Glycosylated Minor Components of Human Adult Hemoglobin

PURIFICATION, IDENTIFICATION, AND PARTIAL STRUCTURAL ANALYSIS

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Human hemolysate contains several minor components designated Hb A₁₅, Hb A₁₀, Hb A₆, which are post-translational modifications of the major hemoglobin component A₄. Individuals with diabetes mellitus have elevated levels of Hb A₁₅, 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 A₁₀, Hb A₁₅, but also resolution of Hb A₁₀ into two components, designated Hb A₁₀₁ and Hb A₁₀₂. Carbohydrate determinations with the thiobarbituric acid procedure revealed that Hb A₁₀₁ and Hb A₁₀₂, as well as Hb A₆, were glycylated. Total phosphate analysis revealed 2.06 and 1.01 mol of phosphate for Hb A₁₀₁ and Hb A₁₀₂ respectively; Hb A₁₀ and Hb A₆ contained no detectable phosphate. Hemoglobin incubated with n-[¹⁴C]glucose-6-P co-chromatographs precisely with Hb A₁₀₁, strongly suggesting that Hb A₁₀₁ is glucose-6-P hemoglobin. Levels of Hb A₁₀₁, and Hb A₁₀₂ 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 A₆. Instead Hb A₆ 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 A₁₀₁, A₁₀, and A₆ are negatively charged minor components found in normal human red cells. Structural analysis of Hb A₆, the most abundant minor component, has shown that the NH₂-terminus of the β chains forms a Schiff base linkage with glucose and then undergoes an Amadori rearrangement to a stable ketoamine linkage (2-4). In contrast to Hb A₁₀, there is virtually no information to date on the structure of Hb A₁₀₁ and Hb A₁₀₂.

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Hemoglobin A₁₀₁, A₁₀, and A₆ are negatively charged minor components found in normal human red cells. Structural analysis of Hb A₆, the most abundant minor component, has shown that the NH₂-terminus of the β chains forms a Schiff base linkage with glucose and then undergoes an Amadori rearrangement to a stable ketoamine linkage (2-4). In contrast to Hb A₁₀, there is virtually no information to date on the structure of Hb A₁₀₁ and Hb A₁₀₂.

In vivo studies have shown that all three minor hemoglobins (Hbs A₁₀₁, A₁₀, and A₆) are formed slowly and continuously throughout the 120-day lifespan of the human red cell (5). The slow conversion of the major component Hb A₄ to Hbs A₁₀₁, A₁₀, and A₆ suggests that the reactions take place nonenzymatically. If so, it should be possible to form Hb A₆ from Hb A₄ in vitro, by simply incubating the major hemoglobin component with glucose.

Under physiological conditions D-glucose-6-P serves as an affinity label, forming a covalent linkage specifically at the NH₂-terminus of each β chain while the reaction with glucose was much slower and less specific (7). Other sugar phosphates also formed covalent adducts with hemoglobin (8). These results raise the possibility that naturally occurring minor components may be adducts of hemoglobin with sugar phosphates. Both Haney and Bunn (7) and Stevens et al. (8) proposed that glucose-6-P Hb may serve as a precurser, undergoing dephosphorylation to form Hb A₆.

Flückiger and Winterhalter (9) found that when a higher concentration of n-[¹⁴C]glucose was incubated with Hb A₄, a [¹⁴C]-labeled n-glucose-hemoglobin product was formed which has the same chromatographic behavior as Hb A₆. Furthermore, nearly all of the radioactive label in this synthetic hemoglobin component was localized to the NH₂-terminus of the β chains.

In this report, we present a new chromatographic procedure for purifying the minor components of human hemolysate. This method allows positive identification of both phosphorylated and glycosylated minor hemoglobin components in normal and diabetic individuals, thus providing information on the biosynthesis of Hb A₆, and the mechanism for its elevation in patients with diabetes mellitus.

EXPERIMENTAL PROCEDURES

Chromatography on Bio-Rex 70—Six grams of human hemolysate was converted to the proper pH and ionic strength either by passage through a Sephadex G-25 coarse (Pharmacia, Inc.) column (5 x 40 cm) previously equilibrated with 0.05 M potassium phosphate buffer, pH 6.60, or by dialysis against this buffer overnight with one change. The hemolysate was then loaded onto a Bio-Rex 70 (Bio-Rad, Inc.) column (5 x 40 cm) equilibrated with at least one column
volume of 0.05 M potassium phosphate buffer, pH 6.0. The starting pH of this column varied between pH 6.58 and pH 6.82. The flow rate was always between 100 to 120 ml/h, being controlled by a Varian Peristaltic pump (LRK Produkter). Nine-milliliter fractions were collected. After the elution of the non-hemoglobin peak and hemoglobins A₂₅ and A₃₂₇, a linear gradient of 0.0 to 0.1 M NaCl in starting buffer (1000 ml total) was used to elute Hb A½. Then 0.1 M NaCl was continued until the Hb A₅ peak was eluted. At this point the column could be stripped of Hb A with 1 M NaCl. Hb A (nGd) was also eluted with Hb A. All chromatography was done in the cold room at 4°C. Fractions containing purified hemoglobin components were pooled and concentrated in an Amicon ultrafiltration apparatus with a PM-10 membrane prior to structural analysis.

For purposes of identifying the minor components, hemolysate was also purified according to the method of Schnek and Schroeder (10) as modified by Trivelli et al. (11).

The remaining experimental procedures used in this study are described in the adjacent miniprint. Figures included in the supplementary miniprint are identified by “S.”

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 A₁₂, Hb A₁₃, and Hb A₂₅, but also the separation of “Hb A” into two components which we have designated Hb A₁₁₅ and Hb A₁₂₅. 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 A₁₃ hemoglobins, which are retained somewhat by the column, can be separated from the non-hemoglobin protein peak as well as from each other. Hemoglobin A₁₃ is eluted as a well defined peak. Finally, hemoglobins A₁₃ and A₁₅ 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, from the major hemoglobin peak and from minor components Hbs A₁₀ and A₁₅, and the non-hemoglobin peak. Note that the separation of Hb A and A₁₅ 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 as a single peak, and may be eluted by their relative electrophoretic mobility. The peaks of Hbs A₁₀, A₁₅, and A₁₅ 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 A₁₅, is resolved into two components which we have designated Hb A₁₃ and Hb A₁₅. Pool 49–55 is clearly Hb A₁₅ and pool 65–85 is clearly Hb A₁₅.

Alkaline Denaturation Studies — Allen et al. (1) found that under their conditions Hb F co-chromatographed with A₁₅ and acetylated Hb F co-chromatographed with A₁₅. Since it was possible that either Hb A₁₃, A₁₅, or A₁₅ is or contains fetal hemoglobin, these components were subjected to alkaline denaturation according to the method of Huehns 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) resembled Hb A₁₅ in their rapid denaturation by alkali, in contrast to Hb F in cord hemolysate. These results indicate that both Hb A₁₁₅, A₁₃, A₁₅, nor A₁₅, contains a significant amount of fetal hemoglobin.

Identification of Modified Subunit — Analysis of hybrid hemoglobins formed from mixtures of the purified hemoglobin components with canine hemoglobin revealed that Hbs A₁₁₅, A₁₅, and A₁₅, all owe their low isoelectric points to modifications on the β chain only. These results are depicted in Table I.

Presence of Carbohydrate — Analysis of the minor hemoglobin components by the colorimetric assay of Flickiger and Winterhalter (9) is shown in Fig. 4A. All the minor components assayed showed absorbance spectra typical of the complex that 5-hydroxymethylfurfural forms with thiobarbituric acid, with an ε₅₄₅ at 443 nm. Our results for Hb A₁₅ were consistent with those reported by Flickiger and Winterhalter (9). The absorbance spectra obtained on Hbs A₁₁₅, A₁₅, and A₁₅ are quantitatively different from that for A₁₅. The amount of color formed with these hemoglobin components was only about 20% of that formed from Hb A₁₅. Likewise, fructose 6-P and fructose 1,6-diphosphate, formed considerably less color than did fructose (Fig. 4B). Thus, both Hb A₁₁₅, and Hb A₁₅ 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 A₁₁₅, A₁₅, and A₁₅ contain carbohydrate groups attached to hemoglobin by a ketomamine linkage like that of Hb A₁₅. Neither a simple glycosidic linkage nor an aldime 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 moiety in Hbs A₁₁₅, A₁₅, or A₁₅, or on 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 A₁₅ (4).

Phosphate Analysis — As shown in Table I, Hb A₁₅ contains 2 mol of phosphorus/mol of αβ dimer, while Hb A₁₅ contains 1 mol. In contrast, no phosphorus could be detected in Hbs A₁₁₅, A₁₃, and A₁₅. These results in conjunction with the colorimetric assays presented above provide strong evidence that Hb A₁₁₅ and A₁₃ contain sugar phosphates. Phosphorus analysis of the non-hemoglobin protein peak ruled out the possibility that Hbs A₁₅ and A₁₅ were contaminated by overlap with some phosphatase rich 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 A₁₅. In our new chromatographic system 5-[1³C]-glucose-6-P labeled hemoglobin co-chromatographed precisely with A₁₅ (Fig. 5A). In view of the other data including chain localization, phosphate analysis and the thiobarbituric acid color test, we tentatively conclude that Hb A₁₅ is glucose-6-P hemoglobin. The possibility that Hb A₁₅
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fig. 1. elution profile of normal adult hemolysate on bio-rex 70.

fraction number

contains other sugar phosphate derivatives of hemoglobin, such as fructose-6-p-hemoglobin as well, cannot be ruled out.

fraction number

conclusion that hba, is an adduct between hemoglobin and glucose-6-p. they obtained a crude mixture of non-hemoglobin protein and hbs a, and a, and a, 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 hba, and a, respectively. in our opinion, their first peak is probably non-hemoglobin protein, which has strong absorp-
Characterization of Glycosylated Minor Hemoglobin Components

Table I

| Hemoglobin component | Modified chain | Phosphate content (per \(\alpha\beta\) dimer) \(N = 3\) |
|----------------------|---------------|----------------------------------|
| Hb A\(_{im}\)        | \(\beta\)      | 2.06 ± 0.340                     |
| Hb A\(_{is}\)        | \(\beta\)      | 1.01 ± 0.163                     |
| Hb A\(_{ia}\)        | \(\beta\)      | 0                                |
| Hb A\(_{ic}\)        | \(\beta\)      | 0                                |
| Hb A\(_{ib}\)        | \(\beta\)      | 0                                |
| Glucose-6-P-Hb\(^a\) | \(\beta\)      | 0.98                             |

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

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\(_{ic}\). 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\(_{is}\) was elevated in diabetics. Table II shows the quantitation of minor components from six chromatographic runs. It is clear that the amount of Hb A\(_{is}\) and Hb A\(_{ic}\) (the sugar phosphate derivatives of native hemoglobin) is the same in normal and diabetic individuals. We suggest that the reported increase in Hb A\(_{ic}\) seen in diabetic hemolysate, may be due to an increase in levels of Hb A\(_{is}\), or possibly to an increase in the amount of "non-hemoglobin" protein. The amount of heme absorbance in the "non-hemoglobin" 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.

![Absorbance spectra of 5-hydroxymethylfurfural formed during the thiobarbituric acid assay (see "Experimental Procedures" for details). A, the following hemoglobin components were assayed: (a) Hb A\(_{im}\) (31 \(\mu\)M in heme), (b) Hb A\(_{is}\) (62 \(\mu\)M in heme), (c) Hb A\(_{ia}\) (62 \(\mu\)M in heme), (d) Hb A\(_{ic}\) (62 \(\mu\)M in heme), and (e) Hb A\(_{ib}\) (62 \(\mu\)M in heme). B, the following sugar standards were assayed: (a) fructose (3.1 \(\mu\)M), (b) fructose-6-P (62 \(\mu\)M), (c) fructose 1,6-diphosphate (62 \(\mu\)M), (d) glucose (33 \(\mu\)M).](http://www.jbc.org/)

| Hemoglobin component | Per cent of total hemolysate \(^a\) |
|----------------------|----------------------------------|
| Normal \(\pm\) S.D.    | Diabetic \(\pm\) S.D.             |
| A\(_{im}\)            | 0.19 ± 0.017                     | 0.29 ± 0.022                       |
| A\(_{is}\)            | 0.19 ± 0.040                     | 0.22 ± 0.040                       |
| A\(_{ia}\)            | 0.48 ± 0.155                     | 0.67 ± 0.261                       |
| A\(_{ic}\)            | 3.3 ± 0.361                      | 7.5 ± 2.18                         |
| A\(_{ib}\)            | 96 ± 0.577                       | 91 ± 2.65                          |

\(^a\) Although the percentages of minor components reported in this table are somewhat lower than those reported elsewhere (11, 18), the importance of this study is a comparison of yields with the same chromatographic technique. In fact, the results include two sets of normal-diabetic pairs which were run simultaneously under identical conditions of buffer and pH.

CONCLUSIONS

The normal levels of Hb A\(_{im}\) 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\(_{im}\). Although our studies have not ruled out the possibility that some Hb A\(_{ic}\) is derived from glucose-6-P Hb (Hb A\(_{im}\)), clearly the bulk of Hb A\(_{ic}\) 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\(_{im}\) 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-
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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 &mu Ci 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 &mu Ci of d-[14C]glucose) at 37°C for 6 days. After passage through Sephadex G-25 the incubation mixture was combined with 1 ml of hemolysate (5.4 mM in heme) and chromatographed on a Bio-Rex column (2.5 x 20 cm). The fraction size was 3.4 ml and the flow rate was 37 ml/h.

nism for the formation of the minor hemoglobin components studied here is that they are synthesized nonenzymatically by condensation between hemoglobin and intracellular sugars.

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Additional Refs. 1S-6S are found on p. 2332.
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Supplementary Material for
Glycosylated Minor Components of Human Adult Hemoglobin

Preparation of Hemoglobin

Blood from normal, non-diabetic subjects was collected into 3.8% NaCitrate (9:1, volume/volume) and collected into 3.8% NaCitrate (9:1, volume/volume) 

Preparation of Hemoglobin

Hemoglobin was purified by differential centrifugation and chromatography on DEAE-cellulose. The purified hemoglobin was further purified by gel filtration on a Sephadex G-100 column. The purified hemoglobin was then lyophilized and stored at -20°C.

Alkaline Denaturation

The purified hemoglobin was denatured by incubation at pH 12.5 for 10 minutes at 4°C and then rapidly cooled to 0°C.

Purification of Glycosylated Hemoglobin

The glycosylated minor components were purified by affinity chromatography on a DEAE-cellulose column. The purified glycosylated minor components were then analyzed by gel electrophoresis and identified by a specific antigen.

Hemoglobin Determination

The absolute content of the native purified hemoglobin component was determined after the protein had been purified using the method of Davis et al.

Supplement References

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Fig. 1. The curves of alkaline denaturation for the components of normal hemoglobin and for cell blood. The following solutions: 1M NaOH (a, b); 0.1 M NaOH (c, d), 0.01 M NaOH (e, f), and 0.001 M NaOH (g, h) were dialyzed in Amicon filters. The dialysis was performed at pH 12.5 in the presence of 0.1 M NaOH. The curves were then plotted as a function of the time of the reaction.

Fig. 2. Elution profile of hemoglobin from a Sephadex G-100 column. The elution was performed with buffer A, 0.1 M NaCl, 0.01 M NaOH, and 1 mM EDTA. The elution was monitored at 280 nm.

Fig. 3. Concentration of glucose-6-P and fructose-6-P in red cells of normal and diabetic subjects. The mean levels of glucose-6-P were 0.78 ± 0.24 nmol/g Hb in the normal (a) and 3.0 ± 0.5 nmol/g Hb in the diabetic (b) red cells. The mean levels of fructose-6-P were 0.68 ± 0.17 nmol/g Hb in the normal (c) and 2.0 ± 0.4 nmol/g Hb in the diabetic (d) red cells, respectively. The P value was determined to be 0.05 for the normal subjects and 0.01 for the diabetic subjects.
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