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Some viscoelastic properties of human erythrocyte spectrin networks end-linked in vitro

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We have succeeded in making macroscopic networks of end-linked human erythrocyte spectrin. The network junctions were made using erythrocyte protein 4.1 irreversibly attached to 5 nm (diameter) colloidal gold particles. Rotary shadowing electron microscopy verifies that the protein 4.1-labelled colloidal gold particles bind only to the tail end of the spectrin molecules. Electron micrographs of protein 4.1-labelled colloidal gold particles incubated at 4°C with spectrin dimers reveal that 1–5 spectrin dimers attach to each protein 4.1-labelled colloidal gold particle yielding a spider-like appearance of these complexes. Incubation with a low concentration of spectrin tetramers instead of dimers leads to extensive formation of spectrin microaggregates whereas use of spectrin concentrations higher than 3 mg/ml and a molar ratio between spectrin tetramers and protein 4.1/Au of 4 leads to formation of macroscopic spectrin networks. We have quantitated the viscoelastic properties of such end-linked macroscopic spectrin networks using a gravitational pendulum viscoelastometer. We find that in vitro end-linked spectrin networks can be described by linear viscoelastic theory. The dynamic storage modulus increases almost linearly with the spectrin-protein 4.1/gold particle concentration when the spectrin concentration exceeds about 3 mg/ml and the molar ratio between spectrin tetramers and protein 4.1/Au is 4. At a spectrin concentration of 6 mg/ml and the same ratio between spectrin and protein 4.1/Au, we find a dynamic storage modulus at low frequency of about 80 dyn/cm^2. This is in adequate agreement with what is predicted by simple elastomer theory.

Introduction

The human erythrocyte has a uniform membrane skeleton which extends over the entire cytoplasmic surface of the plasma membrane lipid bilayer. It is generally recognized that the membrane skeleton is crucial in maintaining the normal elastic properties of the human erythrocyte membrane. The protein network constituting the membrane skeleton is made up mainly of spectrin present as tetramers and possibly higher oligomers. The highly flexible and elongated spectrin tetramer is believed to be end-linked into a locally two-dimensional network by binding at each end to actin oligomers. Protein 4.1 also binds specifically to the tail ends of spectrin tetramers and modulates the spectrin-actin association. The membrane skeleton is kept in close proximity of the membrane lipid bilayer by specific associations between spectrin and the protein ankyrin and between the latter and the integral membrane protein named band 3 (for recent reviews, see Refs. 1–5).

Despite the human erythrocyte plasma membrane being the most studied and best characterized of all cell membranes, the molecular mechanisms which give rise to the viscoelastic properties of the erythrocyte membrane skeleton are still unknown. Until recently it was commonly believed that the membrane of the highly special-

ized human erythrocyte was very different from membranes of other cells [6]. However, it is now becoming increasingly clear that although the membrane skeleton of other plasma membranes may be significantly more complex than that of the erythrocyte plasma membrane, they all appear to have important structural features in common [7–9]. An increased understanding of the molecular mechanisms giving rise to the mechanical properties of the erythrocyte membrane skeleton can therefore be expected also to increase our understanding of the molecular basis for the mechanical properties of plasma membranes in general.

Up till now all measurements of the collective properties of the erythrocyte membrane skeleton have been done on the whole cell membrane (for reviews, see Refs. 10–12). Such measurements have their advantages, but from both a theoretical and experimental point of view it would be of great help if one could make synthetic, macroscopic three-dimensional networks consisting of the main components of the membrane skeleton. We have now succeeded in making such macroscopic networks where spectrin is the main constituent. In this text we describe how this is achieved and also report on the results of measurements of some viscoelastic properties of these spectrin networks.

Materials and Methods

Isolation of spectrin dimers

Human erythrocyte ghosts were either prepared from 300–400 ml human blood collected in Fenwal JF 15 bags (Travenol Laboratories, S.A., Belgium) containing 63 ml citrate/phosphate/dextrose/adenine anticoagulant solution (327 mg citric acid monohydrate, 2.63 g sodium phosphate monohydrate, 2.90 g dextrose (anhydrous) and 27.5 mg adenine per 100 ml), or from packed red blood cells in phosphate-buffered saline with adenine, glucose and mannitol (887 mg NaCl, 16.9 mg adenine, 819 mg glucose and 525 mg mannitol per 100 ml). The preparation started 2–5 weeks after the blood had been drawn from healthy adults. Unless otherwise stated, all preparative steps were carried out at pH 7.6 and 4°C.

Human erythrocyte ghosts were prepared according to Stokke et al. [13] using the filtration set-up for hemoglobin removal. To remove protein 6 (glyceraldehyde-3-phosphate dehydrogenase), phenylmethylsulphonyl fluoride (PMSF) and NaCl were added to concentrations of 20 μg/ml and 155 mM, respectively, and stirred overnight at 4°C. Protein 6 depleted ghosts were pelleted at $19000 \times g$ (14000 rev./min, Beckman JA 14 rotor) for 45 min, washed once in 10 mM phosphate buffer, and pelleted again, Spectrin dimers were extracted in low ionic strength buffer (1 mM Tris/0.1 mM EGTA/0.05 mM dithiothreitol/0.02 mg $\cdot$ ml^{-1} PMSF) at 37°C, recovered by ammonium sulphate precipitation, dialyzed and fractionated on a Sepharose CL-4B gel-filtration column (900×36 mm) equilibrated with 130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol as described earlier [13]. Spectrin tetramers were extracted by dialysis against 3×1000 ml low ionic strength buffer at 4°C, as described by Elgsaeter [14], and purified by gel-filtration as described for spectrin dimers.

Spectrin containing fractions from the gel filtration column were pooled and dialyzed against 2×1000 ml low ionic strength buffer for 24 h. The spectrin solution was then concentrated gently to a given volume using the electrophoretically two-step concentrator designed recently by Stokke et al. [15] using the low ionic strength buffer as electrophoresis buffer. This procedure yields approximately 1 ml of spectrin solution with a concentration 10–20 mg/ml.

Isolation of protein 4.1

Protein 4.1 was purified essentially according to Becker et al. [16]. Spectrin-actin depleted vesicles were further washed by resuspension in 1000 ml low ionic strength buffer pre-equilibrated to 37°C and incubated at 37°C for 30 min. The vesicles were pelleted at $19000 \times g$ (14000 rev./min, Beckman JA 14 rotor) for 30 min, and the supernatant discarded. To further wash the erythrocyte ghost free of spectrin and actin, the vesicles were twice suspended in 2 volumes of 0.6 mM sodium azide/0.5 mM EGTA (pH 8.0), stirred for 20 min on ice and pelleted at $20000 \times g$ (17000 rev./min, Beckman JA 17 rotor) for 30 min. Finally, the vesicles were diluted with an equal volume of 0.2 M glycine/2% Tween 20/2 mM sodium tetrathionate/1 mM EGTA (pH 9.8) and stirred for

10–14 h at 0°C. The suspension was then centrifuged at 130 000 × *g* (45 000 rev./min, Beckman 50Ti rotor) for 60 min. The protein containing supernatant was immediately applied to a 10 mm (i.d.) × 100 mm ion exchange column of DEAE-cellulose equilibrated with 50 mM glycine/0.5 mM EGTA/0.5 mM dithiothreitol (pH 9.8). The column was washed with the equilibration buffer at a flow rate of 30 ml/h, and the proteins eluted with a continuous gradient from 0 to 0.5 M NaCl in the equilibration buffer. Flow rate during elution was 20 ml/h, and proteins in the effluent were monitored for absorbance at 280 nm. Sodium dodecyl sulphate gel electrophoresis was used to identify protein 4.1-containing fractions. The DEAE-column was washed with 1 M NaCl in equilibration buffer after each protein 4.1 purification, and re-equilibrated with the equilibration buffer.

Concentration and purity of spectrin and protein 4.1

The spectrin dimer and protein 4.1 concentration were determined by measuring the absorbance at 280 nm using a specific absorbance *A* (1 cm, 1%, 280 nm) = 10.1 [14] for spectrin and *A* (1 cm, 1%, 280 nm) = 8.0 [17] for protein 4.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out in 0.2% sodium dodecyl sulphate using a 5% stacker and 9% separation slab gel together with the discontinuous buffer system according to Laemmli [18]. The polyacrylamide gels were routinely dried between two sheets of uncoated cellophane. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out on most fractions from the gel filtration column to test the purity of the spectrin fractions, and on most fractions from the DEAE-ion exchange column to identify and test purity of the protein 4.1-containing fractions.

Preparation of colloidal gold-protein 4.1 complex

Colloidal gold solutions were prepared by reduction of chloroauric acid ($HAuCl_4$) with white phosphorus [18]. 1.0 ml of 0.5% $HAuCl_4$ solution was added to 50 ml distilled water, and the pH of this solution was adjusted to 10.0–10.2 with K_2CO_3 (approx. 1.5 ml of a 0.1 N solution). 0.5 ml of white phosphorus in diethylether solution was added beneath the surface of the gold solution during rapid stirring. The stirring was stopped just after the phosphorus solution was added, and the preparation left at room temperature for 15 min. The phosphorus solution was prepared by adding 3 parts of ether to 1 part of ether saturated with white phosphorus, and mixed well. This gold-containing solution developed a purple-brown colour when left at room temperature due to the formation of colloidal gold particles. The suspension was then heated over a Bunsen burner to almost boiling resulting in a change of colour to wine-red. The suspension was then rapidly cooled on ice. The pH was adjusted to 6.5 using HCl. A paper pH indicator was used to avoid colloidal gold contamination of the standard glass pH electrode.

10 μg protein 4.1 was then added per ml of gold suspension and left on ice for 60 min. An equal amount of bovine serum albumin in 1 mM Tris/0.1 mM EGTA/0.05 mM dithiothreitol (pH 7.5) was subsequently added to assume that the gold particles were completely coated with proteins. The saturation of the protein binding to the colloidal gold in this suspension was tested by adding 1 part of 10% NaCl to 1 part of suspension. Below the saturation point the suspension turned blue, whereas it remained wine-red when sufficient proteins were added to cover the gold particles. We found that the saturation point was reached at a final protein concentration around 10 μg/ml in the gold particle suspension. The protein 4.1-Au complex was pelleted at 64 000 × *g* (25 000 rev./min, Beckmann SW 25 rotor) for 90 min, the supernatant carefully discarded and the loose pellet resuspended in a small volume of the supernatant.

Concentration of colloidal gold - protein 4.1 complex

The maximum absorbance of the colloidal gold solution was at 510 nm. The absorbance was measured using a Zeiss PMQ3 spectrophotometer. We used this absorbance to determine the concentration of the suspension of protein 4.1-Au particle complexes. The specific absorbance *A* (1 cm, 1%, 510 nm) was determined from measurements of the absorbance at 510 nm of gold particle suspensions with known gold particle concentrations. The mean gold particle weight was estimated assuming that cloroauric acid was completely reduced [20], that the gold particles had the same density as pure gold, and using the mean particle diameter obtained from electron microscopy.

Rotary shadowing electron microscopy

Aqueous glycerol solutions (70%) were added to the spectrin, protein 4.1-Au suspensions yielding a final glycerol concentration of 50% and spectrin concentration 10–40 μg/ml. Spectrin-glycerol samples were sprayed onto freshly cleaved 5 mm (diameter) mica disks and dried in vacuum as described by Tyler and Branton [21]. Rotary shadowing with platinum from an angle of 5° and carbon supporting film deposition were carried out in the freeze-etch unit described by Elgsaeter [22]. Replicas were floated off on distilled water, washed in clorox and picked up on 400 mesh copper grids. The electron micrographs were obtained using a Philips EM 400 electron microscope operated at 80 keV.

Viscoelastometry

Concentrated spectrin from the electrophoretic concentrator and concentrated suspensions of protein 4.1-Au complex were mixed at 4°C to give the desired concentration. Aqueous solution containing 1300 mM KCl/200 mM NaCl/50 mM Tris/50 mM sodium phosphate/1 mM EGTA/0.5 mM dithiothreitol was then added gently to a final concentration of 130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol. Measurement of the viscoelastic properties of this mixture was carried out using a pendulum viscoelastometer designed for meaurements of the rheological properties of soft viscoelastic gels or concentrated biopolymer solutions [23]. This instrument yields the viscoelastic properties using shear deformation geometry by monitoring the motion of a thin metal blade through the specimen. The metal blade is attached to a gravitational pendulum. The specimen cuvette is attached to a pendulum house. Both the pendulum and the pendulum house pivot about the same line. The angular position of the pendulum house is controlled by a minicomputer that also monitors the angular position of the pendulum. Experimental determination of specimen dynamic shear storage and loss moduli, stress relaxation and shear creep-creep recovery are possible using of this instrument. Details of construction, analysis of operation, and calibration data are presented elsewhere [23].

Results and Discussion

Protein purification

The elution profile from the Sepharose CL-4B gel filtration column of the erythrocyte ghost low ionic extract was similar to that obtained previously [13]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed spectrin and no contaminating actin or other proteins in the spectrin dimer and spectrin tetramer fractions. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of spectrin after electrophoretic concentration showed no changes in electrophoretic mobility.

Fig. 1 shows a typical elution profile from the DEAE-cellulose ion exchange column (Fig. 1A), and sodium dodecyl sulphate polyacrylamide electrophoresis gels of aliquots from the major peak in the elution profile (Fig. 1B). The elution profile is similar to that obtained by Becker et al. [16]. Coomassie blue stained sodium dodecyl sulphate polyacrylamide gels reveals the typical 2-band appearance of protein 4.1 [24] essentially uncontaminated by other proteins (Fig. 1B).

Concentration of protein 4.1-Au complex

The specific absorbance of the colloidal gold suspension was found to be $A(1\text{ cm}, 1\%, 510\text{ nm}) = 150 \pm 15$. This is about 15% lower than the specific absorbance that can be calculated from the data of Horrisberger et al. [19]. The local maximum absorbance at 510 nm did not change in magnitude nor shift in wavelength after saturation with protein 4.1. The measured absorbance at 510

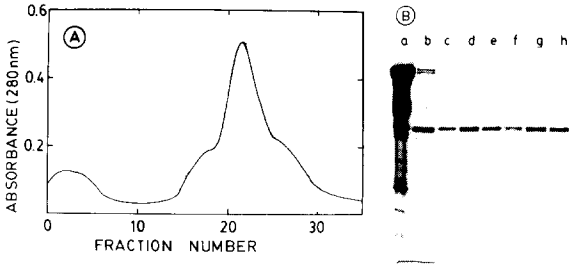

Fig. 1. Elution profile of protein 4.1 containing Tween 20 erythrocyte membrane extract off a DEAE-cellulose ion exchange column (A) and Coomassie blue stained sodium dodecyl sulphate polyacrylamide gels of standard ghost (a), Tween 20 extract (b), and fractions 19, 20, 21, 22, 23 and 24 (c)–(h) from the major peak of the elution profile (B).

nm was proportional within experimental error to the gold concentration for absorbance up to 2.4 (the maximum absorbance which could be measured with the Zeiss PMQ3 spectrophotometer). The concentration of the pelleted colloidal gold particles was estimated from three different dilutions of the suspension having absorbance in the range 0.1–2.0. Fig. 2 shows a typical electron micrograph of the colloidal gold particles on a formvar-carbon coated grid (A), and the diameter size distribution of these particles (B). Because gold particles are electron dense, there was no need for any staining to obtain contrast in the electron microscope, and the measured diameter (B) is not affected by carbon depositions. The measured size distribution shows a relatively homogenously population of the gold particles with an average diameter of 5.0 nm (S.D. 19%). Using the measured diameter and assuming that the gold particles were spherical pure gold particles with density 19.3 g/cm^3, gave an estimate of $7.9 \cdot 10^{15}$ gold particles/ml in a 1% solution. This corresponds to 13.1 $\mu M/\%$.

Association of spectrin to the protein 4.1-Au complex

Association of spectrin to protein 4.1 bound on gold particles was visualized by rotary shadowing electron microscopy. Electron micrographs of spectrin dimers (Fig. 3A) and spectrin tetramers (Fig. 3B) show the well known floppy, elongated

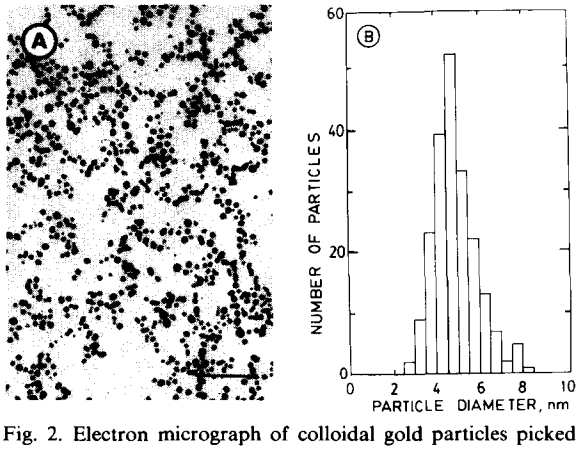

Fig. 2. Electron micrograph of colloidal gold particles picked up on a formvar-carbon coated 400 mesh copper grid and air dried in (A), bar = 50 nm. Size distribution of colloidal gold particle diameter determined from electron micrographs (B).

nature of spectrin molecules [25]. When spectrin dimers were incubated with protein 4.1-Au complexes for 100 min and prepared for electron microscopy as described in Materials and Methods, we found that 1–5 spectrin dimers were attached to each gold particle. Most of the spectrin molecules were end-linked by the gold particles (Fig. 3C). The actual number of spectrin molecules bound specifically to each gold particle depends both on accessability of the protein 4.1 binding site and the concentration ratio between spectrin and protein 4.1/Au. We observed no binding of spectrin molecules to colloidal gold particles coated only with bovine serum albumin (not shown). Fig. 3C shows that protein 4.1 binds at the end of spectrin dimer, but from this type of electron micrograph it is not possible to determine whether the tail end, the head end or both are involved. Fig. 3D shows an electron micrograph of spectrin tetramers incubated with the protein 4.1-Au complex. The electron micrograph reveals that the binding site of protein 4.1 is located at the tail end of spectrin as reported by others [24]. The microaggregate in Fig. 3E is believed to be the precursor of the macroscopic spectrin network. The major associations of a unit cell in the reconstituted spectrin network are shown schematically in Fig. 3F. Protein 4.1 is bound to colloidal gold particles, and spectrin is associated with the protein 4.1 bound to the gold particles.

Viscoelastometry

Kinetics of the gelation. Fig. 4 shows the time-course of the measured dynamic viscosity of 5.5 mg/ml spectrin dimer in 130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol/0.02 $mg \cdot ml^{-1}$ PMSF after temperature adjustment to 37°C. The dynamic storage modulus is less than 0.05 dyn/cm^2 (the lower limit of the instrument), and the increase in viscosity was completed within 50 min. Assuming a solvent viscosity of water at 37°C of 0.69 cP [26], the observed increase in viscosity corresponds to a change in relative viscosity from 1.5 to 2.2. The relative uncertainty of this values are approx. 5%. These observed relative viscosities are only slightly higher than what is estimated using the intrinsic viscosity spectrin dimers and tetramers, respectively [27,28]. Together with the

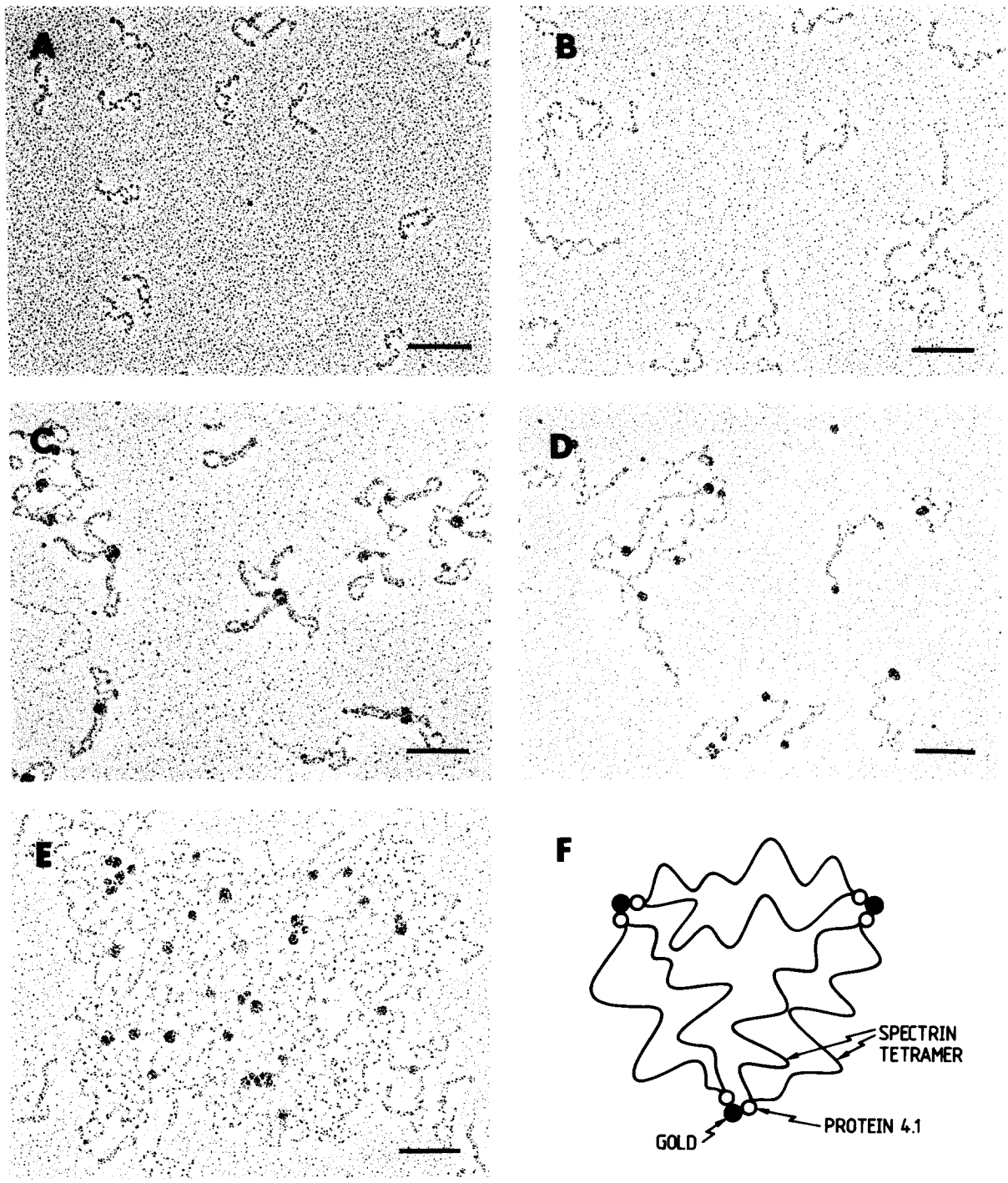

Fig. 3. Electron micrographs of isolated human spectrin dimers (A) and tetramers (B) vacuum dried from glycerol solutions and rotary shadowed with platinum from an angle of 5° according to Tyler and Branton [21]. Rotary shadowing electron micrographs of network junctions incubated at 0°C for 100 min with spectrin dimers (C) or spectrin tetramers, (D) and (E). Schematic illustration of the associations in the macroscopic spectrin networks (F). Bar = 100 nm.

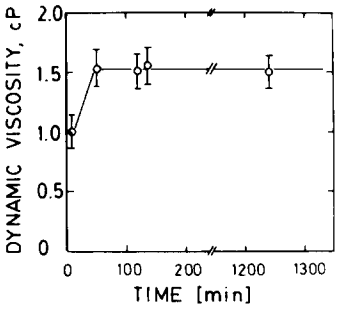

Fig. 4. The dynamic viscosity, $\eta'(\omega = 3.0\ s^{-1})$, of a solution containing 5.5 mg/ml spectrin dimers in 130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol/0.02 mg·ml^{-1} PMSF versus incubation time at 37°C. The viscosity measurements were carried out using a recently designed pendulum viscoelastometer [23] and a pendulum-cuvette combination with instrument parameter $C = 0.38$ dyn/cm^2.

finding that the observed dynamic storage modulus was less than 0.05 dyn/cm^2, this suggests that the increase at 37°C in the viscosity of pure spectrin solutions is caused mostly by conversion from spectrin dimers to tetramers and possibly higher oligomers [29–31]. It is possible that spectrin denaturation also is of some importance [32]. However, it is important to note that spectrin dimers alone do not give rise to any extensive gel formation at the spectrin dimer concentration used.

Fig. 5 shows the time dependence of the dynamic moduli as the gelation takes place at 20°C in samples containing 5.5 mg·ml^{-1} spectrin dimer/1.4 μM protein 4.1-Au complex/130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol/0.02 mg·ml^{-1} PMSF (pH 7.5). The energy stored during the oscillatory deformation 2000 min after the onset of gelation, is approx. 6-times larger than the dissipated energy. This indicates that the spectrin molecules and protein 4.1-Au complex form a cross-linked network under these conditions. The dynamic shear storage modulus shows a small increase when the excitation frequency is increased (Fig. 5, inset), whereas the dynamic loss modulus is essentially constant over the frequency range studied (not shown). The kinetics of gelation was approximately the same when spectrin tetramers were used instead of dimers in the start solution (data not shown). Macroscopically, the in vitro end-linked spectrin networks are highly elas-

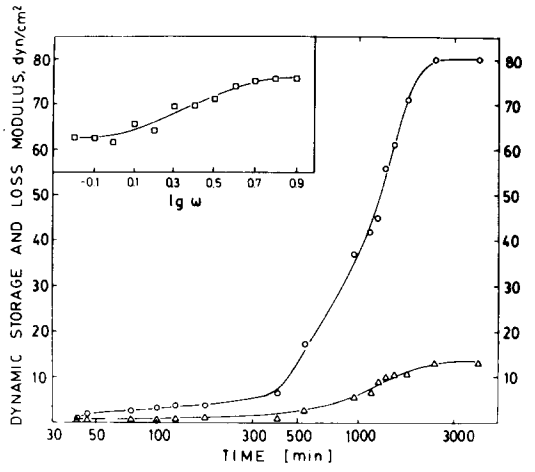

Fig. 5. The dynamic storage and loss moduli, $G'(\omega = 3.0\ s^{-1})$ (○) and $G''(\omega = 3.0\ s^{-1})$ (△) in 5.5 mg/ml spectrin dimer, 1.4 μM protein 4.1-Au in 130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol/0.02 mg·ml^{-1} PMSF versus incubation time at 20°C. The frequency dependence of the dynamic storage modulus $G'(\omega)$ at $t = 1400$ min (□) (insert).

tic, wine-red because of the local maximum absorbance of colloidal gold at 510 nm and nearly self-supported when placed for instance on a glass plate.

Concentration and temperature dependence. We found that the gelation in our system is critically dependent on total spectrin concentration and the ratio between spectrin and protein 4.1-Au complex. Starting from spectrin tetramers, we observed no gelation at spectrin concentrations below 3.5 mg/ml when the molar ratio of spectrin tetramers and protein 4.1-Au was 4 (Fig. 6A). When the start solution contained spectrin dimers instead of tetramers (Fig. 6A), gelation was only observed for spectrin concentration higher than approx. 5 mg/ml (the same molar ratio of spectrin tetramer to protein 4.1-Au complex). Formation of a continuous gel extending across the whole specimen cuvette is not expected to take place when the end-to-end distance of the spectrin tetramer is less than the average centre-to-centre distance of neighbouring tetramers. Assuming a cubic lattice, the average neighbour centre-to-centre distance of spectrin tetramers can easily be calculated as a function of spectrin concentration (Fig. 6B). The reported root mean square end-to-end distance of spectrin tetramers at room temperature is approx.

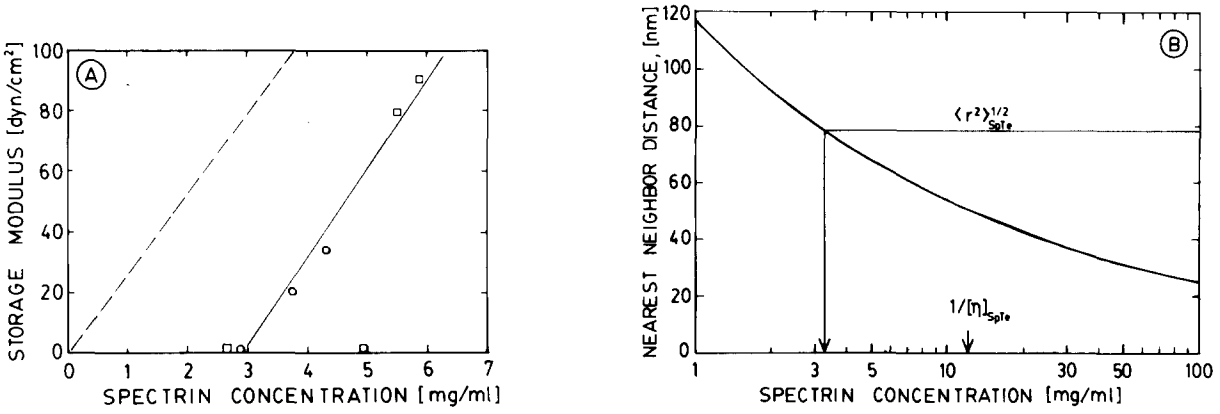

Fig. 6. The dynamic storage modulus $G'(\omega = 3.0\ s^{-1})$ versus spectrin concentration when the molar ratio of spectrin tetramer to network junctions is 4 (A). The dynamic storage modulus was measured at 20°C for solutions containing initially spectrin tetramers (○) or dimers (□). The predicted concentration dependence from the simple elastomer theory for the equilibrium storage modulus for spectrin tetramers is depicted (broken line). The mean centre-to-centre nearest neighbour distance for spectrin tetramers versus concentration (B). The mean centre-to-centre distance was calculated assuming a molecular weight of 920000 for spectrin tetramers [32]. The root mean square end-to-end distance of spectrin tetramers [13] indicates that the concentration where the mean centre-tocentre distance becomes equal to the end-to-end distance is about 3 mg/ml.

80 nm [13]. This suggests that the lowest possible spectrin concentration compatible with formation of a continuous, macroscopic network extending across the specimen cuvette is approx. 3 mg/ml (Fig. 6B) which is very close to the lowest concentration for which gelation was observed (Fig. 6A). However, the spectrin concentration corresponding to a perfect 6-fold topologically replicating network where the mean junction distance equals the root mean square end-to-end distance of spectrin tetramers, is about 9 mg/ml. Because the spectrin concentration in our gels is less than this value, this indicates that the average functionality of our macroscopic spectrin networks is less than 6. It is not expected that entanglement coupling contributes significantly to the measured elasticity because such coupling is generally expected to be important only when the macromolecule concentration exceeds $10/[\eta]$ [34] which is significantly higher than the spectrin concentrations used here (Fig. 6A). The statistical theory of rubber elasticity predicts that the equilibrium storage modulus essentially is given by the density of network strands times kT, where k is the Boltzman constant and T the absolute temperature [35,36]. Fig. 6A shows the shear modulus predicted theoretically when it is assumed that each spectrin tetramer constitutes one network strand and ignoring that at low spectrin concentrations it is no longer possible to obtain a network where each tetramer constitute one network strand. The observed shear modulus is substantially lower than what is predicted theoretically from the elastomer theory, but this is to be expected because of the highly simplified procedure used to estimate theoretically the number of network strands for the spectrin concentrations being used. Despite the over-simplification in the theoretical analysis, the results suggest that the statistical theory of rubber elasticity can account for the elastic properties in shear deformation of the spectrin gels.

Fig. 7A shows the effect on the dynamic storage modulus of lowering the temperature from 20°C to 4°C with subsequent return to 20°C. There was a large increase in the dynamic storage modulus as a result of reducing the temperature to 4°C. We observed a significant hysteresis in the mechanical properties of the spectrin gel (Fig. 7A). However, the dynamic storage modulus was restored to its initial value (from 2 to 1 in Fig. 7A) after about 18 h. We observed no kinetic effects on the storage modulus within 60 min at each temperature below 20°C. The observed temperature coefficient of the storage modulus in this 6 mg/ml spectrin gel is $\Delta G'/\Delta T = -5.9\ \text{dyn} \cdot \text{cm}^{-2} \cdot \text{K}^{-1}$. The effect of increasing the temperature above 20°C on the

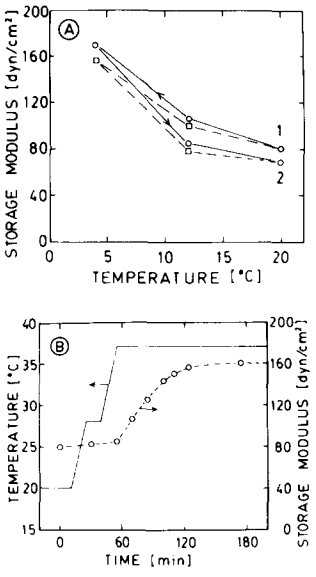

Fig. 7. The dynamic storage modulus of a 6 mg/ml spectrin - 1.5 μM protein 4.1-Au gel first allowed to equilibrate at 20°C for 2000 min and then exposed to other temperatures. The equilibrium values of $G'(\omega\eta = 3.0$ cP/s) (□) and $G'(\omega = 3.0$ s^{-1}) (○) from 20°C to 4°C (A), and the time-course of the dynamic storage modulus $G'(\omega = 3.0\ s^{-1})$ when the temperature was increased to 37°C (○) (B).

spectrin gel elastic properties is shown as a time-course in Fig. 7B. There was a small increase in the storage modulus when the temperature was increased to 37°C. After 37°C was reached, we observed a substantial increase in the dynamic storage modulus. The rate of change in the dynamic modulus from 60 min to 100 min in Fig. 7B was estimated to be $\Delta G'/\Delta t = 1.4$ dyn · cm^{-2} · min^{-1}. This temperature effect may be due to two molecular events, both leading to an increased number of network strands. Either the number of associations between spectrin may increase as the temperature is increased, or the associations between spectrin and protein 4.1 may be temperature dependent. Ungewickell and Gratzer [29] presented data indicating that under thermodynamic equilibrium conditions dimers are more favoured than tetramers at 37°C than at 20°C. This alone suggests a decrease and not an increased dynamic shear modulus with increasing temperature. Marchesi and co-workers [30,31] on the other hand, have shown in vitro that there is an increased tendency for spectrin oligomer formation when the temperature is increased from 21°C to 37°C when the total spectrin concentration is 7 mg/ml. This increased oligomer formation with elevated temperature may account for the observed increase in storage modulus. It has recently been reported that spectrin oligomers may exist in vivo as polyskelions with up to 7 legs [37]. This suggests that polyskelion formation may also contribute to increased elasticity either by increasing the number of network junctions or introducing entanglement coupling. However, the latter possibility is not expected to be of significant importance at the spectrin concentrations used in the current work (Fig. 6B). We do not know of any reports on temperature effects of the spectrin - protein 4.1 association.

Shear stress relaxation, shear creep and creep recovery. Fig. 8 shows the dynamic storage and loss moduli (A), shear stress relaxation modulus (B) and the creep compliance and creep recovery (C) measured at 20°C in a 6 mg/ml spectrin gel with a spectrin tetramer to protein 4.1-Au complex molar ratio equal to 4. The measurements were carried out after the equilibrium value of the elastic properties at 20°C had been reached. The equilibrium creep compliance was found to be independent of the gel deformation (data not shown). The creep compliance-creep recovery (C) show a tme-course characteristic of a soft viscoelastic solid [34]. The shear stress relaxation spectrum and the spectrum of time constants describing the creep recovery using the Fourier deconvolution method [38] are shown in Fig. 8D. The creep compliance was not analysed by the Fourier deconvolution technique because a small steady-state flow at long times may introduce uncertainties in the equilibrium value. The Fourier deconvolution method is sensitive to the equilibrium value, and a small error in the estimates of this value may introduce long relaxation times in the spectra if there are residual values at long times. The shear stress relaxation modulus is smaller than the dynamic storage modulus (Fig. 8A and 8B). However, at short times corresponding to the period of deformation, the shear stress relaxation modulus is not significantly different from the dynamic storage modulus. The product of the equilibrium shear stress modulus and the equilibrium creep compliance is measured to be $J_e \cdot G_e$

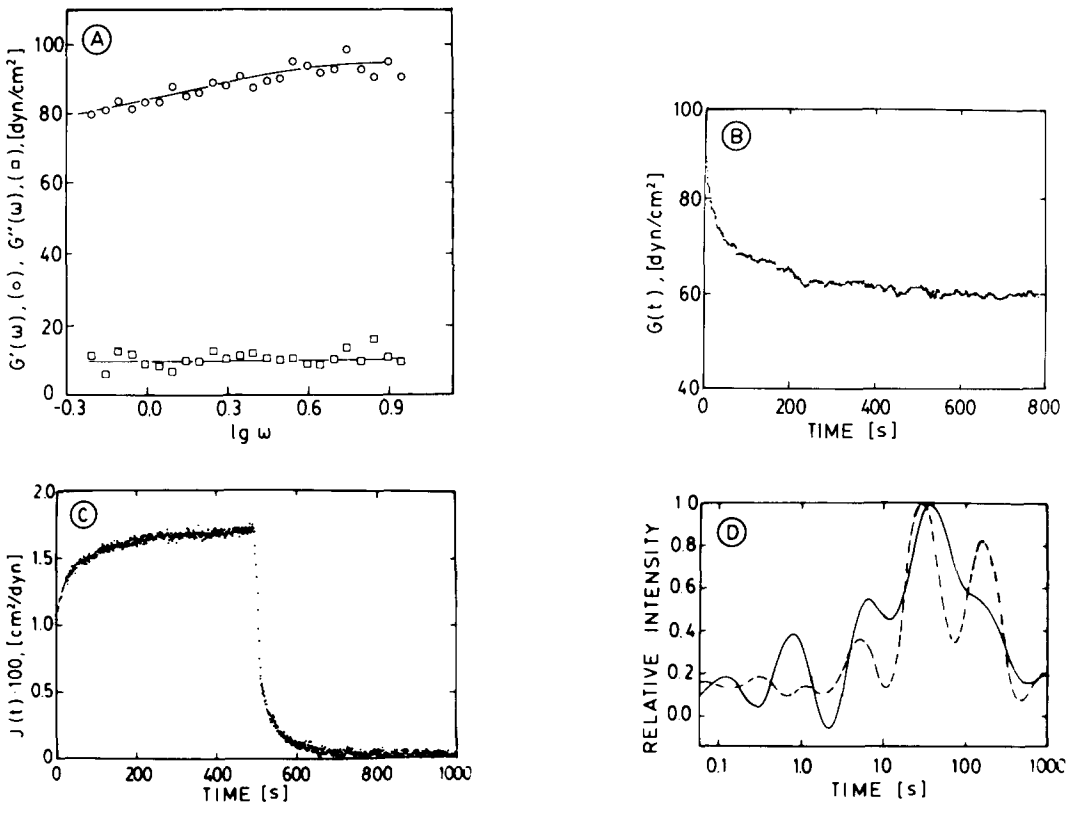

Fig. 8. Dynamic storage modulus $G'(\omega)$ (○), and dynamic loss modulus $G''(\omega)$ (□) as function of the exitation frequency ω (A) at 20°C for a spectrin gel consisting of 6 mg/ml spectrin- 1.5 μM protein 4.1-Au in 130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol/0.02 mg·ml^{-1} PMSF. The shear stress relaxation modulus $G(t)$, (B), and the time-course of the creep compliance - creep recovery (C). The relaxation spectrum (broken line) and spectrum of time constants describing the creep recovery time-course (full line) were calculated using the Fourier deconvolution technique [37] (D).

= 1.0, as expected for a linear viscoelastic solid. The stress relaxation spectrum and the spectrum of time constants describing the creep recovery are manifestations of molecular motions or rearrangements within the spectrin network. The observed time constants are several orders of magnitude larger than the rotational time for spectrin as measured by relaxation of electrically induced birefringence [39]. The stress relaxation data therefore most likely are manifestations of microaggregates slipping by one-another indicating that the network is not perfect topologically replicating throughout the specimen. This is consistent with the observation that the dynamic storage modulus is lower than the value obtained theoretically assuming that the number of network strands equals the number of spectrin molecules in the specimen. The two main time constants observed in stress relaxation, 30 s and 120 s, are significantly smaller than the time constant associated with force relaxation of erythrocyte membranes in vivo determined using the micropipette aspiration technique [40].

Concluding remarks

Fowler and Taylor [41] observed gel formation in a low ionic strength extract from human erythrocytes after addition of purified rabbit muscle G- or F-actin to sufficiently high concentrations. However, these authors were not able to determine quantitatively the elastic properties of these gels because they were observing the gelation using a rolling ball viscometer and therefore could only presented their results in terms of an apparent viscosity. The present work reports

for the first time quantitative measurements of the elastic properties of erythrocyte spectrin end-linked in vitro into a macroscopic gel. Although the concentration range of spectrin used in this study is much lower than on the erythrocyte plasma membrane, and the lipid-hemoglobin environment at the cytoplasmic side of the plasma membrane is different from the aqueous solution used, the observed elastic features of spectrin end-linked in vitro indicate strongly that the spectrin molecules have elastic properties that must be taken into consideration in order to account for the erythrocyte membrane elasticity. Our measurements indicate further that the spectrin network has an elasticity that can be described by linear viscoelastic theory and that the value of the dynamic storage modulus at low frequencies may be accounted for by simple elastomer theory.

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