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Note

Isoelectric focusing, gel chromatography and electrophoresis of pyridoxalated and polymerized stroma-free haemoglobin

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New variants of oxygen transporting blood substitutes are based on irreversibly pyridoxalated and polymerized stroma-free human haemoglobin (SFH)[1–9]. Little is known about the heterogeneity of the above complex preparations. Electrophoresis on cellulose acetate strips makes it possible to separate the pyridoxalated and native haemoglobin into two fractions[3]. We have observed previously[9] one zone of "reversibly" pyridoxalated oxyhaemoglobin of p*I* 6.7 among about eight subfractions of native haemoglobin during flat bed electrofocusing in isoelectric focusing (IEF) agarose gels.

In the present model experiments we used isoelectric focusing together with gel filtration and gel electrophoresis to investigate the heterogeneity of protein molecules in SFH derivatives prepared by covalent binding of pyridoxal 5-phosphate to deoxyhaemoglobin in the presence of borohydride and by subsequent polymerization with glutaraldehyde[1–4,10].

MATERIALS AND METHODS

SFH (90 g/l of haemoglobin) was prepared from outdated human erythrocytes by a standard technique[11]. The reaction of deoxyhaemoglobin with pyridoxal 5-phosphate (Fluka, Buchs, Switzerland) in a molar ratio 1:4 was performed in the presence of potassium borohydride (Lachema, Brno, Czechoslovakia) in 0.1 *M* Tris–HCl buffer, pH 7.4, under nitrogen gas at 5–10°C as described by Sehgal *et al.*[3], with minor modifications. The intermediate product (SFH-P) was treated with 10–100 mg glutaraldehyde (E. Merck, Darmstadt, G.F.R.) per 1 g of haemoglobin for 3 h under the above conditions and the reaction was then stopped by the addition of 20–200 mg of lysine per 1 g of haemoglobin to form the final product (SFH-P-G)[4]. Comparative deoxyhaemoglobin samples were treated with pyridoxal 5-phosphate in the absence of borohydride (SFH···P)[6]. When necessary, the low-molecular-weight reagents were removed by gel chromatography on Sephadex G-25.

Analytical thin-layer isoelectric focusing was performed in the usual manner[8] on IEF agarose with Pharmalyte 3-10 (Pharmacia, Uppsala, Sweden) or with Am-

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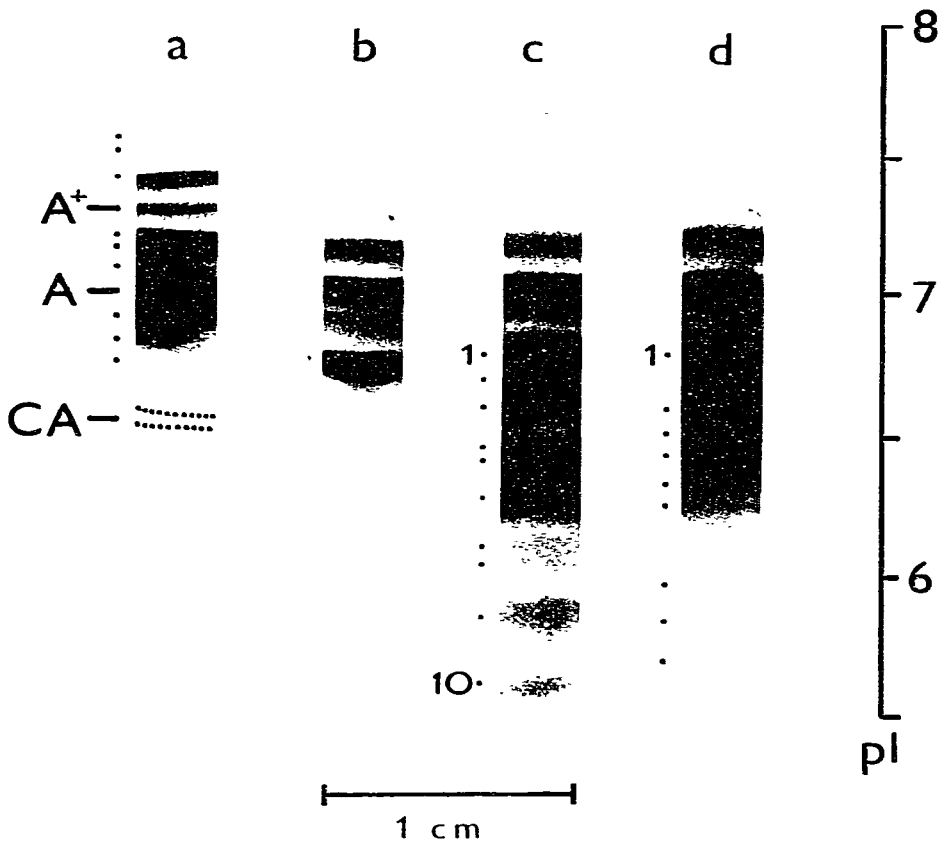

Fig. 1. Electrofocusing of stroma-free haemoglobin samples in IEF agarose gels. a, Stroma-free haemoglobin (SFH); b, SFH + pyridoxal 5-phosphate (SFH···P); c, SFH + pyridoxal 5-phosphate + borohydride (SFH-P); d, SFH-P + 10 mg glutaraldehyde per 1 g of haemoglobin (SFH-P-G/10). A = Haemoglobin A; A^+ = methaemoglobin A; CA = carbonanhydrase. The main pyridoxalated fractions in the patterns b, c and d are indicated by arabic numerals. Pharmalyte pH range 3–10. Staining with Coomassie blue.

pholine 3-10 (LKB, Bromma, Sweden). Both ampholytes led to similar results. Pharmacia as well as Serva kits of protein standards for electrofocusing were used together with previous data[9] to establish an approximate p*I* scale.

The relative molecular mass, M_r, distribution of proteins in SFH derivatives was estimated by gel chromatography on a calibrated Sepharose 6B column as before[9]. Lyophilization of SFH in the presence of 5% sucrose as lyoprotector was performed[12]. The formation of pentyl cross-links was detected by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS)[9]. Haemoglobin and methaemoglobin were estimated as their cyano derivatives[9].

The oxygen dissociation curves were estimated automatically by biotonometry[13,14] at 37°C, pH 7.40, in an isotonic phosphate buffer. The values of P_{50} and the Hill's coefficient of sigmoidity, n, were deduced from the oxygenation curves by means of simple graphical constructions[13,14].

RESULTS AND DISCUSSION

Fig. 1 shows the marked differences between native SFH, reversibly pyridoxalated SFH···P (where the binding is predominantly a Schiff-base like), irreversibly pyridoxalated SFH-P (where the Schiff base was reduced by borohydride to form stable imino bridges) and glutaraldehyde-treated SFH-P-G. Visual evaluation of the patterns before staining with Coomassie blue yielded three or four brown-red zones of various methaemoglobins with p*I* above 7.1 and three more red zones in SFH, four in SFH···P, eight in SFH-P and three in SFH-P-G. After staining, we usually found eleven zones in SFH, seven in SFH···P, fifteen in SFH-P and twelve in SFH-P-G (treated with 10 mg glutaraldehyde). Three to six faint non-haemoglobin zones were present, mostly between p*I* 4.5 and 5.5. About 60–70% of the total haemoglobin was modified during the above reactions. The unreacted haemoglobin subfractions remained at their original positions. The acid shift of the pyridoxalated fractions is evidently due to the induction of phosphate groups. Glutaraldehyde invoked a stepwise smearing of the patterns and disappearance of certain zones in the p*I* region below 6.7. However, these changes were less profound in comparison with the marked changes in the IEF pattern of native SFH after reaction with 10 mg glutaraldehyde alone[7,9]. Evidently, pyridoxalation of SFH has lowered the amount of determinant groups available for the reaction with glutaraldehyde[15] in our samples. The question whether the large number of zones in pyridoxalated SFH might be artefacts due to interaction of SFH-P with the components of the ampholyte remains open. Anyway, the patterns are reproducible and typical for the given SFH derivative.

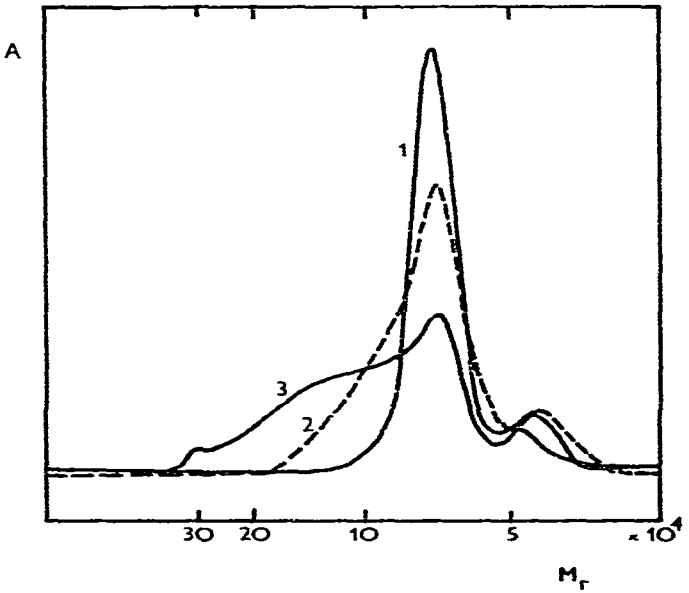

Fig. 2. Gel chromatography of pyridoxalated haemoglobin (SFH-P) treated with different amounts of glutaraldehyde. Sepharose 6B column (27 × 1.5 cm); isotonic phosphate buffer, pH 7.4, saturated with CO; absorbance measured at 254 nm; flow-rate 4 ml/cm² · h. The samples were treated with CO before chromatography. Curves: 1, SFH-P and SFH-P-G/10; 2, SFH-P-G/33; 3, SFH-P-G/66 (the index indicates mg of glutaraldehyde per 1 g of haemoglobin).

Fig. 2 is an example of the dependence of particle size distribution in SFH-P-G on the amount of glutaraldehyde used during polymerization. SFH-P and SFH-P-G samples treated with 10 mg glutaraldehyde yield a relatively uniform fraction having M_r = 64,000 (about 90%) and a smaller fraction corresponding to M_r = 44,000–50,000. At higher amounts of glutaraldehyde, *e.g.*, 30 mg and 60 mg, diverse populations of particles were found in a broad interval of M_r between about 40,000 and 330,000. The use of 100 mg glutaraldehyde caused gel formation.

Similar conclusions can be made from the results of polyacrylamide gel electrophoresis with sodium dodecyl sulphate, Fig. 3. In untreated SFH and SFH-P, haemoglobin is split into monomers and (SFH) into dimers. After treatment with glutaraldehyde, higher fragments corresponding to tri- and tetramers are also present.

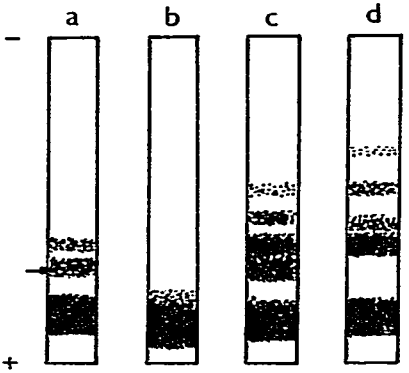

Fig. 3. Gel electrophoresis of fragments of modified SFH after splitting with sodium dodecyl sulphate. Polyacrylamide gel (50 g/l); phosphate buffer, pH 7.2, with SDS (1 g/l). Haemoglobin samples were split for 2 h at 37°C in SDS (20 g/l). Staining with Coomassie blue. a, SFH; b, SFH-P; c, SFH-P-G/10; d, SFH-P-G/50. The position of bromophenol blue is indicated by the arrow.

Table I summarizes the oxygen transporting and offloading ability of the given haemoglobin derivatives. Only SFH-P and to a lesser extent also SFH-P-G samples with covalently bound pyridoxal phosphate had a permanently lowered mean affinity to oxygen (as compared to unmodified SFH), even after removal of the unbound reagents by Sephadex G-25. Freshly prepared samples with methaemoglobin contents below 7% gave sigmoidal dissociation curves and the Hill's coefficients, n, indicated a fair cooperation between the subunits within the SFH-P and SFH-P-G tetramers. However, elevated concentrations of glutaraldehyde and of methaemoglobin resulted in lower P_{50} and n values.

The above parameters decreased slightly (by about 10%) in lyophilized samples (with 5% sucrose)[12] when stored dry at 20°C for 1 week and redissolved in distilled water.

Certain differences in the oxygen affinity and mean molecular mass of our SFH-P-G model samples as compared to those of Sehgal *et al.*[4], Rozenberg *et al.*[1] and Bogomolova *et al.*[2] seem to be due to the different protein and glutaraldehyde concentrations used.

The present results show that thin-layer isoelectric focusing in IEF agarose gels reveals several new details and yields substantially more information on the hetero-

TABLE I

COMPARISON OF OXYGENATION AND SUBUNIT COOPERATION CHARACTERISTICS OF DIFFERENT HAEMOGLOBIN SAMPLES

Standard deviation, S.D. ≤8%. SFH = Stroma-free haemoglobin; SFH···P = deoxyhaemoglobin (1 mole) + pyridoxal 5-phosphate (4 moles); SFH-P = as above, pyridoxal phosphate bound covalently in the presence of borohydride; SFH-P-G/*a* = the above sample treated with *a* mg of glutaraldehyde per 1 g of haemoglobin (see text) (*a* = 10, 20, 33 and 66).

Sample	P_{50} *(kPa)*	*Hill's coeff. n*
Fresh human blood	3.6	2.7
Banked blood after 24 days	2.3	2.6
SFH	1.8	2.6
SFH···P	4.0	2.5
SFH···P purified by Sephadex G-25	2.2	2.5
SFH-P purified by Sephadex G-25	2.9	2.4
SFH-P-G/10	2.8	2.2
SFH-P-G/20	2.7	2.0
SFH-P-G/33	1.9	1.7
SFH-P-G/66	1.4	1.5

geneity of pyridoxalated and polymerized haemoglobins than, *e.g.*, zone electrophoresis[3]. In combination with other techniques it seems to be most suitable for further investigation and routine quality tests of the above blood substitutes, especially of the pyridoxalated ones.

The complexity of the above samples has recently been confirmed by chromatofocusing which will be described elsewhere.

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