BINDING OF HAEMOGLOBIN TO SPECTRIN OF HUMAN ERYTHROCYTES

P. CHAIMANEE and Y. YUTHAVONG
Department of Biochemistry, Faculty of Science, Mahidol University, Rama VI Rd, Bangkok 4, Thailand

Received 10 February 1977
Revised version received 5 April 1977

1. Introduction

The possibility that haemoglobin is a component of the erythrocyte membrane has been raised by several authors [1–5]. It has been shown, for example, that human haemoglobin of various types can bind with haemoglobin-free membrane to various saturable levels under suitable condition [1]. Hanahan [2], however, has emphasized the difficulty in assessing the possible role of haemoglobin as a membrane component, due to complex changes which occurred with membrane preparations by various procedures. Regardless of definition of the 'true' erythrocyte membrane, the fact that reproducible amounts of haemoglobin are retained in membrane prepared by hypotonic lysis at acidic pH [5] indicates that, at least under these conditions, specific interaction exists between haemoglobin and other components in the preparation. This paper is concerned with identification of membrane components from hypotonic lysis which bind with haemoglobin. It is shown that spectrin is a component responsible for a portion of the binding.

2. Materials and methods

Chemicals for electrophoresis were obtained from Eastman. Trypsin (twice crystallized), and soybean trypsin inhibitor were purchased from Sigma.

Normal human blood was collected in citric acid–dextrose solution in the ratio 10:3 v/v. Erythrocytes were washed free of plasma and buffy layer by centrifugation at 2000 × *g* for 10 min at 4°C in 140 mM sodium phosphate buffer, pH 7.4. After 3 washes, the cells were lysed at 4°C in 9 mM phosphate buffer pH 7.4 [6]. The membranes were washed repeatedly in the cold hypotonic buffer until pale-cream in color, and were collected by centrifugation at 22 000 × *g* for 20 min 4°C. In each wash, care was taken to remove the pale-pink pellet underlying in packed membranes. In proteolytic digestion the membranes (1 mg protein/ml) were treated with 10 μg trypsin/ml in 2.5 mM phosphate buffer, pH 8.0, as in ref. [7]. The proteolysis was stopped by addition of excess soybean trypsin inhibitor. Sodium iodide extraction of membrane proteins was as described by Kahlenberg [8]. Spectrin was extracted [9] by incubation of ghosts for 60 min at 37°C in 4 vol. 0.1 M ethylenediamine tetraacetate (EDTA), pH 7.0. The mixture was then centrifuged at 250 000 × *g* for 60 min at 4°C. The supernatant was concentrated by ultrafiltration. Haemoglobin was obtained from haemolysate of membrane preparation. After centrifugation at 20 000 × *g* for 30 min at 4°C, the supernatant was concentrated by Aquacide, stored at −20°C and used without further purification. Suspensions containing known amounts of membrane protein were mixed with haemoglobin at different ratios in a total volume of 2 ml. The mixture was then centrifuged at 37 000 × *g* for 20 min at 4°C. The amount of membrane-bound haemoglobin was examined by the pyridine haemochromogen method [6].

Gel filtration of spectrin, or spectrin–haemoglobin mixture, was done at room temperature on a column (1.3 × 90 cm) of Bio-Gel P-300 (Bio Rad Laboratories) which has been previously equilibrated with the appropriate buffer. The void volume and total volume of the column were determined with Blue Dextran

Table 1
Combination of haemoglobin with modified erythrocyte membrane

Previous treatment		Maximum haemoglobin retention in membrane		Fraction of spectrin remaining
		mg/mg membrane protein after treatment	pg/ghost	
(a)	No addition	1.20	6.8	1
	Trypsin, 10 μg/ml	0.52	2.0	0.22
(b)	No addition	1.20	6.8	1
	EDTA, 0.1 mM	0.53	2.1	< 0.05
(c)	No addition	1.20		1
	NaI, 1.0 M	0.76		0.50

Erythrocyte membrane suspension (approx. 1 mg protein/ml) was treated with various reagents under the following conditions (a) 2.5 mM phosphate, pH 8.0, 22°C, 20 min; (b) pH 7.0, 37°C, 60 min; (c) 7.5 mM phosphate, pH 7.5, 0°C, 30 min. The membrane was washed twice with 10 mM phosphate buffer, pH 6.0, and mixed with different quantities of haemoglobin in 10 mM sodium phosphate buffer, pH 6.1, 25°C. Values for haemoglobin retention were obtained from the maxima of hyperbolic binding curves.

2000 and dinitrophenyl lysine, respectively. The samples (0.2 ml) were applied and eluted at a rate of 10 min/h. Fractions of 1.2 ml were collected and the protein content estimated by means of the absorbance at 280 nm and 418 nm. Sedimentation velocity experiments were carried out with a Beckman-Spinco ultracentrifuge, model E, at 56 000 rev/min 20°C.

3. Results and discussion

Haemoglobin binds with normal and modified erythrocyte membranes in a saturable manner; the value of free haemoglobin concentration at half-maximum binding is approximately 1.2 μM in all cases [10]. Table 1 shows that membrane treated with trypsin has only 43% of the maximum binding capacity of normal membrane when calculated per ghost. A number of proteins are degraded on treatment of erythrocyte membrane with trypsin [7,11]. Among these, spectrin is a major component susceptible to the proteolytic attack: in our experiment 78% of spectrin was degraded by the trypsin treatment as judged from the stain-density of dodecylsulphate–polyacrylamide gel. It is therefore possible that the degradation of spectrin on trypsin treatment is responsible for loss of at least a part of haemoglobin binding to the erythrocyte membrane. This possibility is further strengthened by the finding that membranes previously extracted with 0.1 μM EDTA or 1.0 M sodium iodide also lose substantial haemoglobin binding capacity, concomitant with the loss of spectrin (table 1).

In order to show more clearly that haemoglobin is bound to the erythrocyte membrane through its interaction with spectrin, the properties of isolated spectrin were studied with respect to this interaction. Spectrin was prepared by extraction of erythrocyte membrane with 0.1 mM EDTA and applied to a Bio-Gel P-300 column similar to the procedure of Clarke [12]. Figure 1A shows the elution profile for spectrin, which emerged in the void volume. When the solutions of spectrin and the haemolysate were mixed at pH 6.1, and the mixture was applied to the same column with the same buffer as eluent, the elution profile was as shown in fig.1 B. A fraction of haemoglobin was eluted along with spectrin, as witnessed from the absorbance at 420 nm as well as the increase in absorbance at 280 nm. The ratio of absorbance at 420 nm to 280 nm for purified haemoglobin was separately determined to be 2.98; from this value and from the values of $E^{1\%}_{280}$ of 21 for haemoglobin [13] and 8.8 for spectrin [14], the amount of haemoglobin attached to spectrin is calculated to be 1:13 w/w. Dodecylsulphate–acrylamide gel electrophoresis shows the presence of globin in the peak at void volume. From densitometric scans of Coomassie Blue-stained proteins, a ratio of haemoglobin to spectrin is found to be 1 : 7.5. Assuming

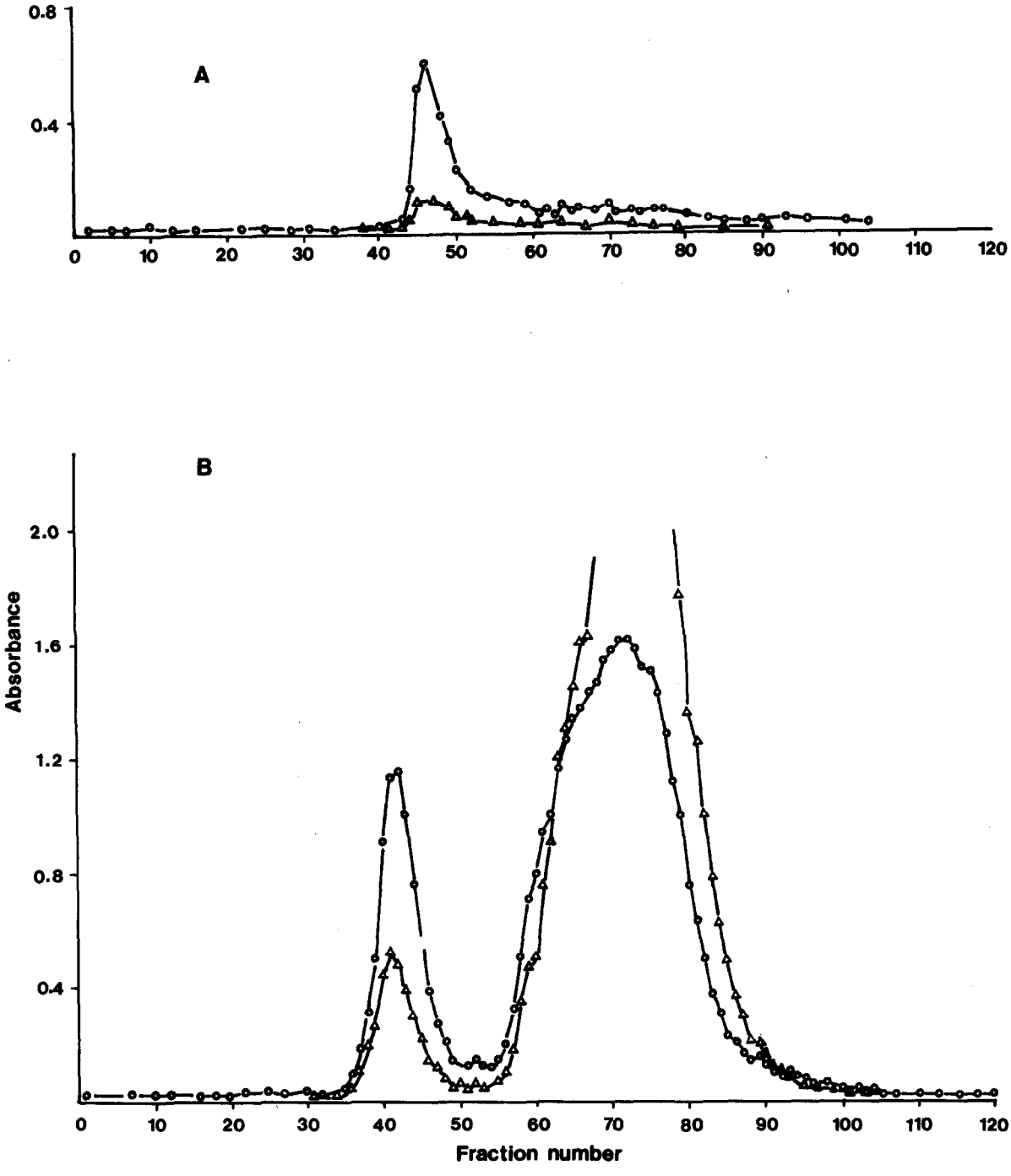

Fig.1. Elution profiles of spectrin and haemoglobin from a Bio-Gel P-300 column (1.3 × 90 cm). Sample solutions (0.2 ml) were applied and eluted with 9 mM phosphate buffer at 10 ml . h^{-1}. Fractions of 1.2 ml each were collected. (A) Spectrin, 5.3 mg, pH 6.1. (B) Spectrin, 7.0 mg and haemoglobin, 8.3 mg, pH 6.1. (○) Optical density at 280 nm. (△) Optical density at 420 nm.

that spectrin in solution is a mixture of monomeric and dimeric molecules [9,15] with an average molecular weight of between 220 000 and 440 000, the molar ratio for the association between spectrin and haemoglobin is calculated from the ratio of extinction coefficients to be between 1.9 and 3.8, and from densitometry to be between 1.1 and 2.2. Clarke [12] also observed a trace amount of haemoglobin in spectrin eluted from Bio-Gel P-300 column, at pH 7.3. Recently, when our work had been essentially completed, Kirkpatrick [16] also reported unpublished observation on co-precipitation of haemoglobin with spectrin and co-migration in the Bio-Gel column.

The sedimentation velocity pattern of freshly

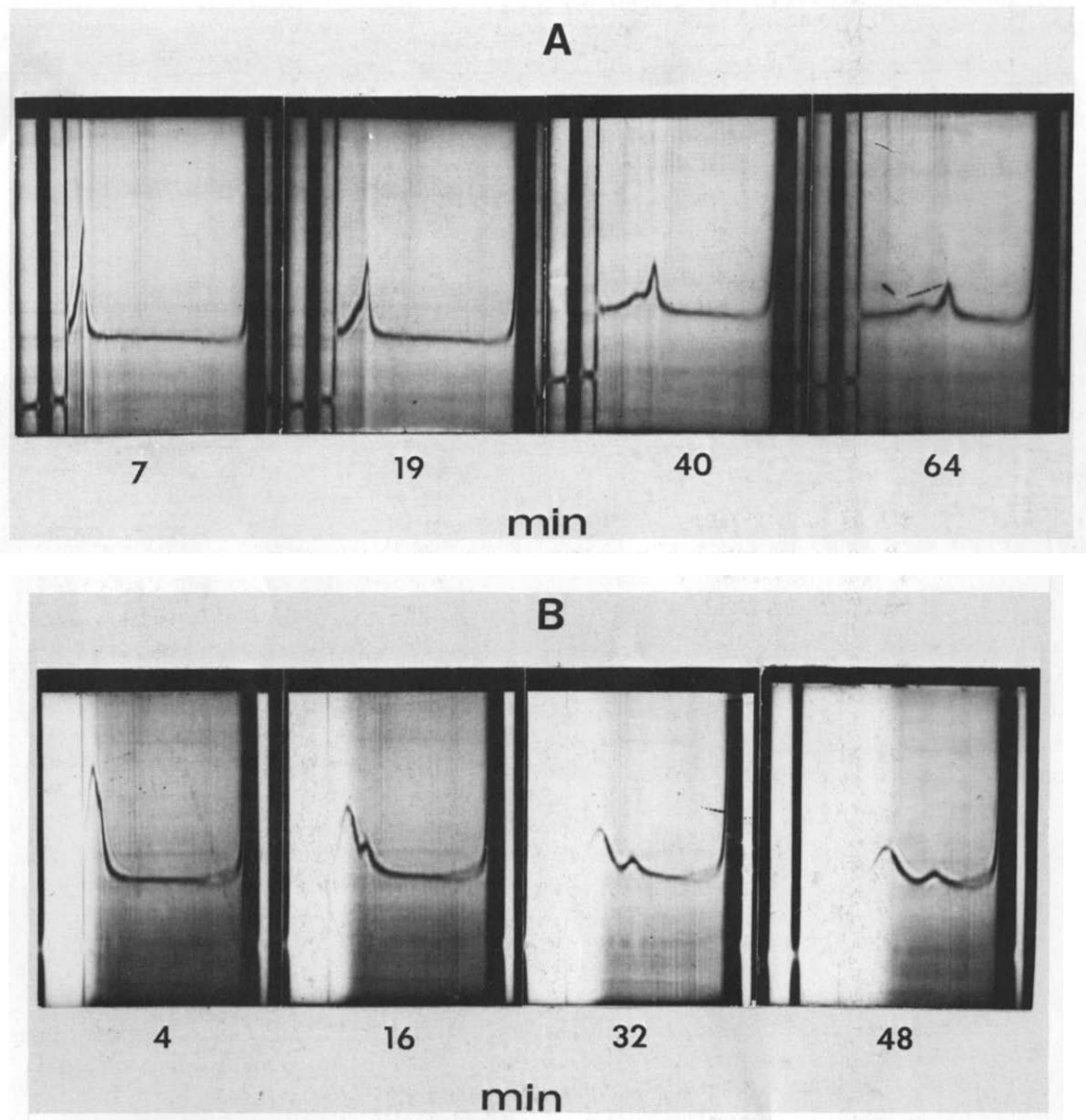

Fig.2. Sedimentation velocity profiles of (A) spectrin (2 mg/ml) in 9 mM phosphate buffer, pH 6.1, at 7 min, 19 min, 40 min and 64 min and (B) spectrin (2 mg/ml) and haemoglobin (1 mg/ml) in the same buffer at 4 min, 16 min, 32 min and 48 min respectively. Both runs were made with an An-D rotor at 56 000 rev/min, 20°C.

prepared spectrin at a concentration of 2 mg/ml shows two peaks corresponding to $s_{20,w}$ of 7.0 S and 4.5 S (fig.2A). These results are somewhat different from those of Gratzer and Beaven [9], Tanford et al. [15] and Clarke [12], the reason for the differences being presumably due to the sensitivity of the *s*-values to variation in preparation of the samples and conditions of the experiments. When haemoglobin is present at a concentration of 1 mg/ml in the spectrin solution (2 mg/ml), two peaks were observed with $s_{20,w}$-values of 9.3 S and 4.4 S (fig.2B). Haemoglobin alone under the same conditions has $s_{20,w}$ of 4.5 S. The shift in $s_{20,w}$-value of the fast-sedimenting component suggests that haemoglobin interacts with and modifies the sedimentation properties of spectrin.

The binding of haemoglobin with spectrin appears to have some specificity, since it was found (results not shown) that neither horse muscle myoglobin nor horse heart cytochrome *c* has any significant binding with spectrin.

The extent of binding of haemoglobin with spectrin as estimated from gel filtration is not at a high enough level to account for the total retention of haemoglobin in the erythrocyte membrane under similar conditions. Indeed, if it is considered that spectrin represents 30% of the total erythrocyte membrane protein by weight, as found from scanning of the staining density of the dodecylsulphate–polyacrylamide electrophoretic pattern, the amount of haemoglobin bound at pH 6.1 would be only 0.023–0.04 mg/mg total membrane protein, calculated from the extent of binding to soluble spectrin. This value is only 2–4% of that actually found. This discrepancy may be explained partly by assuming an increased capacity of membrane-bound spectrin to bind with haemoglobin, compared with soluble spectrin, and partly by the binding of other membrane proteins than spectrin. Wang and Richards [17] found from chemical crosslinking studies that in the intact erythrocytes haemoglobin is in the vicinity of spectrin and a few other membrane components. Our results show that for isolated membrane under suitable conditions, haemoglobin is not only within the vicinity of, but also interacts with, spectrin and other components.

Acknowledgements

We would like to thank the Wellcome Trust (Britain) for a research grant and the University Development Commission of Thailand for providing a studentship (to P.C.). We thank Professor J. A. Lucy, Dr Prapon Wilairat and Dr Bhinyo Panijpan for helpful discussions. The secretarial assistance of Ms Thitika Vadjarodaya and Ms Siriwan Bangthamai is gratefully acknowledged.

References

[1] Fischer, S., Nagel, R., Bookchin, R. M., Roth, E. F. and Tellez-Nagel, I. (1975) Biochim. Biophys. Acta 375, 422–433.
[2] Hanahan, D. J. (1973) Biochim. Biophys. Acta 300, 319–340.
[3] Juliano, R. L. (1973) Biochim. Biophys. Acta 300, 341–378.
[4] Weed, R. I., Reed, C. F. and Berg, G. (1963) J. Clin. Invest. 42, 581–588.
[5] Mitchell, C. D., Mitchell, W. B. and Hanahan, D. J. (1965) Biochim. Biophys. Acta 104, 348–358.
[6] Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130.
(1963) Arch. Biochem. Biophys. 100, 119–130.
[7] Triplett, R. B. and Carraway, K. L. (1972) Biochemistry 11, 2897–2903.
[8] Kahlenberg, A. (1976) J. Biol. Chem. 251, 1582–1590.
[9] Gratzer, W. B. and Beaven, G. H. (1975) Eur. J. Biochem. 58, 403–409.
[10] Chaimanee, P. (1976) M. Sc. Thesis, Mahidol University, Bangkok.
[11] Steck, T. L., Ramos, B. and Strapazon, E. (1976) Biochemistry 15, 1154–1161.
[12] Clarke, M. (1971) Biochem. Biophys. Res. Commun. 45, 1063–1070.
[13] Antonini, E. and Brunori, M. (1971) in: Hemoglobin and Myoglobin in their Reactions with Ligands, p. 18, North-Holland, Amsterdam.
[14] Marchesi, S. L., Steers, E., Marchesi, V. T. and Tillack, T. W. (1970) Biochemistry 9, 50–57.
[15] Schechter, N. M., Sharp, M., Reynolds, J. A. and Tanford, C. (1976) Biochemistry 15, 1897–1904.
[16] Kirkpatrick, F. H. (1976) Life Sci. 19, 1–18.
[17] Wang, K. and Richards, F. M. (1975) J. Biol. Chem. 250, 6622–6626.