IN VITRO GLYCOSYLATION OF HEMOGLOBINS BY DIFFERENT SUGARS AND SUGAR PHOSPHATES

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1. Introduction

Hemoglobins A_{Ia+b} (HbA_{Ia+b}) and hemoglobin A_{Ic} (HbA_{Ic}) the so-called minor hemoglobins are regular constituents of human erythrocytes. HbA_{Ic} is formed postribosomally by attachment of glucose and mannose to the N-terminal valine of the β-chains of HbA via a Schiff's base linkage [1–5], while the structure of HbA_{Ia+b} has not yet been studied in comparable detail. The minor hemoglobins are elevated in diabetic patients [6–9], depending on the blood sugar level [9]. In vitro, glucose stimulates in a dose-dependent manner hemoglobin glycosylation in hemolysates from normal erythrocytes [5,9] and also in purified hemoglobin preparations [5,9]. In the present study we show that Hb glycosylation is not specific for glucose but occurs also with other sugars and sugar-phosphates. In every case incorporation took place mainly into the β-chains of the minor hemoglobins.

2. Materials and methods

D(+)-glucose, D(+)-galactose, D(+)-mannose, D(−)-fructose, D(−)-ribose was purchased from Merck, Darmstadt, FRG. Glucose-6-phosphate (G6P) and uridine-5′-diphospho-glucose (UDP-glucose) were from Boehringer, Mannheim, FRG. D-[U-^{14}C]-Glucose, D-[1-^{3}H]galactose, D-[1-^{3}H]mannose, D-[U-^{14}C]-fructose, D-[U-^{14}C]glucose-6-phosphate, UDP-D-[6-^{3}H]glucose came from The Radiochemical Centre Amersham, Braunschweig, FRG. Whatman CM 23 cellulose was bought from Vetter KG, Wiesloch, FRG. Other reagents were those described in [9].

2.1. *Incubation of hemolysates and HbA_{II} with unlabelled sugars*

Hemolysate or purified HbA_{II}, prepared as described [9] (corresponding to 1 mmol/liter hemoglobin) was dialyzed for 42 h at 37°C against 250-fold vol. developer no. 6 [7] containing 27.7 mmol/liter of the various sugars or sugar phosphates. After 30 min centrifugation at 40 000 × *g* at 4°C hemoglobin fractions were separated by chromatography on Bio-Rex® [7,9].

2.2. *Incorporation of sugars into hemoglobin chains*

Prior to use the hemolysate was dialyzed at 4°C with three changes in 24 h against 100-fold vol. glucose-free Earle's solution [10] fortified with solid $MgSO_4$ and $NaHCO_3$ to yield final concentrations of 1.2 and 40 mmol/liter, respectively. To 8 ml of hemolysate, placed in plastic tubes (2 × 10 cm) the labelled sugars were added to yield a final concentration of 22.2 mmol/liter and spec. radioact. 2.5 mCi/mmol. The pH was adjusted to 7.5 by gassing for 5 min with carbogen (95:5/O_2:CO_2) before the plastic tubes were stoppered and incubated for 2 h at 37°C in a shaking water bath. The mixture was then immediately dialyzed against 1000-fold vol. developer no. 6 at 4°C with four changes in 36 h, and centrifuged at 40 000 × *g* for 30 min at 4°C. Fractionation and quantification of the minor hemoglobins was carried out as described [9].

Separation of α- and β-chains of hemoglobin was performed by chromatography on CM 23 in 8 M urea according to [11,12]. The peak fractions were pooled and dialyzed at 4°C against 100-fold vol. 0.9% NaCl, pH 7.0, with four changes in 36 h prior to about 50-fold concentration by collodium bags SM

13 200 (Sartorius, Göttingen, FRG). Protein-bound radioactivity was determined by precipitation of 0.5 ml protein solution with 0.1 ml 3 mol/liter trichloroacetic acid (TCA) and washing the precipitates twice with 10% TCA. Radioactivity was determined in a Packard tri-carb liquid scintillation counter model 574 after dissolving the precipitates in 0.5 ml NCS® solubilizer. Protein was determined according to [13], hemoglobin concentration was determined from cyanmethemoglobin derivative A_{545} (Test-Kit, Boehringer, Mannheim, FRG).

3. Results

Incubation of hemolysates and of purified HbA_{II} with various sugars leads to marked changes of the elution pattern of hemoglobin fractions when compared with the sugar-free incubation (fig.1a–h). While

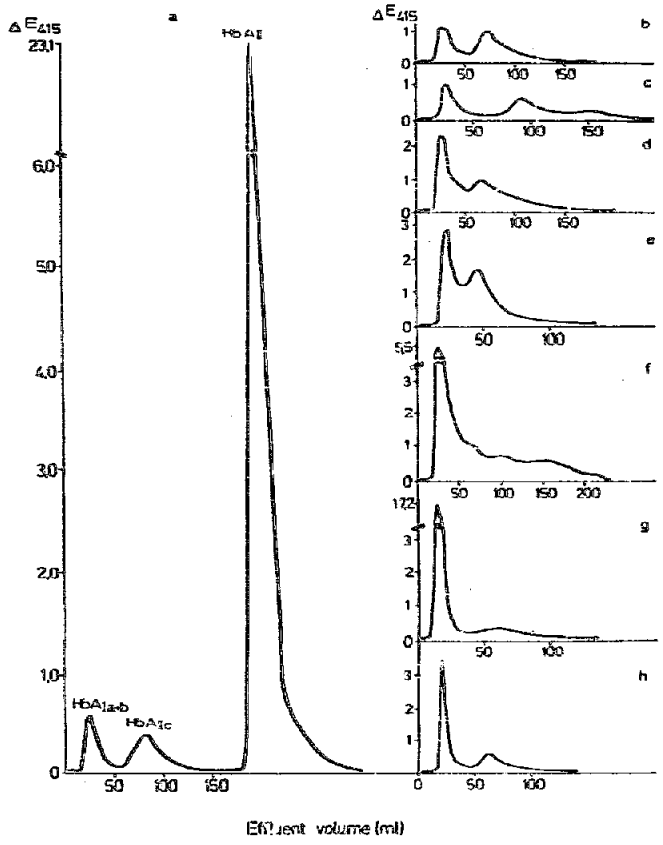

Fig.1. Formation of minor hemoglobins by incubation of hemolysates with various sugars. Hemolysates (corresponding to a hemoglobin concentration of 1 mmol/liter) were dialyzed for 42 h at 37°C against developer no. 6 in the absence (a) or presence of 27.7 mmol/liter glucose (b), fructose (c), mannose (d), galactose (e), ribose (f), glucose-6-phosphate (g), and uridine diphospho-glucose (h), respectively, prior to chromatography on Bio-Rex® (as described in section 2). The HbA_{II} fraction which is eluted after the minor hemoglobins by buffer change has been omitted in diagrams b–h of fig.1.

Table 1

Effect of sugars and sugar phosphates on formation of minor hemoglobins in lysed erythrocytes and purified HbA_{II}

Sugar	Hemolysate			Purified HbA_{II}		
	HbA_{Ia+b} (%)	HbA_{Ic} (%)	Total 'minor hemoglobins' (%)	HbA_{Ia+b} (%)	HbA_{Ic} (%)	Total 'minor hemoglobins' (%)
None	3.3	5.4	8.7	2.9	–	2.9
Glucose	6.5	11.3	17.8	7.3	10.7	18.0
Fructose	5.3	10.2	15.5	5.7	9.9	15.6
Mannose	–	–	34.6	–	–	44.7
Galactose	–	–	27.4	–	–	32.9
Ribose	–	–	58.2	–	–	69.1
Glucose-6-phosphate	37.7	7.4	45.1	39.8	–	39.8
Uridine diphosphate-glucose	9.4	7.4	16.8	7.1	2.0	9.1

Hemolysates and purified HbA_{II} (prepared as described in section 2), corresponding to a hemoglobin concentration of about 1 mmol/liter, were dialyzed for 42 h at 37°C against developer no. 6, containing no sugar or 27.7 mol/liter of the sugars indicated, and analyzed for minor hemoglobin content (see section 2)

in the case of glucose, fructose and UDP-glucose well separated peaks were obtained (fig.1b,c,h) this is not the case with mannose, galactose, ribose and glucose-6-phosphate (fig.1d–g). Data on minor hemoglobin formation from a variety of sugars are given in table 1. As may be seen marked changes were observed, the greatest effect being exerted by ribose, followed by mannose, galactose, glucose and fructose. They all caused an elevation of both HbA_{Ia+b} and HbA_{Ic} albeit in the presence of ribose there appeared a striking shift in favor of the HbA_{Ia+b} peak. Glucose-6-phosphate and UDP-glucose increased almost exclusively the HbA_{Ia+b} region.

Further experiments on the formation of minor hemoglobins from sugars other than glucose were carried out with labelled sugars in order to establish whether these were also covalently incorporated in the β-chain of hemoglobin. Chromatographic separation of the α- and β-chains yielded different protein and radioactivity distribution profiles for HbA_{Ia+b}, HbA_{Ic} and HbA_{II} as illustrated in fig.2.

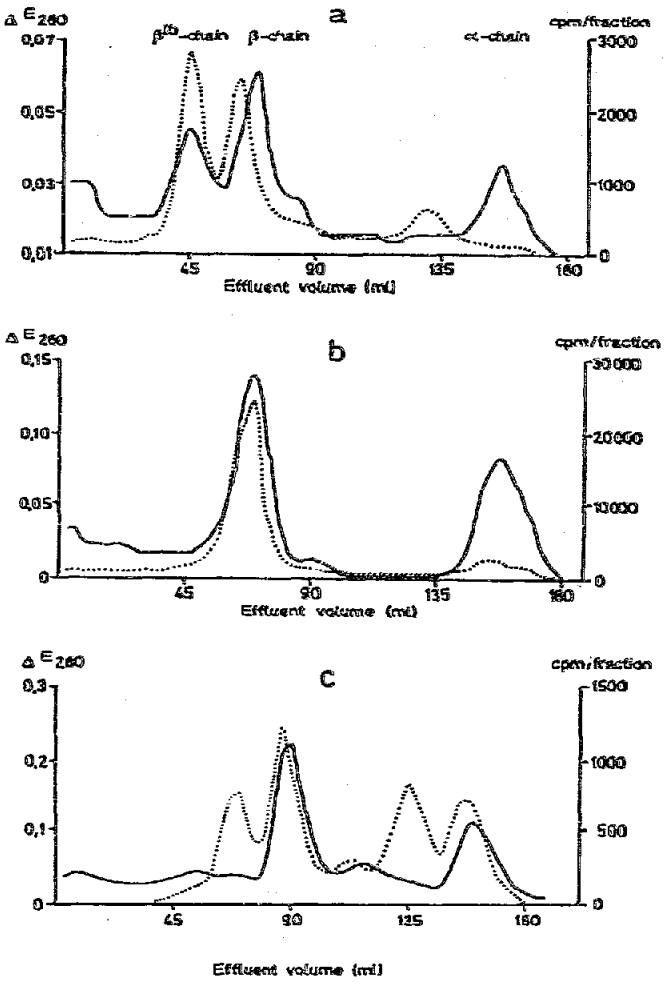

Fig.2. Separation of the α- and β-chains of radioactively-labelled HbA_{Ia+b} (a), HbA_{Ic} (b) and HbA_{II} (c). Hemolysates were incubated with labelled glucose (a), mannose (b), and fructose (c), respectively and treated as described in section 2 prior to chromatography on Bio-Rex 70®. The fractions of the HbA_{Ia+b}, HbA_{Ic} and HbA_{II} region were pooled, concentrated and 2.5, 6.5 and 8.5 mg protein, respectively, were applied on top of a 1 × 10 cm column of CM23 cellulose in 8 M urea. The α- and β-chains were eluted by a linear Na^+ ion-gradient (5 mmol Na_2HPO_4 – 40 mmol Na_2HPO_4) at room temperature in 3 ml fractions at a rate of 1 ml/min. In each fraction E_{280} nm (——) and radioactivity (· · · · ·) were determined.

Table 2
Incorporation of sugars in the α- and β-chains of hemoglobin fractions

	Mol sugar bound per mol α- or β-chain					
	HbA_{Ia+b}		HbA_{Ic}		HbA_{II}	
	β-chains ($\beta+\beta^{Ib}$)	α-chain	β-chain	α-chain	β-chain	α-chain
Glucose	0.1	0	0.06	< 0.005	0.005	0.004
Fructose	0.04	0	0.05	0.009	0.007	0.005
Mannose	0.5	0.27	1.1	0.11	0.11	0.11
Galactose	0.11	–	0.33	< 0.024	0.023	< 0.01
Glucose-6-phosphate	0.05	< 0.002	0.013	0.001	0	0
Uridine diphospho-glucose	0.02	< 0.003	0.016	0	0	0

In the case of HbA_{Ia+b} the chains emerged in three protein peaks, two of them at the position of the normal α- and β-chain, and a third peak called β^{Ib} [14] in front of the β-chain. Except with mannose (table 2) radioactivity was mainly associated with the β- and β^{Ib}-chains a small peak appearing in front of the α-chain (fig.2a).

Unlike HbA_{Ia+b}, HbA_{Ic} displayed only one β-chain besides the α-chain. Radioactivity was almost exclusively confined to the β-chain and only traces were found in the α-chain (fig.2b). The protein profile of the HbA_{II} subunits closely resembled that of HbA_{Ic}. The radioactivity pattern however, differed from the other hemoglobin fractions, as the α- and the β-chains contained the label in nearly the same amounts (fig.2c). Further, two additional radioactive peaks were observed in HbAII in front of the α- and the β-chain, respectively, the nature of which remains to be elucidated. This pattern was obtained with fructose, glucose, mannose and galactose but not with glucose-6-phosphate and UDP-glucose which were not incorporated at all into HbA_{II}.

From these studies there is little doubt that the formation of minor hemoglobins is not specific for glucose and can occur with a variety of other sugars. Quantitatively, sugar incorporation into the β-chains is highest in the case of mannose, followed by galactose, glucose, fructose and the sugar phosphates, as summarized in table 2.

4. Discussion

HbA_{Ia+b} and HbA_{Ic} are formed on incubation of hemolysates or purified HbA_{II} with glucose [9]. The present study shows that other sugars can also give rise to these minor hemoglobins, ribose, mannose, and galactose being even more effective than glucose. Incorporation of galactose and glucose into hemoglobins A and S was reported [15], however, neither the hemoglobin fractions nor the α- and β-chains were separated.

Our studies strongly support covalent incorporation of the sugars studied and furthermore demonstrate that, in the minor hemoglobins the linkage is specifically located on the β-chains. While these studies were in progress glucose was found to be incorporated into the β-chain of HbA_{Ic} [5]. The N-terminal valine residue was identified as the binding site.

In accordance with [16,17] glucose-6-phosphate appeared to be a much better substrate than glucose as far as the formation of HbA_{Ia+b} is concerned (table 1). However, after dialysis, TCA precipitation and urea treatment the β-chains contained little glucose-6-phosphate, even less than glucose (table 2). The high amount of protein-bound glucose-6-phosphate in HbA_{Ia+b} found [17] is probably due to the stabilisation of a Schiff's base linkage by reduction with $NaBH_4$ prior to the separation of the α- and β-chains.

Since we were more interested in the spontaneously-formed TCA-stable hemoglobin–sugar adducts we have omitted the $NaBH_4$ reduction step.

Also with respect to HbA_{Ic} formation it appears from table 2 that the uptake of glucose is considerably higher than that of glucose-6-phosphate. These data do not support the view that hemoglobin glycosylation occurs via the incorporation of glucose-6-phosphate and subsequent removal of the phosphate group rather than by direct uptake of free glucose [17]. Also the fact that the concentrations of 2,3-diphosphoglycerate and P_i exceed that of glucose-6-phosphate in the red cell by 2—3 orders of magnitude does not favour this concept, as these compounds compete with glucose-6-phosphate for a common binding site on the β-chain of hemoglobin [17,18]. That the free sugars rather than the sugar phosphates are involved in minor hemoglobin formation is also consistent with the finding [14] that the glucose-6-phosphate content of native HbA_{Ib} is unmeasurably low, and also is in agreement with the statement [19] that the red cell lacks glucose-6-phosphatase. Furthermore, it is compatible with the observation that the levels of HbA_{Ic} and of glucose-6-phosphate during the life span of the erythrocytes show no correlation. While HbA_{Ic} increases with the age of the red cell [20] the glucose-6-phosphate concentration is highest in the reticulocytes [21].

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