Heterogeneity of the Haemoglobin-A_{1c}-band in Isoelectric Focussing

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Summary. A double HbA_{1c}-band was demonstrated in isoelectric focussing of blood from diabetics with a fasting blood glucose above 11 mmol/l. The same band-doubling was also demonstrated after incubation of erythrocytes in glucose/saline solutions. This finding may reflect the presence of the initial condensation product between haemoglobin and glucose, the Schiff-base intermediate.

Key words: Glycosylated haemoglobin, isoelectric focussing, rapid glycosylation, Schiff-base intermediate.

Rapid glycosylation of haemoglobin (Hb) has been demonstrated in vitro as well as in vivo by Svendsen et al. [1]. When haemolysates containing rapidly formed glycosylated haemoglobin were analysed by ion exchange chromatography, a change in elution volume for the HbA_{1c}-fraction towards earlier elution was noticed. To test whether this was due to co-chromatography of a compound with an isoelectric point close to that of HbA_{1c}, an optimised isoelectric focussing technique [2] was adapted for further study of rapid glycosylation of Hb.

Materials and Methods

1) Preparation of haemolysates and chromatographic determination of HbA_{1c} was performed as previously described by Svendsen et al. [3].

2) For isoelectric focussing the gels were made of 5% Cyanogum 41 (95% acrylamide, 5% bis-acrylamide), 2% Ampholine®, pH 7–9, 0.1% Ampholine®, pH 3.5–10, 2% β -alanine and 0.02% riboflavin, all w/v.

The 1 mm thick gels were photopolymerised for 1–2 h and prefocussed for 1 h at 500 V constant. On the surface of the gel

near the cathode 5, 10 or 15 μ l haemolysate (1 mmol Hb/l) was applied. The voltage was raised and maintained constant at 100 V to the end of the run which was indicated by clearly visible separation between HbA and HbA_{1c}. After focussing, the gel was fixed for 1 h in 12.5% (w/v) trichloroacetic acid (TCA) and stained overnight in a mixture of 1 part 1% (w/v) Coomassie Brilliant blue G-250 in water and 19 parts 12.5% TCA. Destaining was performed in 12.5% TCA and the gel was kept in 5% (v/v) glycerol in water.

The identity of the fractions was confirmed by electrofocussing HbA and HbA_{1c} purified by column chromatography [3].

To verify the presence of the double HbA_{1c}-band, visual inspection of the gels took place without knowledge of the origin of the sample.

3) For in vitro experiments 0.15 ml blood was mixed with 1.0 ml saline (0.154 mol/l) or saline containing 20, 40 or 80 mmol/l glucose and incubated at 37°C for 2 h. Thereafter haemolysates were prepared and analysed immediately.

Blood glucose concentration was determined by a modification of the Trinder glucose oxidase method [4].

4) Patient details. With informed consent studies were performed on:

A) Six outpatient diabetics having a fasting blood glucose value above 20 mmol/l (mean 24.2). They were all insulin-treated and received a mean dose of 0.64 IU/kg/day. The patients' ages ranged from 11–74 years (mean 49.7 years) with diabetes for 3–9 years (mean 5.3 years). Body weights were within 84–127% (mean 98.2%) of ideal [5, 6].

B) A group of 50 random diabetics from whom blood samples were taken at visits to the outpatient clinic of the Steno Memorial Hospital. Data for these patients are given in Table 1.

C) Non-diabetic subjects from the hospital staff.

Results

Figure 1 shows the isoelectric focussing of haemolysate from one of six diabetics having a fasting blood glucose value above 20 mmol/l (patient group A). Besides bands corresponding to HbA and HbA_{1c} an

Table 1. Clinical data for 50 random outpatient diabetics

Treatment	Number		Age, years		Diabetes duration, years		% ideal body weight		Insulin dose, IU/kg/day	
	M	F	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Diet alone	3	4	53.7	30–62	3.9	1–10	126.4	102–176		
or diet + sulphonylurea										
Insulin	25	18	33.8	8–71	13.7	1–41	100.4	83–126	0.64	0.22–1.06

Fig. 1. Isoelectric focussing of 5, 10 and 15 µl haemolysate from a diabetic with a blood glucose concentration of 22.4 mmol/l at time of sampling**Table 2.** Changes in percentage HbA_{1c} during a 2 h in vitro incubation at 37°C of red blood cells from a normal and a diabetic subject

Glucose concentration in saline mmol/l	% HbA _{1c} in normal haemolysate	% HbA _{1c} in diabetic haemolysate
0	4.29	8.85
20	4.83	9.10
40	5.20	9.57
80	5.85	10.18

additional, less intense, anodal band is noticed (unstable HbA_{1c}). This additional band was found in haemolysates from all six patients.

Thereafter 50 random samples from diabetics were analysed by isoelectric focussing. By comparing records of visible band-doubling in HbA_{1c} and blood glucose concentration it was found that 11.1 mmol/l glucose constituted a borderline value: 23 patients with a blood glucose concentration above 11 mmol/l all showed a double HbA_{1c}-band similar to the one

shown in fig. 1. In haemolysates from 25 diabetics with a blood glucose concentration below 11 mmol/l no double-band was seen and in the two remaining subjects having a glucose concentration of 11 mmol/l one showed a double HbA_{1c}-band, one a single band.

Finally, erythrocytes from diabetics and normal subjects were incubated for two hours at 37°C in saline (0.154 mol/l) and saline containing 20, 40 and 80 mmol glucose/l and the haemolysates were analysed by isofocussing and chromatography. In the non-diabetic as well as in the diabetic erythrocytes the additional HbA_{1c}-band was found in the 40 and 80 mmol/l glucose-incubated samples but could not be detected in 0 and 20 mmol glucose/l samples. Results from the chromatographically determined HbA_{1c} are given in Table 2. It is seen that the absolute amount of glycosylated haemoglobin formed during the incubation in glucose for 2 hours was similar in the normal and the diabetic erythrocytes. Clearly visible detection of the double HbA_{1c}-band was possible when rapidly formed HbA_{1c} was present in concentrations of 0.7% of the total Hb or higher,

but not if erythrocytes were incubated in saline (0.154 mol/l) at 37°C for 6 h before preparation of haemolysate.

Discussion

HbA_{1c} is considered to be a condensation product between haemoglobin and glucose, and the amount of HbA_{1c} in the blood is thought to be a measure of the mean blood glucose concentration over a period of time. The first step in the reaction leading to HbA_{1c} is the formation of a Schiff-base which undergoes an Amadori rearrangement to give a ketoamine compound [7, 8]. None of the rate constants involved are known, but the ketoamine is supposed to be stable.

The present study shows that if the concentration of glucose is raised, either due to diabetes or during incubation in vitro, an additional HbA_{1c}-band is seen after separation by isofocussing. Such a band-doubling has been described by Spicer et al. [9] after high-voltage zone sharpening in focussing experiments with haemolysate which had been incubated for 4 h at 30°C in 50 mmol/l glucose in a bicarbonate buffer. They suggested that the double band consisted of HbA with 1 and 2 glycosylated β -chains, respectively. This may not be the explanation for the intermediate HbA_{1c}-band described here. It would be expected that each glucose bound to the valine-NH₂-group would lower the pK-value equally. As HbA_{1c} is said to contain 2-2 glycosylated β -chains, a product with 1 glycosylated β -chain would have an isoelectric point between that of HbA and HbA_{1c} and would not be anodal to HbA_{1c}, as we have found.

Bunn et al. [7] examined the composition of chromatographic fractions from Bio-Rex-70 columns by the thio-barbituric acid test. They found ketoamine-linked carbohydrate in the HbA_{1c}-peak and also in front of the HbA-peak. They suggested that glycosylation might occur at other NH₂-groups than NH₂-valine in the β -chains, i.e. the terminal NH₂-groups in the α -chains and ϵ -NH₂-groups in lysine. The attachment of glucose to these amino-groups does not result in the same reduction in the pK-value of the amino-groups as when glucose is bound to β -valine-NH₂ in HbA_{1c}. Therefore HbA_{1c} is the only minor glycosylated HbA-derivative which can be detected by chromatography or electrophoresis.

The intermediate HbA_{1c} described here is hardly such a "HbA-derivative" since its ΔpI (relative to HbA) is greater than that for HbA_{1c}, and it is not found when the HbA-fraction from Bio-Rex-70-chromatography is analysed by isoelectric focussing.

The composition of the rapidly glycosylated fraction described is, at present, not known, but the fraction probably represents an intermediate compound placed somewhere in the reaction scheme leading from HbA + glucose to stable HbA_{1c}, perhaps one of the two possible Schiff-base compounds.

The observed borderline for recognition of doubled HbA_{1c}-band in diabetics (blood glucose concentration above 11 mmol/l) reflects the sensitivity of our isoelectric focussing procedure. Using a better focussing technique (a more narrow pH-gradient, more sensitive staining, etc.) the presence of a double HbA_{1c}-band would probably be observed in samples from subjects with a lower blood glucose concentration and perhaps even in normal subjects.

Since it was possible to separate the unstable form of the glycosylated haemoglobin from stable HbA_{1c} by isoelectric focussing this method seems superior to chromatographic determination. It may be valuable for clinical purposes and in studies of the rate constants involved in the glycosylation reaction.

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