*Biochimica et Biophysica Acta*, 1066 (1991) 1–5
© 1991 Elsevier Science Publishers B.V. 0005-2736/91/$03.50
*ADONIS* 000527369100252L

BBAMEM 75273



# The role of electrostatic forces in the interaction between the membrane and cytoskeleton of human erythrocytes

Edith Kahana [1,*], Sara Streichman [1] and Brian L. Silver [2]

[1] *Department of Hematology, Rambam Medical Center and Faculty of Medicine, Technion-Israel Institute of Technology, Haifa (Israel)* and [2] *Department of Chemistry, Technion-Israel Institute of Technology, Haifa (Israel)*

(Received 5 March 1991)

Key words: Erythrocyte; Cytoskeleton; Electrostatic force; Spectrin; (Human)

**Evidence is presented that electrostatic forces play a major role in the interaction between the cell membrane and cytoskeleton of human erythrocytes. Experiments were carried out on the effects of ionic strength variation, $Ca^{2+}$ and $Mg^{2+}$ ions, dimethonium ion and lipophilic ions on the release of spectrin from the erythrocyte ghost. In addition it was shown that the release of spectrin for fixed $Ca^{2+}$ or $Mg^{2+}$ concentration shows a maximum as a function of $Na^+$ concentration. All results are consistent with the existence of a repulsive electrostatic force between membrane and cytoskeleton.**

## Introduction

The human erythrocyte has a characteristic shape determined by the interaction between the cell membrane and a filamentous cytoskeleton composed mainly of the proteins spectrin, actin and protein 4.1 [1]. The structure and dynamic behavior of the cytoskeleton and the spectrin molecule in particular have been the subject of a great deal of research partly because the erythrocyte is a convenient model system for studying the physical and chemical interactions between the cell membrane and the cell contents. Several blood diseases are associated with erythrocyte deformation and defects either in spectrin or its interactions have been implicated [2]. From the theoretical point of view an understanding of the spectrin–membrane interaction provides a challenge for recently developed theories of electrostatic and electrodynamic forces [3].

The cytoskeleton can be almost entirely removed from erythrocyte ghosts by incubating the ghosts in a low ionic strength medium [4]. This long-known fact strongly suggests that electrostatic forces play a major role in holding the cytoskeleton to the cell membrane. Furthermore the disruptive effects of low ionic strength can be counteracted by the presence of small concentrations of $Ca^{2+}$ or $Mg^{2+}$ [5]. This could be due to the strong effect of divalent ions on Debye length and surface potential [6], but specific binding of cations by the membrane [7] cannot be ruled out a priori. The interpretation of the effects of divalent cations on membranes are thus complicated by the need to separate out what can be called the chemical effect of the cations, e.g. complexation, from the purely electrostatic effects of charge [8]. Recently McLaughlin et al. [9] have shown that the divalent organic cation dimethonium $[(CH_3)_3NCH_2CH_2N(CH_3)_3]^{2+}$ fails to bind to negatively charged phospholipid bilayers but effects the electrical double layer in quantitative agreement with the predictions of the Gouy-Chapman equation. In the present paper we describe some experiments on the interaction between the cytoskeleton and the membrane. The results give very strong support to the supposition that electrostatic forces are a major factor in the interaction, and in fact it seems impossible to explain the observations as a whole in any other way. Experiments are reported on the effect of various ions on spectrin release, including dimethonium, $Ca^{2+}$, $Mg^{2+}$, lipophilic anions and solutions containing varying proportions of mono- and divalent cations.

## Experimental

### *Materials*

All salts were analytical reagent grade. Dimethonium chloride was made by reacting tetramethyldiamine with methyl iodide and recrystallizing the

* Present address: MRC Muscle & Cell Motility Unit, King's College London, 26–29 Drury Lane, London, WC2B 5RL, U.K.

Correspondence: B.L. Silver, Department of Chemistry, Technion-Israel Institute of Technology, Haifa, Israel.

product several times from methanol/*t*-butanol solvent [9]. The resulting salt was passed through an ion-exchange column to exchange iodide with chloride. The product was shown to be at least 99% pure by $^1$H-NMR.

*Methods*

*Spectrin extraction.* White ghosts were prepared by hypotonic lysis in 5 mM sodium phosphate buffer pH 8.0, (5P8) including 0.12 mM phenylmethylsulfonyl fluoride (PMSF), according to the procedure of Dodge et al. [10]. Crude spectrin was extracted by incubation of the white ghosts for 30 minutes at 37 °C with an equal volume of 0.5 mM sodium phosphate (pH 8.0) containing 0.12 mM PMSF, following the procedure of Bennett and Branton [5] with some modifications. In those experiments in which $Ca^{2+}$, $Mg^{2+}$ or dimethonium were included, the washing solution contained 0.2–2 mM $Ca^{2+}$, $Mg^{2+}$ or dimethonium in addition to 0.3 mM sodium phosphate at pH 8 and 0.12 mM PMSF. Increasing amounts of NaCl were added to these solutions. It should be noted that the maximum yield of released spectrin in 0.5 mM phosphate (pH 8.0), was approx. 80%, compared to the common procedure of extraction with 0.1 mM phosphate and 0.12 mM PMSF.

*Protein estimation.* Protein estimation was carried out according to Lowry et al. [11].

*Gel electrophoresis.* Sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) was performed in slab gels according to the procedure of Laemmli [12]. Total acrylamide concentration was 7%.

*NMR spectra.* $^1$H-NMR spectra were taken on a Bruker NMR spectrometer operating at 400 MHz.

## Results

### *I. The effects of $Ca^{2+}$, $Mg^{2+}$ and $Na^+$*

Crude spectrin was extracted from ghosts as described in Methods. Inclusion of 2 mM $Ca^{2+}$ or $Mg^{2+}$ in the extraction medium was sufficient to prevent the separation of the cytoskeleton from the membrane, confirming others' data [5] (Table I). When the $Ca^{2+}$ concentration was reduced to 0.2 mM a partial inhibition of the spectrin extraction was observed. If sodium ions in the range 2 mM to 8 mM were added to the 0.2 mM $Ca^{2+}$, the yield of spectrin extraction went through a maximum with increasing $Na^+$ concentration (Fig. 1). To verify that the quantitated extracted protein from the ghosts at all points of Fig. 1 is qualitatively crude spectrin, a PAGE was performed. Similar results were obtained with 0.5 mM $Mg^{2+}$ (Fig. 2).

### *II. The effects of dimethonium*

Dimethonium, as the chloride, at a concentration of above 0.5 mM in 0.3 mM phosphate buffer (pH 8) reduces the yield of spectrin to zero (Table I).

TABLE I

*The effect of divalent cations on the yield of spectrin from human erythrocyte ghosts at low ionic strength*

$DMT^{2+}$, dimethonium ion.

| Solution | % yield [a] |
|---|---|
| 0.3 mM phosphate, pH 8 | |
| +0.3 mN NaCl | 98 |
| +9.75 NaCl | 87 |
| +0.3 mM NaCl | 36 |
| +0.2 mM $DMT^{2+}$ | 16 |
| +0.5 mM $DMT^{2+}$ | 0 |
| +2.0 mM $DMT^{2+}$ | 0 |
| +0.2 mM $Ca^{2+}$ | 22 |
| +2.0 mM $Ca^{2+}$ | 0 |
| +0.2 mM $Mg^{2+}$ | 0 |
| +2.0 mM $Mg^{2+}$ | 0 |

[a] Expressed as a percentage of the yield in 0.3 mM phosphate (pH 8).

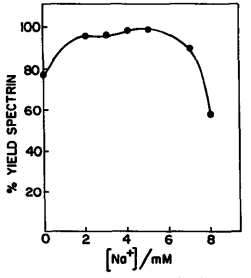


Fig. 1. The yield of spectrin as a function of $[Na^+]$ for fixed $[Ca^{2+}]$, 0.2 mM.

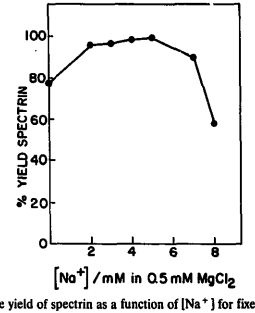


Fig. 2. The yield of spectrin as a function of $[Na^+]$ for fixed $[Mg^{2+}]$, 0.5 mM.

TABLE II

*The yield of spectrin as a percentage of that obtained in 2 mM NaCl*

| Solution | % yield |
|---|---|
| 2 mM NaCl | 100 |
| 2 mM NaI | 176 |
| 2 mM KSCN | 140 |
| 2 mM sodium acetylsalicylate | 170 |

### *III. The effects of lipophilic anions*

The presence of 2 mM $I^-$, $SCN^-$ or acetylsalicylate ion strongly increased the yield of spectrin compared to the corresponding solutions containing $Cl^-$ (Table II).

## Discussion

The simplest model capable of explaining the experimental results is one in which only electrostatic and dispersion forces are taken into account. At physiological pH values both the erythrocyte membrane [13] and the spectrin-actin complex are negatively charged [14]. The resultant repulsive force is modified by the presence of electrolytes in the cell fluids and opposed by attractive dispersion (Van der Waals') forces. The distance dependence of these two forces can result in a static equilibrium separation between the membrane and cytoskeleton in which the forces are balanced. The so-called hydration or hydrostructural force [15] is repulsive but only significant at distances of less than about 20 Å. Neither hydration nor Van der Waals are strongly dependent on the ionic composition of the aqueous medium [16]. Electrostatic forces are, however, extremely sensitive to ionic concentrations [3]. In general a reduction in ionic strength will increase the repulsive force between spectrin and the membrane. The physical basis for this effect is the decrease in the shielding of the surface charge. The standard method for separating spectrin from the erythrocyte membrane is almost certainly a reflection of the increase of electrostatic repulsion at low ionic strength. Variations in the ionic composition of the medium result in variations in the yield of spectrin and, in terms of the present model the yield should follow the same general behaviour as the electrostatic repulsion. In fact, the qualitative form of the graphs in Figs. 1 and 2 can be accounted for in terms of electrostatic forces, if it is assumed that the inner surface of the membrane and the cytoskeleton are both negatively charged.

For a pair of parallel negatively charged surfaces carrying homogeneously smeared negative charge and separated by an aqueous solution of $Z^{+1}/Z^{-1}$ salts the repulsive force between the surfaces is a monotonically decreasing function of salt concentration [17]. This is a reflection of the decrease in Debye length with increasing concentration of ions.

The cytoskeleton is primarily composed of spectrin and actin. The number of spectrin dimers per unit area of erythrocyte membrane has been estimated at 780 $\mu m^{-2}$. At physiological pH values there are about 200 negative charges as estimated from titration [20]. These values give $-0.025$ C $m^{-2}$ for the negative charge density on the cytoskeleton. By way of comparison, the estimated charge density on the inner and outer surfaces of thylakoid membranes is $-0.034$ and $-0.025$ C $m^{-2}$, respectively [21]. The inner surface of the erythrocyte membrane also appears to be negatively charged [13]. Thus, the membrane–cytoskeleton interaction is between two similarly charged surfaces. The occurrence of a maximum in the repulsive force between two such surfaces when the (small) divalent ion concentration is fixed and the monovalent ion concentration is increased has been demonstrated by the calculations of Duniec et al. [18] and Barber et al. [19]. Divalent ions have a comparatively strong effect on the Debye length but if only $Z^{+2}/Z^{-2}$ or $Z^{+1}/Z^{-1}$ salts are present the repulsion between two similarly charged surfaces is again a monotonically decreasing function of concentration. When both monovalent and divalent cations are present the repulsive force can exhibit a maximum when the concentration of monovalent cation is increased [18,19]. The same qualitative behaviour can be expected for a negative charged membrane adjacent to a similarly charged molecular network and indeed the yield of spectrin for fixed $[M^{2+}]$ and increasing $[Na^+]$ shows a maximum (Figs. 1 and 2) for both $Ca^{2+}$ and $Mg^{2+}$. Preliminary results indicate that a similar maximum is observed for the dimethonium ion. A simple physical interpretation is that $M^{2+}$ ions strongly shield the surface charge. Addition of $M^+$ ions progressively replace $M^{2+}$ from the surface thus *reducing* the shielding. When the $M^+$ concentration approaches higher values the $M^+$ ions *increase* the shielding in the manner predicted by Gouy-Chapman theory. The only previously reported experimental evidence for this maximum is for the case of the thylakoid membranes of chloroplasts [21], but there is no reason to suppose that the phenomenon is not general.

The experimental results and theoretical considerations very strongly implicate electrostatic forces in the binding of the cytoskeleton to the membrane and further support is provided by the effects of dimethonium, an ion which does not bind to negatively charged phospholipid bilayers. Using a variety of methods, McLaughlin et al. [9] have shown convincingly that the effects of the ion on surface potential are accounted for by the Gouy-Chapman equation. In the present study dimethonium was shown to qualitatively mimic the effect of $Ca^{2+}$ and $Mg^{2+}$ by counteracting the spectrin-releasing effect of low ionic strength. This

suggests that in the present context, $Ca^{2+}$ and $Mg^{2+}$ ions act predominantly through their electrostatic charges rather than by complexation of anionic groups on the membrane and/or the cytoskeleton. The suggestion is supported by the fact that the yield of spectrin in low ionic strength buffer is reduced to zero by concentrations of dimethonium of 0.5 mM or higher, simulating the effect of $Ca^{2+}$ or $Mg^{2+}$. It is not to be expected that the effects of $Mg^{2+}$, $Ca^{2+}$ and dimethonium will be quantitatively identical. The point is that the qualitative effect of all these ions, including one that does not bind to membranes, is consistent with the action of electrostatic forces.

Lipophilic ions are strongly adsorbed to phospholipid bilayers and modify the electrical properties of the bilayer, including the surface potential [22]. The addition of lipophilic anions to erythrocyte ghosts should thus result primarily in adsorption into the bilayer part of the membrane although there may be adsorption onto certain areas of membrane protein and the cytoskeleton. If electrostatic repulsion has a significant role, lipophilic anions should affect the ease of separation of membrane from cytoskeleton. This is what is in fact observed. It should be noted that the anions are effective at very low concentrations and that the increased yield of spectrin compared to solutions containing the same concentration of $Cl^-$ ions is not attributable to changes in ionic strength but can be rationalized in terms of the chaotropic series [22]. Lowering ionic strength or adding divalent cations are ways of increasing or decreasing the surface potential for fixed surfaces charge. Lipophilic ions change the surface potential by changing the charge close to the surface.

In summary there are now five independent lines of evidence relating to the effect of ions on the release of spectrin:

(a) The effect of lowering of ionic strength [4].

(b) The effect $Ca^{2+}$ and $Mg^{2+}$ [5].

(c) The effect of the dimethonium ion, present communication.

(d) The functional dependence on $[Na^+]$ for fixed $[Ca^{2+}]$ or $[Mg^{2+}]$, present communication.

(e) The effect of lipophilic anions, present communication.

The essential involvement of electrostatic interactions in coupling the skeletal network with the inner leaflet of the phospholipid bilayer was recently demonstrated: Maksymov et al. [23] have shown that spectrin dimers actually absorb model membranes provided they contain the negatively charged phosphatidylserine.

It should be stated that whilst the direct electrostatical repulsion described above is, undoubtedly, essential in the process of spectrin release, other salt-dependent events probably take place. Viscometric studies [24], as well as sedimentation measurements [25] have shown that the spectrin molecule undergoes conformational changes with salt concentration. These changes are associated with spectrin expansion in low ionic strength medium and thus alter locally the more specific interactions [26,27] between spectrin and the anchor proteins. Increased repulsion of the negatively charged phospholipid groups head as a result of lowering the ionic strength of the medium, cannot be excluded.

**Acknowledgements**

Some of the ideas contained in this paper have their roots in a conversation held between one of the authors (B.L.S.) and Professor J. Barber of the Imperial College of Science and Technology, London, U.K. Help with programming was provided by Jonathan Silver. This research was supported by the fund for the V.P.R. Fund/New York Metropolitan Research Fund and the Mitchell-Cope Foundation. This work is in partial fulfillment of the requirements for the D.Sc. degree of E. Kahana.

**References**

1 Palek, J. (1983) Semin. Hematol. 20, 139.
2 Palek, J. and Lux, S.E. (1983) Semin. Hematol. 20, 189.
3 Silver, B.L. (1986) The Physical Chemistry of Membranes, Chapter 7, George Allen & Unwin.
4 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 11, 220.
5 Bennett, V. and Branton, D. (1977) J. Biol. Chem. 252, 2753.
6 Ninham, B.W. and Parsegian, V.A. (1971) J. Theor. Biol. 31, 405.
7 McLaughlin, S.G.A., Szabo, G. and Eisenman, G. (1971) J. Gen. Physiol. 58, 667.
8 McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G. and McLaughlin, A. (1981) J. Gen. Physiol. 77, 445.
9 McLaughlin, A., Eng, W.-K., Vaio, G., Wilson, T. and McLaughlin, S. (1983) J. Membr. Biol. 76, 183.
10 Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119.
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 256.
12 Laemmli, U.K. (1970) Nature (Lond) 227, 680.
13 Lin, G.S.B., Macey, R.I. and Melhorn, R.J. (1983) Biochim. Biophys. Acta 732, 683.
14 Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) J. Cell. Biol. 63, 1018.
15 Ninham, B.W. (1980) J. Phys. Chem. 84, 1423.
16 Barnes, C. and Davies, B. (1975) J. Chem. Soc. Far. Trans II 71, 1667.
17 Verwey, E.J.W. and Overbeck, J.Th.G. (1948) Theory of the Stability of Lyophobic Colloids, Elsevier, Amsterdam.
18 Duniec, J.T., Sculley, M.J. and Thorne, S.W. (1979) J. Theor. Biol. 79, 473.
19 Rubin, B.T., Chow, W.S. and Barber, J. (1981) Biochim. Biophys. Acta 634, 174.
20 Stokke, B.T., Mikkelsen, A. and Elgsaeter, A. (1986) Biophys. J. 49, 319.
21 Barber, J. (1982) Annu. Rev. Plant Physiol. 33, 261.
22 McLaughlin, S. (1976) Curr. Topics, Membr. Transport 9, 71.

23 Maksymin, R., Sen-fang, S., Gaub, H. and Sackmann, E. (1987) Biochemistry 26, 2983.
24 Stokke, B.T. and Elgsaeter, A. (1981) Biochim. Biophys. Acta 640, 640.
25 Ralston, G.B. and Dunbar, J.C. (1979) Biochim. Biophys. Acta 579, 20.
26 Laermonth, R.P., Woodhouse, A.G. and Sawyer, W.H. (1989) Biochim. Biophys. Acta 987, 124.
27 Bennett, V. (1982) J. Cell. Biochem. 18, 49.