FURTHER IDENTIFICATION OF THE NATURE AND LINKAGE OF THE CARBOHYDRATE IN HEMOGLOBIN A_{1c}

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Summary: We have found that Hb A_{1c} contains neutral sugars which are only partially hydrolyzed from the N-termini of β chains. In both normal and diabetic Hb A_{1c}, 0.2-0.3 equivalents of hexose were released, composed primarily of glucose and mannose in a 3:1 ratio. When Hb A_{1c} was reduced with ^3H-Na BH_4 and then treated with periodic acid, most of the radioactivity was recovered as ^3H-formic acid with much lesser amounts of ^3H-formaldehyde. From these results, we propose that in the red cell, glucose binds to the α-amino position of hemoglobin β-chains (valine) in an aldimine (Schiff base) linkage. This aldimine can then partially rearrange in a reversible manner to form a ketoamine linkage which is stable to acid hydrolysis. This Amadori-type rearrangement accounts for the formation of mannose, the C-2 epimer of glucose, as well as the inability to demonstrate ^3H-Na BH_4 reduction at the C-1 position.

Over 90% of the soluble protein within adult human red cells is hemoglobin A ($\alpha_2\beta_2$). In addition, several minor hemoglobin components have been described, the most abundant of which is Hb A_{1c}, comprising 4-6% of the total hemoglobin (1). Holmquist and Schroeder (2) showed that Hb A_{1c} is structurally identical to Hb A except that an N-terminal amino group of a β chain is attached to a carbonyl group of an aldehyde or ketone by a Schiff base. Subsequently, Bookchin and Gallop (3) confirmed the Schiff base linkage to both β chains (1 mole/mole αβ dimer) and showed that ^3H-hexitol valine could be isolated from ^3H-$NaBH_4$ reduced Hb A_{1c} which indicated that the N-terminal

valine residues of both β chains were linked to a hexose. Their methods could not distinguish between various hexose isomers and their results did not rule out the presence of additional groups attached to the sugar by hydrolyzable linkages. Interest in Hb A_{1c} has been enhanced by the recent demonstration that this component is increased about two-fold in patients with diabetes mellitus (4-7). Additional assessment of possible pathophysiological and diagnostic implications of Hb A_{1c} requires further establishment of its structure in more chemical detail. Accordingly, we have employed a variety of techniques in order to identify the nature and mode of linkage of the carbohydrate moiety in Hb A_{1c} from normal and diabetic individuals. We have also attempted to understand the difficulties observed in efforts to quantitatively hydrolyze the linkage between the carbohydrate and the N-terminal valine residues.

MATERIALS AND METHODS

Blood was obtained from normal adult donors and from patients with juvenile-onset diabetes mellitus. Specimens were collected in heparin or EDTA. Hb A_{1c} was prepared from fresh hemolysate by the method of Schnek and Schroeder (8) as modified by Trivelli et al (6). Up to 5.5 g of hemoglobin was applied to large BioRex 70 columns (5.0 x 50 cm). In this chromatographic procedure, the minor components A_{1a}, A_{1b} and A_{1c} are eluted before the main hemoglobin A peak, designated herein as hemoglobin A_{II}.

Hydrolysis of Hemoglobins: Hemoglobin specimens were treated with 1-4 N CH_3COOH, HCOOH, HCl and H_2SO_4 at 100° C in sealed test tubes for various lengths of time. The following conditions provided the highest yield of free reducing sugar from Hb A_{1c}: Incubation of 30 mg/ml hemoglobin in 4N acetic acid at 100°C for 4 hours. Solutions were then freed of remaining protein by the addition of trichloroacetic acid to final concentration of 5%. Excess trichloroacetic acid was removed by 4 extractions with equal volumes of ethyl ether. These hydrolysates were then passed through a cation exchange resin (Dowex 50X2, 200-400 mesh H+ form) followed by an anion exchange resin (Dowex 1X8, 200-400 mesh, formate form) (9) and then evaporated to dryness and stored at 4°C.

Analysis of Monosaccharides: Free reducing sugars were measured colorimetrically by the ferricyanide test (9). Glucose, fructose, mannose and galactose were assayed enzymatically. An aliquot of neutralized test samples was added to a mixture containing 2 mM ATP, 2 mM Mg, 5 mM NADP in 0.1 M Tris-HCl buffer, pH 8.0, at room temperature. Optical density at 340 nm was followed on a Gilford recording spectrophotometer equipped with an automatic cuvette changer. The sequential addition of G6PD, hexokinase, phosphoglucose isomerase and phosphomannose isomerase (all 1 U/ml) provided direct assays for glucose, fructose and mannose respectively. In addition, glucose and galac-

tose were measured by glucose oxidase and galactose oxidase respectively (Sigma Technical Bulletin 510, 7-73).

Sugars in the hydrolysates were also analyzed by gas liquid chromatography as their trimethylsilyl-derivatives (10). After adding 100 nmoles of internal standard, α-methylmannopyranoside, the test samples and controls were dried by lyophilization. To this, 50 µl of N-trimethylsilylimidazole (Pierce Chemical Co.) was added. The samples were vigorously mixed, heated at 85°C for 20 minutes and vigorously mixed again. Then 1-10 µl was injected onto a 6 foot glass column packed with 3% SE-30 on Chromosorb W (HP) run at 175°C in a Hewlett-Packard 5830A gas chromatograph with a flame ionization detector.

Borohydride Reduction and Periodate Oxidation: 25 mCi (5.1 mg) of ^{3}H-$NaBH_4$ (New England Nuclear Co.) was mixed thoroughly with 75 mg of unlabeled carrier $NaBH_4$ with the use of an agate mortar and pestle. It was calibrated by reduction with 4-p-nitro-benzamidobutyraldehyde (11). The reagent was stored in a dessicater over Drierite at room temperature. Hemoglobins A_{1c} and A_{II} were treated with a 100-fold molar excess of ^{3}H-$NaBH_4$ in phosphate buffer, pH 7.5 for one hour at room temperature. The hemoglobin was then separated from excess $NaBH_4$ on a G-25 Sephadex column in 0.1M NaCl. Following the preparation of globin in acid acetone, the α and β chains of ^{3}H-$NaBH_4$ reduced-Hb A_{1c} were separated by chromatography on CM-Cellulose in 8M urea (12). The subunits were then desalted by chromatography on G-25 Sephadex and lyophilized. Test specimens were reacted with 0.4M periodic acid in H_2O, pH 0-1, for one hour at room temperature in the dark. Excess periodate was destroyed with 5-fold molar excess fucose or ethylene glycol.

Identification of Tritiated Formaldehyde and Formic Acid: "Dimedone" derivatives were prepared to isolate ^{3}H-formaldehyde (13). To each of the test samples, 1000-fold excess of cold formaldehyde was added as carrier. The dried recrystallized derivatives and the supernates were counted in a liquid scintillation counter. The latter was then distilled first at alkaline pH to trap formic acid as formate and then at acid pH to liberate the formic acid.

RESULTS

In specimens of normal adult blood, Hb A_{1c} comprised about 4-6% of the total hemoglobin content. At least twice this amount was obtained from several patients with diabetes mellitus. These results are in general agreement with the data of Trivelli et al (6).

Analysis of Monosaccharides: Following hydrolysis, 0.2-0.3 mole of reducing sugar was obtained per mole of αβ dimer. The analysis of this carbohydrate is shown in Table 1. In all of our experiments, Hb A_{II} and Hb A_{II} plus an equimolar[1] amount of added standard hexose were also carried through the hydrolysis procedure. Both enzymatic analysis and gas chromatography

[1] 1 mole hexose per mole αβ dimer.

TABLE 1

ANALYSIS OF THE CARBOHYDRATE RELEASED FROM HEMOGLOBIN A_{1c}

Sample	Total Reducing Sugar µmole/µmole αβ dimer	Enzymatic Assay µmole/µmole αβ dimer glucose	mannose	fructose	galactose	Gas Chromatography (% of total carbohydrate[1]) glucose	mannose
Hb A_{1c}-Normal[2]	0.18 ± .02	0.145 0.105	0.024 0.026	0	0	60.0 ± 2.8	24.5 ± 1.0
Hb A_{1c}-Diabetic	0.32 0.28	0.161 0.159	0.051 0.049	0 0	0	75.5 69.5	24.4 30.5
Hb A_{II}-Control	0.0	0.0	0.0	0	0	No significant carbohydrate peaks	
HB A_{II} +glu	0.75[3]	0.72[3]	0.0	0	0	97.6 79.1	2.4 3.2

1. The recovery of sugars by gas chromatography was 48 ± 12% of reducing sugar values.

2. Mean ± SEM of 5 determinations.

3. µmoles recovered/µmoles added.

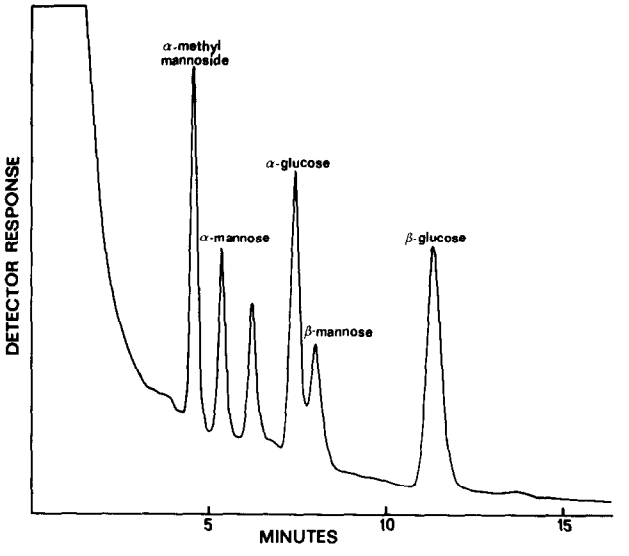


Fig. 1: Gas chromatography of carbohydrates isolated from diabetic Hb A_{1c}. α-methyl mannoside was used as an internal standard. Peak appearing between α-mannose and α-glucose is unidentified. See text for method.

(Figure 1) indicated that the reducing sugar in Hb A_{1c} of both normals and diabetics was about 70% glucose and 30% mannose. No fructose or galactose was detected by enzymatic assay. Gas chromatography revealed a small amount

of galactose in normal Hb A_{1c} and Hb A_{II}. In addition, a small unidentified peak between α-mannose and α-glucose was seen on some specimens.

Analysis by the resorcinol reaction (14) failed to detect ketoses. No amino sugars were found by the Elson-Morgan reaction (14) in the hydrolysate, prior to absorption on Dowex 50.

^{3}H-Sodium Borohydride Reduction and Periodate Oxidation: Following treatment with sodium ^{3}H-borohydride, 90% of the radioactivity of Hb A_{1c} was localized in the β chains. In a parallel incubation, only 5% as much radioactivity was detected in Hb A_{II}. The ^{3}H-$NaBH_4$ reduced β A_{1c} chain was then reacted with periodic acid. ^{3}H-$NaBH_4$ reduced mannose and fructose served as model compounds and were carried through the same procedure in parallel fashion. Excess borohydride was destroyed with 0.1 M HCl. The relative amounts of radioactivity recovered as ^{3}H-formaldehyde and ^{3}H-formic acid are shown in Table 2. As expected, the radioactivity recovered after periodic acid treatment of mannose was formaldehyde rather than formic acid since it is derived from the reduced C-1 carbon atom. In contrast, ^{3}H-βA_{1c} subunit released primarily ^{3}H-formic acid.

TABLE 2

IDENTIFICATION OF TRITIATED FORMALDEHYDE AND FORMIC ACID

Sample	Dimedone Derivative Total CPM	Dimedone Derivative CPM/mmole	Dimedone Supernate Total CPM	Distillation of Dimedone Supernate Total CPM Base	Distillation of Dimedone Supernate Total CPM Acid
^{14}C-HCHO	575,132	1,593,906	50,414	28,542	33,790
^{14}C-HCOOH	565	1,757	820,514	7,518	153,966
Mannose	13,870	35,373	21,060	4,640	8,140
Fructose	674	1,642	155,880	3,478	69,112
A_{1c}-Normal	1,195	2,395	665,800	34,661	296,993
A_{II}-Control	236	579	4,750	-	-

This experiment indicates that the borohydride reducible carbon (C=N or C=O) in Hb A_{1c} is not primarily at C-1, but at another position, probably C-2 as a result of an Amadori rearrangement (see Discussion).

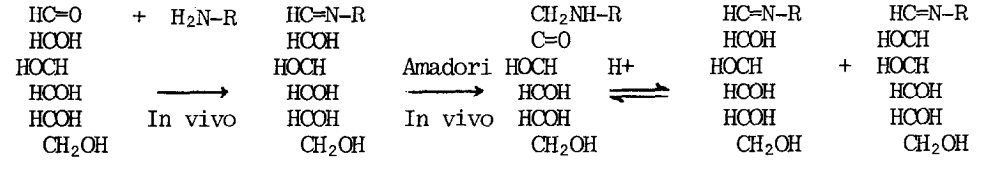
DISCUSSION

These results provide a direct demonstration of about 20-30% of the carbohydrate moiety in Hb A_{1c}. The detection of glucose and mannose is fully consistent with the proposal of a hexose attached to N-termini of β chains (3). Under the various conditions which were tested for hydrolysis, we could recover in the order of 0.25 moles of reducing sugar per mole of αβ dimer. It was likely that the bulk of the sugar was attached to globin by a linkage which is resistant to acid hydrolysis. Furthermore, the high yield of labeled formic acid following periodate oxidation of ^{3}H-$NaBH_4$ reduced Hb A_{1c} is not compatible with an aldose remaining attached to Hb A by a Schiff base at C-1.

These observations are consistent with the following scheme:

1. The N-termini of the β chains of Hb A_{II} combine with glucose to form a Schiff base (aldimine) attachment.

2. During the circulation of the human red cell, much of the aldimine undergoes an Amadori rearrangement (15) to form a more stable ketoamine.



(glucose) (aldimine) (ketoamine) (R=βA)

3. Any aldimine remaining or reformed at C-1 may be hydrolyzed during acid treatment. As a result of the rearrangement about the epimeric C-2 atom, a mixture of glucose and mannose is obtained by hydrolysis of the aldimine

The above scheme accounts for the low overall yield of neutral sugar on hydrolysis as a result of the stability of the ketoamine. It also accounts for the results obtained after periodate oxidation of reduced Hb A_{1c}.

The aldimine-ketoamine linkage of sugar to protein in Hb A_{1c} is unique among glycoproteins encountered to date. However, the likelihood of a post synthetic rearrangement and the low yield of sugar following hydrolysis complicate the analysis of this type of glycoprotein. There may be other examples which have, thus far, escaped detection.

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