylfurfural forms with thiobarbituric acid, with an \(\varepsilon_{\text{max}}\) at 443 nm. Our results for Hb A\(_{2p}\) are consistent with those reported by Fluckiger and Winterhalter (9). The absorbance spectra obtained on Hbs A\(_{1ps}\), A\(_{1ps}\), and A\(_{1ps}\) are quantitatively different from that for A\(_{1ps}\). The amount of color formed with these hemoglobin components was only about 20% of that formed from Hb A\(_{2p}\). Likewise, fructose-6-P and fructose 1,6-diphosphate, formed considerably less color than did fructose (Fig. 4B). Thus, both Hb A\(_{1ps}\) and Hb A\(_{1ps}\) gave color consistent with that expected of a phosphorylated sugar derivative.

The close similarity of the absorbance spectra in Fig. 4A provides qualitative evidence that Hbs A\(_{1ps}\), A\(_{1ps}\), and A\(_{1ps}\) contain carbohydrate groups attached to hemoglobin by a ketamine linkage like that of Hb A\(_{1ps}\). Neither a simple glycoaldehyde linkage nor an aldamine linkage (Schiff base) would produce products capable of forming this type of colored complex with thiobarbituric acid (13). However, our results provide no information on the structure of the carbohydrate moity in Hbs A\(_{1ps}\), A\(_{1ps}\), or A\(_{1ps}\) on or its site of attachment. We are currently attempting to recover, quantify, and identify reducing sugars following acid hydrolysis of these hemoglobins, using procedures similar to those which we employed for the analysis of Hb A\(_{2p}\). (4)

Phosphorylation Analysis - As shown in Table I, Hb A\(_{1ps}\) contains 2 mol of phosphorus/mol of \(\alpha\) dimer, while Hb A\(_{1ps}\) contains 1 mol. In contrast, no phosphorus could be detected in Hbs A\(_{1ps}\), A\(_{1ps}\), and A\(_{1ps}\). These results in conjunction with the colorimetric assays presented above provide strong evidence that Hb A\(_{1ps}\) and A\(_{1ps}\) contain sugar phosphates. Phosphorus analysis of the non-hemoglobin protein peak ruled out the possibility that Hbs A\(_{1ps}\) and A\(_{1ps}\) were contaminated by overlap with some phosphoric acid non-hemoglobin protein.

Co-chromatography of Natural and Synthetic Components - Although not a rigorous proof of structure, co-chromatography of the natural and synthetic components can provide important independent evidence. Haney and Bunn (7) showed that synthetically prepared glucose-6-P hemoglobin, chromatographed by the procedure of Allen et al. (1), migrated in a position close to that of Hb A\(_{1ps}\). In our new chromatographic system \(\Delta^{14}\)C-glucose-6-P labeled hemoglobin co-chromatographed precisely with A\(_{1ps}\). Fig. 5A. In view of the other data including chain localization, phosphate analysis and the thiobarbituric acid color test, we tentatively conclude that Hb A\(_{1ps}\) is glucose-6-P hemoglobin. The possibility that Hb A\(_{1ps}\)
characterization of glycosylated minor hemoglobin components

Fig. 1. Elution profile of normal adult hemolysate on Bio-Rex 70. Eighty-two milliliters of hemolysate (5 mM in heme) of proper pH and ionic strength were loaded onto a Bio-Rex 70 column (6 x 40 cm) which had been previously equilibrated with 0.05 M potassium phosphate buffer, pH 6.60. The column was developed as described under "Experimental Procedures" using a flow rate of 117 ml/h. The fraction size collected was 8.8 ml. By taking advantage of the large change in extinction coefficients for oxyhemoglobin in the Soret (\(e_{540}^\text{Soret} = 131 \text{ M}^{-1} \text{ cm}^{-1}\)) and visible (\(e_{40}^\text{Visible} = 14.37 \text{ M}^{-1} \text{ cm}^{-1}\)), and far-visible (\(e_{40}^\text{Far-visible} = 6.08 \text{ M}^{-1} \text{ cm}^{-1}\)), it is possible to display the entire human hemolysate profile.

Fig. 2. Rechromatography of the minor components of normal hemolysate. A, elution profile of Bio-Rex 70 developed according to several modifications of the method of Allen et al. (1). Forty-one milliliters of carboxyhemoglobin (6.7 mM in heme) were loaded onto a column (6 x 40 cm). The flow rate was 57 ml/h and the fraction size was 9.6 ml. Hb A\(_{1a}\) (Fractions 42 to 47), Hb A\(_{1b}\) (Fractions 49 to 55), and Hb A\(_{1c}\) (Fractions 65 to 85) were pooled and concentrated by ultrafiltration. B, rechromatography of each of the peaks (Hbs A\(_{1a}\), A\(_{1b}\), and A\(_{1c}\)) on Bio-Rex 70 (5 x 40 cm) using the method described in Fig. 1. The elution profiles for Hb A\(_{1a}\) (O--O), Hb A\(_{1b}\) (O--O), and Hb A\(_{1c}\) (A--A) are all shown. Nine-to ten-milliliter fractions were collected. A linear salt gradient of 0 to 0.1 M NaCl (500 ml total) was started at Fraction 120 for the "Hb A\(_{1a}\)" column and at Fraction 121 for the "Hb A\(_{1b}\)" column.

contains other sugar phosphate derivatives of hemoglobin, such as fructose-6-P-hemoglobin as well, cannot be ruled out. In like manner, the more negatively charged Hb A\(_{1b}\) is likely to be an adduct of a sugar moiety containing two phosphates on the beta chain of hemoglobin. Among the phosphorylated intermediates within the red cell, the only two candidates are glucose 1,6-diphosphate and fructose 1,6-diphosphate. The former compound does not form a stable adduct when incubated with Hb A\(_{1a}\), while the latter does (8). Unlike synthetic glucose-6-P hemoglobin, our preparations of \(p-^\text{[14C]}\)fructose 1,6-diphosphate hemoglobin have not produced a single major monodisperse chromatographic peak. Only a portion of this synthetic hemoglobin co-chromatographed with Hb A\(_{1a}\). Thus we have less convincing evidence concerning the structure of Hb A\(_{1b}\).

Our results are in direct opposition to those of Stevens et al. (8) who concluded that Hb A\(_{1a}\) is an adduct between hemoglobin and glucose-6-P. They obtained a crude mixture of non-hemoglobin protein and Hbs A\(_{1a}\), A\(_{1b}\), and A\(_{1c}\) and rechromatographed it by carboxymethylcellulose chromatography using a stepwise elution. A small heme-containing peak was obtained in the void volume and second peak (4-fold larger) was obtained immediately following the step to a buffer of higher pH. They interpreted these peaks as being Hb A\(_{1a}\) and A\(_{1b}\), respectively. In our opinion, their first peak is probably non-hemoglobin protein, which has strong absorp-
Characterization of Glycosylated Minor Hemoglobin Components

Table I

Structural analysis of minor hemoglobin components

The various hemoglobin components were purified and analyzed as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Hemoglobin component</th>
<th>Modified chain</th>
<th>Phosphate content (per αβ dimer) N = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A$_{aa}$</td>
<td>β</td>
<td>2.06 ± 0.340</td>
</tr>
<tr>
<td>Hb A$_{ab}$</td>
<td>β</td>
<td>1.01 ± 0.163</td>
</tr>
<tr>
<td>Hb A$_{bb}$</td>
<td>β</td>
<td>0</td>
</tr>
<tr>
<td>Hb A$_{bc}$</td>
<td>β</td>
<td>0</td>
</tr>
<tr>
<td>Hb A$_{ac}$</td>
<td>β</td>
<td>0</td>
</tr>
<tr>
<td>Glucose-6-P-Hb$^a$</td>
<td>β</td>
<td>0.98</td>
</tr>
</tbody>
</table>

$^a$ Glucose-6-P-hemoglobin has been shown previously to be a β chain modification (7). The phosphate analysis was done only on one preparation and is included for sake of comparison.

The normal levels of Hb A$_{ab}$, A$_{ac}$, and A$_{bc}$ in diabetic individuals. Our values for normal and diabetic individuals are very close to those previously reported (11, 18). The reason for this discrepancy is not clear. Theoretically, diabetic red cells would be expected to have normal levels of glucose-6-P since even in normal red cells, hexokinase is operating at its $V_{max}$, and thus the rate of formation of glucose-6-P should be no higher in hyperglycemic individuals.

The results shown in Fig. 5B are in excellent agreement with the results found by several groups (11, 14, 15). It has also been reported (16-18) that the quantity of Hb A$_{ab}$ had radioactivity associated with it. These results are currently under investigation.

Minor Hemoglobin Components in Diabetic Hemolysate - The elution profile of diabetic hemolysate on Bio-Rex 70 is shown in Fig. 6S. The most notable difference between the profiles of normal (Fig. 1) and diabetic hemolysate is the increase in the quantity of Hb A$_{bc}$. This increase is in agreement with the results found by several groups (11, 14, 15). It has also been reported (16-18) that the quantity of Hb A$_{ab}$ was also elevated in diabetics. Table II shows the quantitation of minor components from six chromatographic runs. It is clear that the amount of Hb A$_{ab}$ and Hb A$_{ac}$ (the sugar phosphate derivatives of native hemoglobin) is the same in normal and diabetic individuals. We suggest that the reported increase in Hb A$_{ab}$ seen in diabetic hemolysate, may be due to an increase in levels of Hb A$_{ab}$ or possibly to an increase in the amount of "non-hemoglobin" protein. The amount of heme absorbance in the "non-heme" peak is always elevated in the diabetic. Spectral monitoring of this peak in the visible and Soret regions and, in the presence and absence of dithionite, revealed that the heme absorbance of this peak is not due to ferrous hemoglobin but rather is derived from a spectrally distinct heme protein or proteins.

Glycolytic Intermediates in Normal and Diabetic Red Cells - As shown in Fig. 7S, no differences in the levels of glucose-6-P and fructose-6-P were observed in the red cell of normal versus diabetic individuals. Our values for normal individuals, as well as those of Stevens et al. (8) are very close to those previously reported (19). In contrast, Stevens et al. (8) reported about a 1.5-fold increase in these intermediates within diabetic red cells. The reason for this discrepancy is not clear. Theoretically, diabetic red cells would be expected to have normal levels of glucose-6-P since even in normal red cells, hexokinase is operating at its $V_{max}$, and thus the rate of formation of glucose-6-P should be no higher in hyperglycemic individuals.

CONCLUSIONS

The normal levels of Hb A$_{ab}$ and glucose-6-P and fructose-6-P in diabetic red cells provide strong evidence rebutting the proposal (7, 8), that glucose 6-phosphate Hb is the precursor of Hb A$_{ab}$. Although our studies have not ruled out the possibility that some Hb A$_{ab}$ is derived from glucose-6-P Hb (Hb A$_{abc}$), clearly the bulk of Hb A$_{ab}$ does not come from this source.

The concentration of intracellular glucose is about 200-fold that of glucose-6-P, while the rate at which glucose-6-P reacts with Hb A$_{ab}$ is at least 10-fold greater than that observed with glucose (7). It is apparent that glycosylation is a direct reflection of levels of different metabolites within the red cells as well as the rate at which these metabolites react with hemoglobin. Thus, the simplest and most plausible mecha-
Characterization of Glycosylated Minor Hemoglobin Components

Fig. 5. Elution profiles of two synthetic glycosylated hemoglobins chromatographed on Bio-Rex 70. A, purified Hb A, (5.5 mM in heme) was incubated with 15 mM glucose-6-P (containing 30 µCi of d-[14C]glucose-6-P) at 37°C for 3 days. After passage through Sephadex G-25 the incubation mixture was combined with 41 ml of hemolysate (5.4 mM in heme) of proper pH and ionic strength and loaded onto a Bio-Rex 70 column (5 x 40 cm). The column was developed as described in Fig. 1. The fraction size was 9.9 ml. B, purified Hb A, (5 mM in heme) was incubated with 15 mM glucose (containing 50 µCi of l-[14C]glucose) at 37°C for 6 days. After passage through Sephade G-25 the incubation mixture was combined with 1 ml of hemolysate (5.4 mM in heme) and chromatographed on a Bio-Rex column (5.2 x 20 cm). The fraction size was 3.4 ml and the flow rate was 37 ml/h.

Acknowledgments—We are indebted to Dr. Kenneth H. Gabbay for supplying the diabetic samples and for measuring Hb A1c content in individuals whose red cell glycolytic intermediate levels were studied. We also thank Dr. Alan L. Schwartz for his generous gift of cord blood. Furthermore, we wish to acknowledge Dr. Paul M. Gallop for his valuable discussions throughout the course of this work. Finally, we wish to thank Adrianna Morris and Michael Austin for their help in preparing the manuscript.

REFERENCES

Additional Refs. 1S-6s are found on p. 2332.
Characterization of Glycosylated Minor Hemoglobin Components

Supplementary Materials for
Glycosylated Minor Components of Normal Adult Hemoglobin

A. N. C. van der Schans, Robert Guegan, Margaret Honigman, Julius Schier and M. Franklin Bunn

Preparation of Hemolysates

Sera were obtained from normal donors following venipuncture under aseptic conditions. The sera were stored at -20°C until use. The hemoglobin was solubilized by the method of Bearn et al. (19), which involves the addition of 1 vol. of 1.6 M sodium chloride to 1 vol. of serum, followed by centrifugation at 4°C for 20 minutes.

Alkaline Denaturation

The rate of alkaline denaturation of each minor purified hemoglobin component was measured spectrophotometrically by the method of Barlow et al. (20). A sample of each fraction in the minor component and a sample of normal blood were run for comparison.

Determination of Protein-Nucleotides

The purified hemoglobin components were assayed according to the method of Bearn and Treadwell (19) using the proteins and nucleotides as standards. The nucleotides were identified by paper chromatography as described by Hirs (21).

Chromatographic Test

The purified hemoglobin components were separated by electrophoresis on filter papers using a buffer consisting of 0.1 M glycine-0.1 M Tris-HCl-0.1 M NaCl, pH 8.9. The samples were applied as 20-μl aliquots and run for 2 hours at 200 volts. The fractions were then stained with 0.05% of amido black 10B and then immersed in the buffer for 15 minutes. The bands were visualized by ultraviolet light and then photographed. The bands were scanned and the absorbance at 540 nm was measured. The results were expressed as a percentage of the total absorbance.

Figure 1: Time course of alkaline denaturation for the components of normal hemoglobin and for blood. The following samples: 20 mg A<sub>2</sub> (2 g), 10 mg A<sub>2</sub> (2 g) and 5 mg A<sub>2</sub> (2 g) were assayed as described. A<sub>2</sub> was calculated from the results of the reaction.

Figure 2: Concentration of glycine-06 and fructose-06 in red cells of normal and diabetic individuals. The values of glycine-06 and fructose-06 were determined in each individual, and the slope of the line was calculated for each individual. The values of glycine-06 and fructose-06 were then compared with the normal values of glycine-06 and fructose-06. The slopes of the lines were then calculated for each individual.

Figure 3: Electrophoretic patterns of hemoglobin. The electrophoretic patterns were run on filter papers using a buffer consisting of 0.1 M glycine-0.1 M Tris-HCl-0.1 M NaCl, pH 8.9. The samples were applied as 20-μl aliquots and run for 2 hours at 200 volts. The bands were visualized by ultraviolet light and then photographed. The bands were scanned and the absorbance at 540 nm was measured. The results were expressed as a percentage of the total absorbance.
M J McDonald, R Shapiro, M Bleichman, J Solway and H F Bunn


Access the most updated version of this article at http://www.jbc.org/content/253/7/2327

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/253/7/2327.full.html#ref-list-1