Journal of Chromatography, 92 (1974) 391–399

CHROM. 7351

STUDIES ON THE HETEROGENEITY OF HEMOGLOBIN

XVI. SEPARATION OF VARIANTS WITH A GLU→LYS SUBSTITUTION BY CHROMATOGRAPHY ON CM-CELLULOSE

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(Received January 18th, 1974)

SUMMARY

Six β chain variants, two α chain variants, and one δ chain variant each with a substitution of a glutamyl residue for a lysyl residue in different positions, and Hb-C-Harlem, Hb-A_2, and Hb-Miyada have been analyzed by CM-cellulose chromatography. These variants can be divided into two groups as follows: Group I —C, C-Siriraj, A_2-Melbourne, Agenogi, O-Arab, O-Indonesia, and C-Harlem; Group II —Hb-E-Saskatoon, Hb-Chad, Hb-E, Hb-A_2, and Hb-Miyada. The variants of group I are eluted at an elution pH value which differs 0.3–0.43 pH units from that of Hb-A, whereas this difference for the variants of Group II is only 0.2 pH units. These observations have been discussed in the light of our present knowledge of the structure of the hemoglobin molecule. The difference between the elution pH values of many variants and that of Hb-A_2 makes it possible to use this technique for the quantitation of Hb-A_2 in red cell hemolysates containing hemoglobins which have electrophoretic mobilities similar to that of Hb-A_2.

INTRODUCTION

Repeatedly, it has been shown that two variants of human hemoglobin (Hb) in which a glutamyl (Glu) residue is replaced by a lysyl (Lys) residue in position 6 (Hb-C) or in position 26 (Hb-E) behave quite differently on columns of carboxymethylcellulose (CMC) or carboxymethyl Sephadex[1–4]. Hb-C separates completely from Hb-A, Hb-S, and Hb-A_2, because it is eluted last when a mixture of these hemoglobins is chromatographed; however, Hb-E cannot be separated from either Hb-S or Hb-A_2. In this paper, we report the results of similar chromatographic analyses but involving hemolysates with other related hemoglobin variants. Six of the eight glutamyl residues of the β chain, three of the four glutamyl residues of the α chain, and one of the seven glutamyl residues of the δ chain are known to be substituted by a lysyl residue in specific variants. All of these variants but one, Hb-O-Padua or $\alpha_2^{30\ Glu\rightarrow Lys}\beta_2$ (ref. 5), have been studied. The variants are listed in Table I; the

hemoglobins C-Harlem and Miyada are included since their electrophoretic mobility is comparable to that of the others.

MATERIALS AND METHODS

Blood samples were obtained from residents of the state of Georgia, or were mailed, airmail special delivery, from other countries to Augusta, Ga. Table I lists the sources of the different samples. Diagnosis was based on data from starch gel electrophoresis, clinical observations, family studies, and structural analyses. Hemolysates from washed red blood cells were prepared by mixing one volume of cells with one volume of distilled water and 0.5 volume of carbon tetrachloride. Stroma and other debris were removed by centrifugation. Hemolysates were stored at 4°.

TABLE I

GLOSSARY OF HEMOGLOBIN VARIANTS USED IN THIS STUDY

Name	*Abnormal chain*	*Position**	*Type of substitution*	*Reference*	*Supplier of material***
C	β	6-A3	Glu→Lys	6	Own source
C-Siriraj	β	7-A4	Glu→Lys	7	R. Q. Blackwell, Taiwan
E-Saskatoon	β	22-B4	Glu→Lys	8	H. Lehmann, Great Britain
E	β	26-B8	Glu→Lys	9	S. Pootrakul, Thailand
Agenogi	β	90-F6	Glu→Lys	10	I. Takeda, Japan
O-Arab	β	121-GH4	Glu→Lys	11	G. D. Efremov, Yugoslavia
Chad	α	23-B4	Glu→Lys	12	R. Q. Blackwell, Taiwan
O-Indonesia	α	121-GH4	Glu→Lys	11	G. Sansone, Italy
A_2-Melbourne	δ	43-CD2	Glu→Lys	13	R. S. Sharma, Australia
S	β	6-A3	Glu→Val	6	Own source
C-Harlem	β	6-A3 and 73-E17	Glu→Val Asp→Asn	14	M. Hubbard, Atlanta, Ga.
Miyada		Cross-over between Thr 12β and Ala 22δ		15	Y. Ohta, Japan

* In chain and in helix or intrahelical segment.

** As blood or hemolysate of a heterozygous carrier, except for Hb-E which was from subjects with Hb-E-β-thalassemia.

Chromatography on 1.8 × 35 cm columns of CMC (microgranular, preswollen CM-52; Whatman Biochemicals, Springfield Mill, Kent, Great Britain) was done as described before[3]. About 50 to 60 mg oxyhemoglobin, after overnight dialysis against 0.01 *M* sodium phosphate buffer*, pH 6.7, at 4°, were applied to the column, and the chromatogram developed at room temperature with a pH gradient obtained by mixing 0.01 *M* sodium phosphate buffers of selected pH values (7.4, 7.6, and 7.8) with the 0.01 *M* sodium phosphate buffer, pH 6.9, contained in a constant-volume (250 ml) mixing flask. The flow-rate was 16 ml/h and the effluent was collected in 4-ml fractions. Initially, pH 7.4 developer was placed in the supply bottle; this buffer was replaced by the pH 7.6 developer after 24 to 30 h when the total elution volume

* All developers contain 100 mg KCN/1000 ml.

was at least 400 ml. The elution of a few variants required the pH 7.8 buffer as third developer. The development of the pH gradient was assessed through measuring the pH of every tenth tube at room temperature using a Radiometer pH-4 meter (Radiometer, Copenhagen, Denmark). The pH of the tube containing the highest concentration of a specific hemoglobin was considered to be the elution pH value of this protein. Artificially prepared mixtures of hemoglobins, as well as hemolysates, have been analyzed. Fig. 1 presents, as examples, chromatograms of the hemoglobins of red cell hemolysates from a normal adult and from a Hb-Miyada heterozygote.

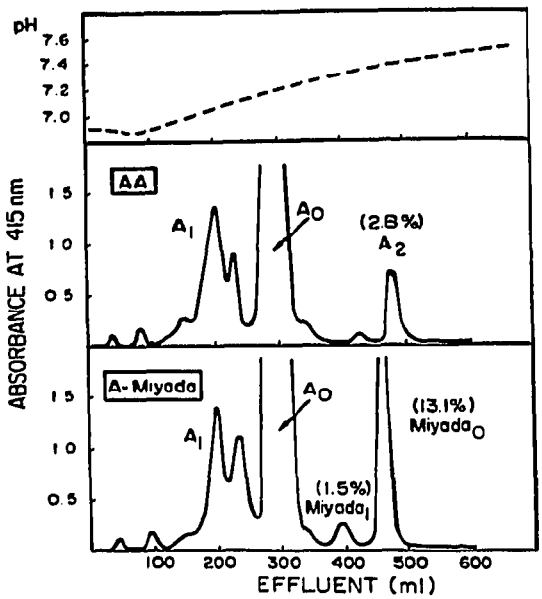

Fig. 1. CM-cellulose chromatograms of red cell hemolysates of a normal adult and of a subject with Hb-Miyada. Hemoglobins A_1 and $Miyada_1$ are normally occurring minor hemoglobins.

Horizontal starch gel electrophoresis in Tris–EDTA–boric acid buffer, pH 9.0, followed a previously described procedure[16]. Many hemolysates were also analyzed by DEAE-Sephadex chromatography; this procedure has been described before[17,18].

RESULTS

Electrophoretic studies

The electrophoretic mobilities of ten variants (eight with a Glu→Lys substitution, Hb-Miyada and the Hb-C-Harlem) have been compared (Fig. 2). The hemoglobins O-Arab, C-Harlem, E, Miyada, C, and E-Saskatoon had nearly identical mobilities. Hemoglobins O-Indonesia and C-Siriraj moved considerably faster towards the anode, whereas hemoglobins Chad and Agenogi had intermediate mobilities. Thus, electrophoresis will aid in the identification of some variants but not of all.

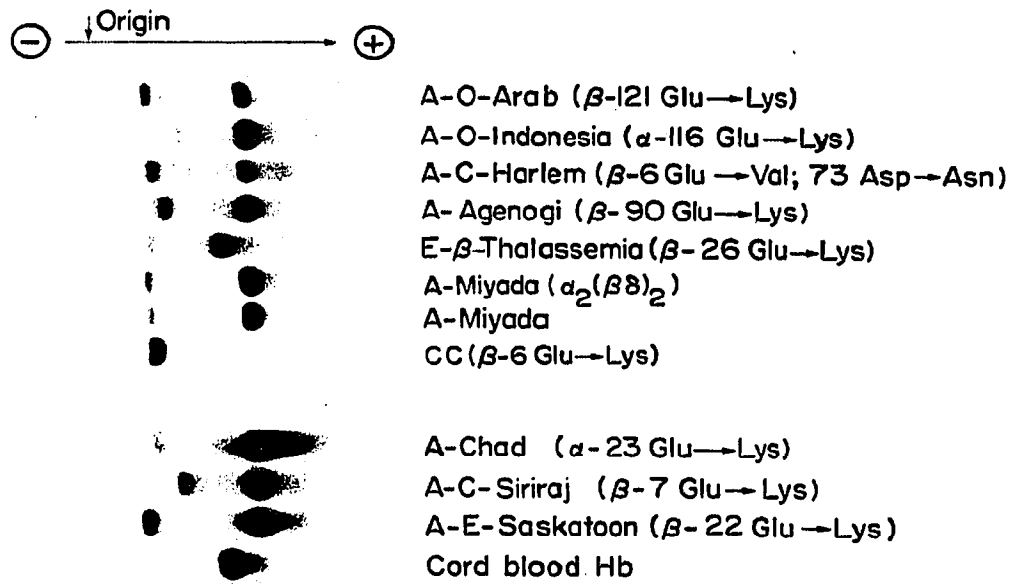

Fig. 2. Starch gel electrophoretic separation of various hemoglobin variants in Tris–EDTA–boric acid buffer at pH 9.0. O-Dianisidine stain. The quantities of some variants in hemolysates cannot be judged from this photograph.

Chromatographic studies

Fig. 3 presents CM-cellulose chromatograms of three artificial mixtures; the major hemoglobins present in these mixtures are indicated in the figure. The data show that despite nearly identical electrophoretic properties of the variants complete separation could be obtained between Hb-C and Hb-C Harlem, between Hb-C and Hb-E, and between Hb-C and Hb-O-Arab. The separation of Hb-O-Arab from Hb-O-Indonesia, and that of Hb-C from Hb-Agenogi are not surprising and were anticipated from the electrophoretic data.

Table II summarizes the elution pH values for all variants. The values are presented as the means of n numbers of analyses, as the ranges of values and as the differences between the elution values of the variants and that of Hb-A_0 (or Hb-A_2 when calculated for the Hb-A_2-Melbourne variant). Data by CM-cellulose chromatography place the eight Glu→Lys variants in two major groups; in Group I the ΔX-A (or A_2) varied between 0.29 and 0.43, and in Group II this value was 0.20. The variability in the ΔpH values of the group I variants was surprising, and allowed the separation of some of the variants from each other. The data obtained for the hemoglobins listed in groups III and IV are given for reason of comparison. The elution pH values of Hb-A_2 and Hb-Miyada were identical but unexpectedly low, whereas that of Hb-C-Harlem places this variant with its two substitutions in the first group together with Hb-Agenogi. It is noteworthy that this type of chromatography does not permit the separation of Hb-F from Hb-A and that of Hb-S from Hb-A_2.

Similar data by DEAE-Sephadex chromatography are also included in Table II. The differences between the elution pH values were relatively small and variants with (nearly) identical electrophoretic mobilities could not be separated from each other. However, the hemoglobins C, O-Arab, E-Saskatoon, E, A_2, Miyada, and C-

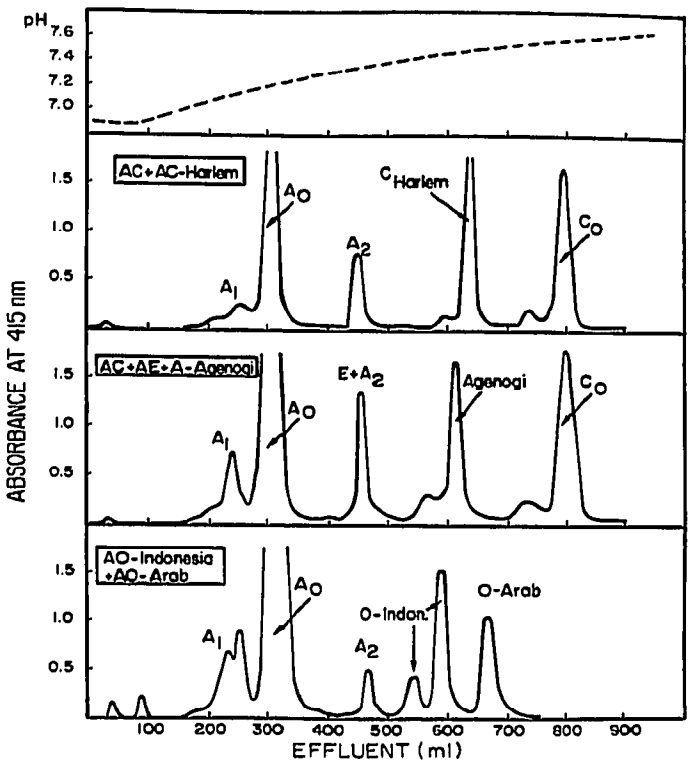

Fig. 3. CM-cellulose chromatograms of artificial mixtures of various hemoglobins. For further explanation see text.

Harlem were eluted from the column ahead of and (nearly) completely separated from the hemoglobins C-Siriraj, Agenogi, O-Indonesia, and Chad.

Quantitative data

Table III summarizes the quantitative data obtained by CM-cellulose and by DEAE-Sephadex chromatography. The percentages of the abnormal hemoglobin as established by these two procedures were comparable. The levels of Hb-A_2 were determined by CMC chromatography only; these data confirm and extend previously reported results[3].

DISCUSSION

CMC is a weak cation exchanger with a small ion capacity of about 1 mequiv./g dry weight and a total hemoglobin binding capacity of about 600 mg/g dry weight at pH 6.5 and in 0.01 *M* sodium phosphate. At this pH, the protein is attached to the functional groups of the CMC mainly through its α-NH_3^+- and ε-NH_3^+-groups. Slight change in pH towards the isoelectric point of the hemoglobin causes it to be desorbed, and indeed Hb-A and Hb-F are eluted from the cation exchanger at pH values between 7.15 and 7.20. The replacement of a glutamyl residue by a lysyl

TABLE II

CHROMATOGRAPHIC DATA ON TWO α-CHAIN, SIX β-CHAIN, AND ONE δ-CHAIN VARIANTS WITH A GLU→LYS SUBSTITUTION, AND ON SOME OTHER HEMOGLOBINS

Group	*Name of variant*	*Abnormal chain*	*Position and type of substitution*	*Elution pH values on CM-cellulose*				*Elution pH values on DEAE-Sephadex*			
				n	*Mean*	*Range*	*ΔX-$A_0(A_2)$*	*n*	*Mean*	*Range*	*ΔX-A_0*
I	C	β	6-A3	17	7.59	7.53–7.65	0.43	8	8.22	8.16–8.27	0.37
	C-Siriraj	β	7-A4	3	7.49	7.46–7.52	0.33	4	8.12	8.10–8.16	0.27
	A_2-Melbourne	δ	43-CD2	1	7.75	–	0.39	–	–	–	–
	Agenogi	β	90-F6	4	7.45	7.43–7.46	0.29	1	8.12	–	0.27
	O-Arab	β	121-GH4	2	7.52	7.50–7.54	0.36	2	8.18	8.18–8.18	0.33
	O-Indonesia	α	116-GH4	2	7.49	7.48–7.50	0.33	2	8.11	8.06–8.16	0.26
II	E-Saskatoon	β	22-B4	3	7.36	7.33–7.38	0.20	1	8.24	–	0.39
	Chad	α	23-B4	2	7.37	7.36–7.38	0.21	3	8.08	8.06–8.12	0.23
	E	β	26-B8	4	7.35	7.32–7.37	0.19	5	8.17	8.14–8.20	0.32
III	A	$\alpha_2\beta_2$		23	7.16	7.12–7.25	0	10	7.85	7.82–7.88	0
	F	$\alpha_2\gamma_2$		25	7.20	7.16–7.24	0.04	12	7.65	7.60–7.70	−0.20
	S	$\alpha_2\beta_2$	6Glu→Val	20	7.33	7.30–7.37	0.18	8	8.04	8.01–8.08	0.19
	A_2	$\alpha_2\delta_2$		26	7.36	7.29–7.42	0.20	11	8.16	8.12–8.18	0.31
	Miyada	$\alpha_2(\beta\delta)_2$		2	7.33	7.32–7.34	0.18	2	8.20	8.18–8.22	0.35
IV	C-Harlem	$\alpha_2\beta_2$	6Glu→Val; 73Asp→Asn	1	7.45	–	0.29	1	8.22	–	0.37

TABLE III

QUANTITATIVE DATA ON RED CELL HEMOLYSATES

Condition	*Hb-A_2 (CMC)*			*Hb-X* (CMC)*			*Hb-X* (DEAE)*		
	n	*%*	*Range*	*n*	*%*	*Range*	*n*	*%*	*Range*
AA	3	2.4	1.9–3.0	—	—	—	—	—	—
AC	4	3.3	2.3–4.2	4	33.8	31.4–35.1	4	36.9§§	32.9–40.9
CC**	9	3.6	2.8–4.8	9	95.7	93.4–97.6	7	99.0§§	98.1–99.6
C-β^+-Thal.	6	5.2	4.2–6.0	6	66.5	56.6–75.9	5	70.0§§	57.7–78.8
C-β^0-Thal.	6	5.7	4.4–7.5	6	89.6	84.3–93.7	2	92.8§§	88.5–97.0
AC-Siriraj	1	3.7		1	22.3		1	31.4§§	
A-Agenogi	2	4.5	3.0–5.9	2	39.2	39.0–39.4	1	45.5§§	
A-O-Arab	2	3.6	3.6–3.6	2	37.8	36.9–38.7	1	43.9§§	
A-O-Indonesia	1	2.0		1	11.6§			n.d.	
AE	1	—		1	36.2§§		1	40.8§§	
A-E-Saskatoon	1	—		1	45.9§§		1	45.7§§	
A-Chad	1	—		1	18.8§§		1	16.1§§	
A-Miyada	2	—		2	15.2§§	14.6–15.8	2	16.1§§	15.9–16.2
A-C-Harlem	1	5.7***		1	23.7			n.d.	

* X denotes the variant present in the particular sample. The amount of X was determined by CM-cellulose chromatography (CMC) or by DEAE-Sephadex chromatography (DEAE).

** One sample with a high level of Hb-F (18%) and a relatively low level of Hb-A_2 (2.2%) has not been included. The possibility that this subject has the Hb-C-HPFH condition[19] instead of a homozygosity for Hb-C could not be excluded.

*** Contains a considerable quantity of Hb-C_1-Harlem.

§ Contains the Hb-A_2 variant O_2 ($\alpha_2{}^0\delta_2$).

§§ Includes Hb-A_2.

residue in the hemoglobin molecule results in an increase in the positive net charge at pH 6.5 which causes this variant to be more strongly adsorbed by the cation exchanger requiring a higher pH for its desorption.

Theoretically, the replacement of a glutamyl residue by a lysyl residue in different positions of either the α or β (or δ) chains would influence the adsorption–desorption properties of the variants in an equal manner unless secondary changes alter the net charge of the protein and its binding to the ion exchanger. This binding is not only dependent on the amount of charge carried by the protein at a given pH and fixed ionic strength of the solvent, but also on the accessibility of the functional groups on the surface of the protein. Assuming that the hemoglobins are not subject to major configurational changes at pH values used in the chromatographic experiments (6.7 to 7.8) the difference in chromatographic affinity of variants with supposedly similar isoelectric points might indeed be caused by differences in accessibility of certain charged groups.

Fig. 4 summarizes the observation made on the eight α and β chain variants with a Glu→Lys substitution, Hb-C-Harlem, Hb-A_2, and Hb-Miyada by comparing elution pH values with relative electrophoretic mobilities. A direct relationship between these parameters is evident for many variants; however, hemoglobins E, E-Saskatoon, and Chad join Hb-A_2 and Hb-Miyada in having a much lower elution pH value than expected.

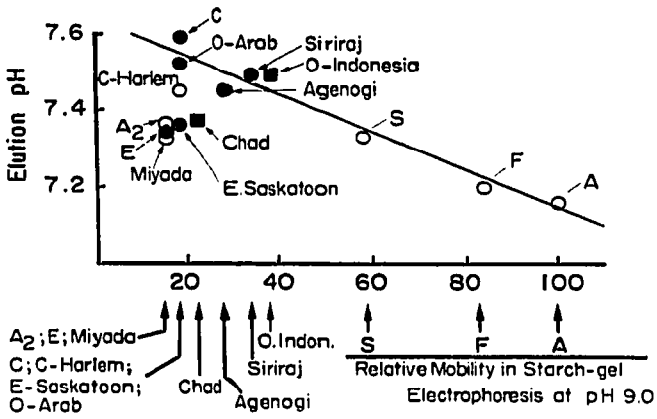

Fig. 4. Relationship between elution pH value and mobility in starch gel electrophoresis. Closed squares and closed circles refer to α and β chain variants with a Glu→Lys substitution, respectively.

Inspection of the model of the hemoglobin molecule, based on observations by Perutz *et al.*[20–25], shows that all glutamyl residues in both types of chain are on the surface of the molecule, and replacement by a lysyl residue would make the charged ε-NH_3^+- groups readily accessible to binding. In the β chain of Hb-A the glutamyl residue at position 26 (B8) forms a salt bridge to the arginyl residue at position 30 (B12). This neutralization is eliminated in hemoglobin E because of the replacement of glutamyl residue at position 26 by a lysyl residue. It is possible, however, that a salt bridge is formed from this lysyl residue to the glutamyl residue in position 22 thus neutralizing the charge on lysine. In Hb-E-Saskatoon (and also in the comparable α chain variant Hb-Chad) the normally occurring salt bridge from glutamyl residue in position 26 to arginyl residue in position 30 could perhaps be formed either between these residues or between glutamyl residue in position 26 and the lysyl residue found in position 22 in this variant. Thus, introduction of a lysyl residue in this part of the β (or α) chain in lieu of a glutamyl residue could lead to (partial) disruption of existing interchain interactions or to the formation of new contacts causing a change in the accessibility of certain charged groups during cation-exchange chromatography. Changes in interactions between residues due to Glu→Lys substitutions in other parts of the molecule are not apparent, and the hemoglobins C, C-Siriraj, O-Arab, O-Indonesia, Agenogi, and A_2-Melbourne should form one group with similar chromatographic properties. Why some of these variants, notably the hemoglobins C-Siriraj, Agenogi, and O-Indonesia, are eluted at lower elution pH values than the other variants is not clear; however, some interference of the Glu→Lys substitution with interchain interactions has to be assumed.

The practical advantage of the observed differences between these variants is obvious. Identification of certain variants is facilitated, and quantitation of Hb-A_2 in samples from subjects with certain variants, notably the hemoglobins C and C-Harlem, will aid in the differential diagnosis of homozygosity and double heterozygosity also involving β-thalassemia.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Drs. Blackwell, Efremov, Lehmann, Ohta, Pootrakul, Sansone, Sharma, Takeda and Miss M. Hubbard for supplying us with samples containing the hemoglobin variants. We thank Dr. Walter A. Schroeder for advice and interest. This work was aided in part by grant HL-05168 from the National Institute of Health, U.S. Public Health Service.

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