Biochimica et Biophysica Acta, 600 (1980) 140—149

BBA 78825

INTERACTION OF DIVALENT CATIONS WITH HUMAN RED CELL CYTOSKELETONS

G.H. BEAVEN and W.B. GRATZER

National Institute for Medical Research, The Ridgeway, London NW7, and Medical Research Council Cell Biophysics Unit, King's College, Drury Lane, London WC2 (U.K.)

(Received January 21st, 1980)

Key words: Ca^{2+} binding; Cytoskeleton; Erythrocyte; Membrane protein; Shape change; Spectrin

Summary

The binding of Ca^{2+} to spectrin from human erythrocytes was investigated by equilibrium dialysis, and the binding of Mn^{2+} by electron paramagnetic resonance. The results led to the conclusion that no binding sites of high affinity (greater than about 10^4 M^{-1}) are present. In the cytoskeletal protein complex isolated from erythrocytes, which (like crude spectrin) contains actin and some other proteins, a set of sites with an association constant of $4 \cdot 10^4$ M^{-1} for Mn^{2+} is observed. These may be divalent cation binding sites on the actin molecules. Weak interactions of Ca^{2+} and Mg^{2+} with spectrin are reflected by self-association of the spectrin heterodimers, which can be followed in the analytical ultracentrifuge and by light-scattering. This self-association is affected by the state of the protein thiol groups. Conditions in which self-association of spectrin occurs have been defined. No aggregation is observed at the Mg^{2+} activity thought to correspond to that in the cytoplasm.

Introduction

On considering the mechanism by which the cytoskeleton of the mammalian erythrocyte controls the shape and rheoviscous properties of the cell, the possibility must be entertained that it involves the binding of intracellular Ca^{2+} by the proteins. The control of shape and movement and of membrane properties by Ca^{2+} is a familiar phenomenon in a variety of systems, and in the erythrocyte a number of striking changes in shape and properties do indeed occur when Ca^{2+} is allowed to enter [1—5]. An effect of Ca^{2+} on the mechanical

Abbreviation: SDS, sodium dodecyl sulphate.

properties of ghosts at sub-micromolar levels has also been reported [6]. That the cytoskeleton is the site of such action has been suggested [6,7], and the binding of Ca^{2+} to specific sites on the major cytoskeletal protein, spectrin, has been claimed [8—10]. On the other hand, in a thorough study of the binding of Ca^{2+} to the human erythrocyte membrane, Cohen and Solomon [11] referred to unpublished data which indicated that there were unlikely to be any sites of high affinity on spectrin. That some interactions between spectrin and Ca^{2+} occur, however, cannot be contested, for at high concentrations of Ca^{2+} or Mg^{2+} aggregation sets in [12—14]. To rule out the presence of a small number of strong sites presents considerable difficulties. Thus, if there is the minimum structurally rational number of one strong site per spectrin heterodimer, maximum uptake at low Ca^{2+} activities would correspond to 1 mol of Ca^{2+} for 500 000 g of protein. For detection at this level, high protein concentrations, very low levels of endogenous Ca^{2+} and sensitive means of estimation are required. We describe here binding measurements by two methods, using purified spectrin. Binding has also been studied on cytoskeletal complexes, isolated from erythrocytes by extraction with non-ionic detergent [15]. Our conclusion is that there is in fact no strong binding site for divalent cations on spectrin. Weaker binding to the cytoskeletal network, however, does occur, and has been measured.

Materials and Methods

Spectrin

Spectrin was prepared as the heterodimer by brief extraction at low ionic strength at 37°C, as previously described [16]. In some instances, the protein was then concentrated and partially purified by precipitation with an equal volume of cold $(NH_4)_2SO_4$ [17], and immediately redissolved in buffer. For purification the spectrin, as extracted, was applied directly to a Sepharose 4B column (90 × 2.5 cm) and eluted with 0.1 M NaCl/0.05 M Tris, pH 7.6 [16]. The fractions containing the dimer (sedimentation coefficient 9.75 S [14]) were bulked and concentrated, either by precipitation with $(NH_4)_2SO_4$ or vacuum dialysis. All samples were screened for purity by gel electrophoresis in the presence of SDS [18]. Spectrin was phosphorylated, either in situ by incubation of the ghosts with ATP, prior to extraction [19], or after extraction with a crude preparation of endogenous cyclic AMP-independent kinase [20], as described elsewhere [21].

Cytoskeletal complexes

These were prepared by extraction of ghosts with solutions containing Triton X-100 by using the method of Yu et al. [15], modified in detail [21]. An attempt was made to reduce the proportion of lipid in this material by increasing the concentration of Triton X-100 used for extraction to 2.5%. Most experiments were performed on such preparations, which were similar to those resulting from extraction with 0.5% Triton X-100 in respect to protein composition, as judged by SDS gel electrophoresis [18]. The protein composition was analysed by microdensitometry of stained gels, using a Joyce-Loebl densitometer with a blue filter. Areas under peaks were determined by planimetry or

cutting out and weighing. The lipid concentration was determined by phosphorus analysis, following ashing [22], and the total concentration of lipid and protein by drying salt-free aliquots to constant weight. Comparisons of concentrations were also made by dissolving aliquots in 1% SDS solutions by heating at 100°C for 5 min and measuring the absorbance at 280 nm. An approximate protein concentration can then at once be obtained by applying a linear scattering correction and assuming the specific absorption of the protein to be that of its preponderant component, spectrin, viz. $E(1\%, 1\text{ cm}) = 10.8$ [23].

Ca^{2+} binding

To prepare spectrin solutions for binding experiments, endogenous divalent metal ions were removed by addition of 1 mM EDTA to the solution (in 0.1 M NaCl/0.05 M Tris, pH 7.6), and dialysing exhaustively against the same buffer, made up in glass-distilled water, checked with an ion-selective electrode (Radiometer, Copenhagen) for Ca^{2+} level. The dialysis tubing was also extensively washed with dilute EDTA, followed by large volumes of glass-distilled water. In some experiments, Dow Chelex chelating resin was added to the dialysate to ensure removal of contaminating divalent metals. Ca^{2+} levels in the dialysate were not higher than about 1 μM. The cytoskeleton preparations were freed of contaminating divalent metal ions by suspension in the buffer containing 1 mM EDTA and collection by centrifugation, as in the initial preparation. They were then several times suspended in a large excess of glass-distilled water and pelleted. In other experiments divalent metal ions were removed by addition of EDTA, followed by dialysis in the same way as for spectrin.

For equilibrium dialysis of spectrin, Perspex (Lucite) cells were used, with a capacity of 1 ml in either chamber. The cells were rotated at 4° for 48 h to reach equilibrium. Solutions were manipulated with glassware rinsed with EDTA and double glass-distilled water, and all apparatus was treated in the same way. Spectrin concentrations were 2—5 mg/ml. Because the endogenous Ca^{2+} levels in these experiments cannot be assumed to be zero, the problem of analysis of the equilibrium levels of Ca^{2+} in solution is not mitigated by the use of a radioactive Ca^{2+} isotope. A method of sufficient sensitivity, requiring relatively low volumes, is the use of the Ca^{2+} indicator, Arsenazo III. This was purified and used as described earlier [24]. The stock solutions of dye were first treated with Chelex resin and filtered through an EDTA-washed sinter. The residual Ca^{2+} was determined by spectrophotometric titration with 0.1 M EDTA, as described [24]. The same method was used to determine Ca^{2+} concentrations in both compartments of the equilibrium dialysis cells, sampled directly into a semimicro 1-cm path spectrophotometer cell, an appropriate volume of the stock indicator solution being added, and its Ca^{2+} content allowed for. Titrations were performed in a Cary 16 spectrophotometer at a fixed wavelength of 654 nm [24].

Mn^{2+} binding

The binding of Mn^{2+} was followed by electron paramagnetic resonance, at X-band frequency. Solutions of spectrin were stirred for 1 h with Chelex resin and then centrifuged. Mn^{2+} was added from a stock solution and the EPR spec-

trum was measured in a 0.5 mm (internal diameter) capillary in a Varian E-3 spectrometer at 20°C. Spectra were run at 9.0 GHz with a modulation amplitude of 4 G and a power input of 230 mW. The binding of Mn^{2+} to cytoskeletal complexes in suspension could not be measured in this way because of the high viscosity of the solution. Instead $MnCl_2$ was added to cytoskeleton suspension at a concentration of about 25 mg/ml and the mixture was agitated by vortex-mixing. After equilibration for 15 min with periodic mixing to ensure equilibrium, the suspension was centrifuged at 10 000 × *g* for 10 min and the supernatant was transferred to the capillary for an EPR spectrum. The Mn^{2+} concentration was referred to the deflection between the two highest-field extrema in the derivative spectrum. An identical determination was made on insoluble spectrin, precipitated by addition of acetate buffer (0.05 M final concentration) to give a pH of 4.8. A calibration with $MnCl_2$ was performed on each occasion. Displacement experiments were performed at constant concentration (generally in the range 10—25 μM) of Mn^{2+}. Small volumes of concentrated Ca^{2+} or Mg^{2+} stock solutions were added to give the desired final concentrations, and the EPR signal amplitudes were measured as before, after mixing and re-equilibration.

Aggregation effects on spectrin were observed in the analytical ultracentrifuge (Beckman Model E) using schlieren optics. $CaCl_2$ or $MgCl_2$ was added to spectrin solutions at 1—5 mg/ml in 0.1 M NaCl/0.05 M Tris, pH 7.6. They were allowed to incubate for various times at 20, 30 or 37°C, and were then run at 60 000 rev./min. Aggregation was also observed in terms of light-scattering at 90°, using a Perkin Elmer MPF-4 spectrofluorimeter, set at a wavelength of 350 nm for both monochromators. The cell housing was thermostatically controlled at 20, 30 or 36°C, and the change of scattering was observed as a function of time after addition of Ca^{2+} or Mg^{2+}. Fluorescence spectra of spectrin solutions at lower Ca^{2+} concentrations were measured with the same instrument, and the circular dichroism as a function of divalent metal ion concentration was observed with a Jasco J-41C instrument.

Results

Binding experiments on spectrin

Binding experiments were performed with both purified and crude spectrin, the latter preparations containing about 10% of actin and other proteins. Equilibrium dialysis led to the results shown in Fig. 1. The maximum protein concentration readily obtainable is equivalent to about 10 μM dimer. The data show that within the limits of precision of the method there is no difference between the concentrations of Ca^{2+} on the two sides of the dialysis membrane. These error limits could possibly conceal the binding of 1 mol of Ca^{2+} per dimer, but not as much as 1 mol per polypeptide chain. It thus seems likely that there is no strong binding of Ca^{2+}. In an attempt to improve the sensitivity of detection of binding still further we have had recourse to EPR. As is well known [25], Mn^{2+} is a good isomorphous replacement for Ca^{2+} in many systems and has been widely used for studies of Ca^{2+}-binding sites. It is readily detectable down to concentrations of well below 10 μM, and it affords the advantage that it does not occur, like Ca^{2+}, as a prevalent contaminant in

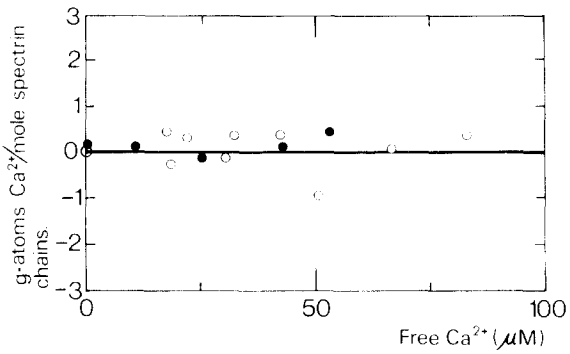

Fig. 1. Equilibrium dialysis of Ca^{2+} binding to spectrin. The open and closed circles refer to two separate spectrin preparations (spectrin concentration 2.9 mg/ml). Conditions are as described in the text.

detectable concentrations.

The EPR spectrum of Mn^{2+} is not detectably changed by the presence of spectrin at comparable molar concentrations, the relative amplitudes of the six peaks which constitute the familiar $Mn^{2+} \cdot 6H_2O$ signal remaining unchanged. Because of the elevated viscosity in the presence of the protein, the measurement of signal amplitudes is hazardous. The most critical test for the presence of binding (in the absence of a change in the shape of the signal such as would betoken the appearance of a new coordination state for Mn^{2+}, giving rise to a different sharp-line spectrum) is therefore an increase in signal when excess Ca^{2+} is added to displace bound Mn^{2+}. In purified spectrin solutions no such effect is observed. In crude spectrin small effects have sometimes been observed, which we attribute (see below) to the presence of actin. Spectrins, either phosphorylated with endogenous kinase, or dephosphorylated, were also examined in the same way, and no difference was found.

We have examined also the conformationally sensitive properties of the protein, namely intrinsic fluorescence, circular dichroism in the peptide region, and in that of aromatic absorption (250—300 nm), where spectrin shows a series of Cotton effects, arising from aromatic side chains in asymmetric environments, and sedimentation velocity. In no case does Ca^{2+} or Mg^{2+} induce any detectable change, as long as the ionic strength is sufficiently high to eliminate binding governed by the Donnan effect, and the divalent cations are not present in such large concentrations as to induce strong aggregation (see below).

Binding experiments on cytoskeletal complexes

Having obtained no indication of strong binding of divalent metal ions to spectrin, we sought to determine whether the spectrin in its cytoskeletal complex with other proteins, or any of the other constituents of the cytoskeleton might reveal themselves to have strong binding sites. The cytoskeletal preparations resulting from extraction with Triton X-100 [15] are devoid of integral membrane proteins, but retain a considerable amount of phospholipid. We attempted to reduce this by increasing the concentration of detergent used for extraction. Phosphorus analysis, combined with dry-weight determinations, led

to the following compositions for the cytoskeleton preparations: preparations made with 2.5% Triton X-100 [15] contained 25—35% by weight of lipid, and those made with 0.5% Triton X-100 somewhat higher proportions. Most experiments were performed with the former. The major protein constituent was found by gel electrophoresis to be spectrin, but actin and several other proteins at lower concentration are also present.

Because of the sensitivity of detection, Mn^{2+} was again used for the binding studies. Because of the bulk viscosity of the cytoskeleton preparations, the binding was measured by centrifugation, followed by sampling of the supernatant. By this means, binding at the level of a few micromolar Mn^{2+} in a suspension containing, say, 25 mg/ml cytoskeletal protein (approx. 3 μM in spectrin dimer) could be detected. Fig. 2b shows a binding profile, defined by the magnitude of the Mn^{2+} signal in the supernatant, compared with that of Mn^{2+} added to the supernatant after its separation from the cytoskeleton, i.e., free $Mn^{2+} \cdot 6H_2O$. Binding clearly occurs. Fig. 2a shows a Scatchard plot, which is consistent with the presence of a set of indistinguishable binding sites (1.4 nmol of sites per mg protein) with an association constant of $4 \cdot 10^4$ M^{-1}. This figure can be used to calculate a binding profile, which as Fig. 2b shows, gives an excellent fit to the experimental points. The evaluation of the densitometric analysis of the protein electrophoresis patterns of the cytoskeletons leads to a molar ratio of actin to spectrin dimer of about 1.5 : 1. The actin concentration is then about 1.5 nmol per mg total protein in the cytoskeleton preparations, which is compatible with an identification of the Mn^{2+}-binding sites with actin. It should be noted that there is no evidence of sites of higher affinity (although a very small number of these could elude detection). The

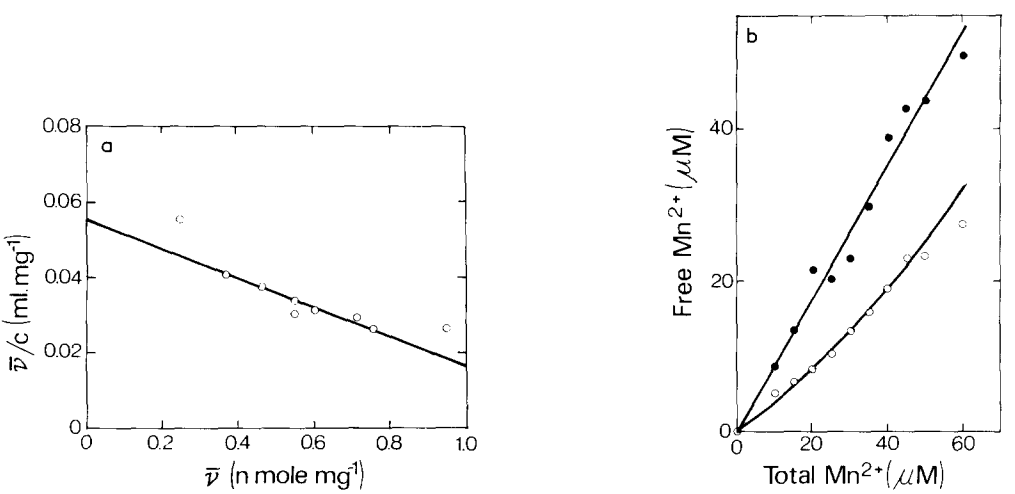

Fig. 2. (a) Scatchard plot for binding of Mn^{2+} to cytoskeletal complexes prepared by extraction of erythrocytes with Triton X-100, observed by EPR spectroscopy as described. Total protein concentration, 22 mg/ml. The slope gives an association constant of $4 \cdot 10^4$ M^{-1}. (b) Binding curves showing the relation between free Mn^{2+} in the supernatant and total concentration. The open circles refer to an experiment in which the total protein concentration was 32 mg/ml. The curve is calculated for a 38 μM concentration of binding sites with an association constant of $4.2 \cdot 10^4$ M^{-1}. The filled circles were obtained under the same conditions, except for the presence of 25 mM Ca^{2+} to displace the Mn^{2+}.

Mn^{2+}-binding sites serve equally for the binding of Ca^{2+} and Mg^{2+}, for when the binding assay is performed in the presence of these ions, correspondingly less Mn^{2+} is bound, and at concentrations in the millimolar range of Ca^{2+} or Mg^{2+} (Mn^{2+} concentration of the order of 10 μM) uptake of Mn^{2+} is suppressed. Thus, these ions have an affinity of a similar order to that of Mn^{2+} for the sites on the cytoskeletons.

Similar experiments as with cytoskeletons were performed on insoluble spectrin, precipitated at pH 4.7 [14]. The results were consistent with those of equilibrium dialysis on spectrin solutions in that they showed (at pH 4.7) no detectable binding of Mn^{2+}, the same signal amplitudes being obtained within instrumental error in the presence and absence of the protein.

Spectrin aggregation at high divalent cation concentrations

We now consider the interaction of spectrin with divalent metal ions in the higher concentration regime. Spectrin is stable as the dimer, for a period of days at least, in the cold at a Ca^{2+} concentration of less than 1 mM. Only above this concentration does self-association set in. Thus, at Ca^{2+} concentrations of 2 mM and above, and Mg^{2+} concentrations of 10 mM and above, rapidly sedimenting boundaries appear in the analytical ultracentrifuge. These form more rapidly if the spectrin is incubated at 30 or 35° C. Progressively, and especially at higher concentrations, large aggregates form and the solutions become turbid. The association can be readily followed by light-scattering from such a solution, using a fluorimeter. The rate of increase of scattering is strongly

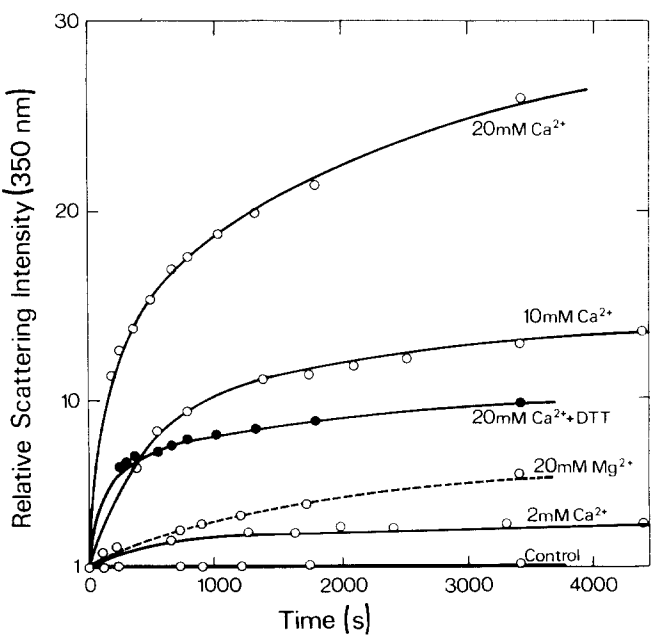

Fig. 3. Kinetics of aggregation of spectrin (0.25 mg/ml) by high concentrations (as indicated) of Ca^{2+} or Mg^{2+}. Scattering intensity was measured at 90° to the incident beam, using a spectrofluorimeter. Temperature 36°C, and other conditions as in the text. Effect of dithiothreitol (DTT) (0.25 mM) is also shown.

dependent on the temperature and on the divalent cation concentration. It also increases as the pH is lowered; at pH 6.7 the rate of development of turbidity is almost an order of magnitude greater than at pH 7.8 and it becomes essentially instantaneous as the pH region of phase separation [14] is approached. At 37° C the rate of turbidity rise is also faster than at 30° C by about 50-fold. At pH 7.6 and 37° C, at which our measurements were routinely carried out, the lower limits of Ca^{2+} and Mg^{2+} concentration, at which aggregation became detectable, were approx. 1 and 5 mM, respectively. Ca^{2+} is thus much more effective in bringing about aggregation than is Mg^{2+}. Fig. 3 shows typical aggregation-rate curves. It is rather surprisingly found that the rate of aggregation is affected by the state of the thiol groups, of which there are about 20 per spectrin dimer [26]. Thus, when a reducing agent, either β-mercaptoethanol or dithiothreitol, is added, the rate of association is considerably diminished. This perturbation of the association reaction by the state of the thiol groups probably accounts for the rather poor reproducibility of aggregation rates under these conditions from one spectrin preparation to another.

Discussion

The equilibrium dialysis results allow us to conclude that there are no strong binding sites for Ca^{2+} on the spectrin molecule, at least at the level of one or more per polypeptide chain. From the EPR experiments we may go further, for the sensitivity of the method is sufficient to detect one site per heterodimer for Mn^{2+}. Working, as we have, at a protein concentration of about 10 μM in dimers, Ca^{2+}-displacement experiments would enable us to detect with confidence a deficit of, say, 5% in the $Mn^{2+} \cdot 6H_2O$ signal at a concentration of 20 μM. The sensitivity might be rather less if bound Mn^{2+} gave rise to a sharp-line signal, but certainly a single site with an association constant in the range 10^4—10^5 M^{-1} should be detectable. We cannot, of course, exclude the possibility that such a site might be highly specific for Ca^{2+}, but the ability of Mn^{2+} to substitute for Ca^{2+} in strong binding sites on a variety of proteins has been demonstrated. The conclusion that strong divalent metal binding sites are absent in spectrin conflicts with preliminary findings, which appeared some years ago in the literature [8,9], and were related to changes in mechanical properties of the erythrocyte ghosts at sub-micromolar Ca^{2+} levels [6,7]. Our results are equally incompatible with the reported presence of a very large number (125) of binding sites with an association constant of 10^4 M^{-1} [10]. These experiments were, however, performed with crude spectrin, containing 20% of other proteins. The actin contaminant might alone then account for at least a part of this binding. Our results, on the contrary, support those of Cohen and Solomon [11] who found that the extraction of spectrin from ghost vesicles does not sensibly diminish the binding of Ca^{2+}, and also refer to data for spectrin, which exclude the presence of Ca^{2+} sites with a binding constant as large as 10^4 M^{-1}.

In the cytoskeleton preparations, which contain the other proteins present in crude spectrin, binding sites for Mn^{2+} exist. The binding constant is $4 \cdot 10^4$ M^{-1} and Ca^{2+} and Mg^{2+} both compete. These sites are not explicable in terms of the phospholipid present in the preparations; apparent binding constants for

divalent metals to phospholipid bilayers are of the order of 10—100 [27]. A likely locus is actin, present, as has been noted, at a concentration in the cytoskeletons which is compatible with the number of Mn^{2+} bound. Actin contains a single divalent cation binding site. The binding of Mn^{2+} to muscle G-actin has been measured, with the conclusion that there are several weak binding sites, but only one strong site, with an association constant of $2 \cdot 10^5\ M^{-1}$, albeit at low ionic strength to prevent polymerisation [28]. The corresponding association constants for the binding of Mg^{2+} and Ca^{2+} under the same conditions were also about $10^5\ M^{-1}$. Since the affinity is expected to fall with increasing ionic strength, our value for the binding constant is in the anticipated range. The binding constants for Mn^{2+}, Ca^{2+} and Mg^{2+} do not differ very greatly from one another [29,30], and consequently the actin in situ (the polymerisation state of which is still uncertain) must be supposed to be normally saturated with Mg^{2+}.

In the absence of any additional, and stronger, binding sites for Ca^{2+} than those on the actin molecules, one may rule out direct binding of Ca^{2+} to the cytoskeleton as the basis for Ca^{2+}-dependent changes in the membrane characteristics. The change in flexibility, reported to occur at free Ca^{2+} concentrations in the vicinity of 10^{-7} M [6] is probably a function of an enzymatic event, such as the activation of the cross-linking enzyme, transglutaminase [31,32]; a membrane-associated protease has also been found to become active when Ca^{2+} is present [33].

As regards the aggregation of spectrin observed at much higher Ca^{2+} or Mg^{2+} concentrations, one must suppose that this is a relatively non-specific consequence of electrostatic free energy changes, which accompany binding at weak sites, comprising probably carboxylate side chains, present in abundance in this rather acidic protein [26]. The effect with Ca^{2+} is clearly much greater than with Mg^{2+}. It is probably not relevant to the state of spectrin in the cell, for much of the intracellular Mg^{2+} is in the form of complexes with nucleotides and other cell constituents. According to a recent determination, the activity of Mg^{2+} in the cell is less than 1 mM [34], and in these circumstances the spectrin is probably little affected. In any case it is improbable that the filamentous lattice of the spectrin, together with its associated proteins, in the cell would afford scope for non-specific self-association.

The effect of reducing agents on the progress of the aggregation reaction is not easily understood, since no intermolecularly linked spectrin molecules can be detected in the purified protein. One cannot exclude the possibility, however, that an undetectably low proportion of disulphide-linked associates of dimers is present and is sufficient to nucleate a self-association reaction. That disulphide links between adjacent spectrin dimers can form in situ has been demonstrated [35,36]. Reactive thiol groups might also form some intramolecular disulphide bonds, and this could conceivably modify the solubility properties of the protein. The relation of the thiol groups of spectrin to its aggregation propensities is being further investigated.

Acknowledgements

We are grateful to Dr. J.F. Gibson for indispensable advice and help on the

use of EPR spectroscopy in measuring low concentrations of Mn^{2+}. We thank Mr. A.C.M. Harris for preparations of spectrin and of cytoskeletons.

References

1 Weed, R.I., La Celle, P.L. and Merrill, E.W. (1969) J. Clin. Invest. 48, 795—809
2 Romero, P.J. and Whittam, R. (1971) J. Physiol. 214, 481—507
3 Dunn, M.J. (1974) Biochim. Biophys. Acta 353, 97—116
4 Palek, J., Curby, W.A. and Lionetti, F.J. (1971) Am. J. Physiol. 220, 19—26
5 Allan, D., Billah, M.M., Finean, J.B. and Mitchell, R.H. (1976) Nature 261, 58—60
6 LaCelle, P.L. and Kirkpatrick, F.H. (1975) in Erythrocyte Structure and Function (Brewer, G.J., ed.), pp. 535—553, Alan R. Liss, Inc., New York
7 LaCelle, P.L. (1970) Semin. Haematol. 7, 355—371
8 LaCelle, P.L., Kirkpatrick, F.H., Udkow, M.P. and Arkin, B. (1972) Nouv. Rev. Fr. Haematol. 12, 789—798
9 La Celle, P.L., Kirkpatrick, F.H. and Udkow, M.P. (1973) in Erythrocytes, Thrombocytes, Leukocytes; Recent Advances in Membrane and Metabolic Research (Deutsch, E., Gerlach, E., Moser, K. and Williams, W., eds.), p. 49, G. Thieme, Stuttgart
10 Brauer, E., Kupka, K.D. and Rudloff, V. (1976) Bioelectrochem. Bioenerg. 3, 509—518
11 Cohen, C.M. and Solomon, A.K. (1976) J. Membrane Biol. 29, 345—372
12 Marchesi, S.L., Steers, E., Marchesi, V.T. and Tillack, T.W. (1969) Biochemistry 9, 50—57
13 Clarke, M. (1971) Biochem. Biophys. Res. Commun. 45, 1063—1070
14 Gratzer, W.B. and Beaven, G.H. (1975) Eur. J. Biochem. 58, 403—409
15 Yu, J., Fischman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233—248
16 Ungewickell, E. and Gratzer, W.B. (1978) Eur. J. Biochem. 88, 379—385
17 Ralston, G.B. (1975) Aust. J. Biol. Sci. 28, 259—266
18 Laemmli, U.K. (1970) Nature 227, 680—685
19 Birchmeier, W. and Singer, S.J. (1977) J. Cell Biol. 73, 647—659
20 Hosey, M.M. and Tao, M. (1977) Biochim. Biophys. Acta 482, 348—357
21 Pinder, J.C., Bray, D. and Gratzer, W.B. (1977) Nature 270, 752—754
22 Jaenicke, L. (1974) Anal. Biochem. 61, 623—627
23 Kam, Z., Josephs, R., Eisenberg, H. and Gratzer, W.B. (1977) Biochemistry 16, 5568—5572
24 Gratzer, W.B. and Beaven, G.H. (1977) Anal. Biochem. 81, 118—129
25 Williams, R.J.P. (1970) Q. Rev. Chem. Soc. 24, 331—365
26 Fuller, G.M., Boughter, J.M. and Morazzani, M. (1974) Biochemistry 13, 3035—3041
27 Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1975) Eur. J. Biochem. 58, 133—144
28 Martonosi, A., Molino, C.M. and Gergely, J. (1964) J. Biol. Chem. 239, 1057—1064
29 Strzelecka-Golaszewska, H. (1973) Biochim. Biophys. Acta 310, 60—69
30 Kasai, M. and Oosawa, F. (1968) Biochim. Biophys. Acta 154, 520—528
31 Lorand, L., Siefring, G.E. and Lowe-Kreutz, L. (1979) J. Supramol. Struct. 9, 427—440
32 Palek, J., Liu, P.A., Lin, S.C., Timura, B. and Crusberg, J. (1978) J. Cell Biol. 79, 244A
33 Triplett, R.B., Wingate, J.M. and Carraway, K.L. (1972) Biochem. Biophys. Res. Commun. 49, 1014—1020
34 Gupta, R.K., Benovic, J.L. and Rose, Z.B. (1978) J. Biol. Chem. 253, 6172—6176
35 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21—32
36 Salhany, J.M., Swanson, J.C., Cordes, K.A., Gaines, S.B. and Gaines, K.C. (1978) Biochem. Biophys. Res. Commun. 82, 1294—1299