Structural and Dynamic States of Actin in the Erythrocyte

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ABSTRACT Analysis of the nucleotide tightly associated with isolated erythrocyte cytoskeletons shows it to be ADP, rather then ATP. This confirms that at least a major part of the erythrocyte actin is in the F-form. A re-evaluation of the stoichiometry of spectrin and actin in the erythrocyte (taking account of a gross difference between the color responses of the two proteins on staining of electrophoretic gels) leads to values of 1×10^5 and 5×10^5 for the number of molecules of spectrin tetramer and actin respectively per cell. It has been found possible to perform spectrophotometric DNAase I assays for actin on lysed whole cells. The concentration of monomeric actin at 0°C is ~16 μg/ml packed cells. After washing the lysed cells the monomer pool is not re-established, indicating that only a small proportion of the actin subunits are free to dissociate. The actin monomer concentration in the cytosol remains unchanged after equilibration of the cells with cytochalasin E. The ability of actin-containing complexes in the membrane to nucleate the polymerization of added G-actin was measured fluorimetrically; it was found that membranes incubated with cytochalasin E were completely inert with respect to nucleating activity under conditions that favor appreciable growth at the slowly-growing ("pointed") ends of free actin filaments. This suggests that these ends of the actin "protofilaments" in the red cell are blocked or sterically obstructed. After treatment of the membranes with guanidine hydrochloride under conditions that dissociate F-actin, the measured concentration of actin monomer rises to ~180 μg/ml of packed cells, which is nearly 70% of the total actin content. On treatment with trypsin in the presence of DNAase, the spectrin and 4.1 are extensively degraded, but the actin remains undamaged. This treatment, followed by exposure to guanidine hydrochloride, causes a further rise in the concentration of actin responsive to the DNAase assay to 250 μg/ml of cells, compared with 270 μg/ml estimated by densitometry of stained gels. The oligomeric complex, consisting of actin, spectrin, and 4.1, that is extracted from the membrane at low ionic strength, generates no detectable actin monomer after the same treatment. From literature data on the number of cytochalasin binding sites per cell and our value for the total actin content, we obtain a number-average degree of polymerization for actin in the membrane of 12–17. The results lead to a model for the structure of the cytoskeletal network and suggest some consequences of metabolic depletion.

Actin was first reported to occur in the human erythrocyte membrane by Ohnishi in 1962 (1). Subsequently, the electrophoretic "band 5" component was identified as actin by Tilney and Detmers (2) and Sheetz et al. (3). The actin is associated with spectrin and two other constituents, proteins 4.1 and 4.9, in a cytoskeletal complex (4, 5), which is implicated in regulation of the shape and mechanical character of the cell (see e.g. reference 6 for review). The absence of discernible actin filaments in the membrane raised the possibility (2, 7) that the actin is monomeric in this complex, and this view received support from the report (8) that the nucleotide associated with it was ATP, rather than ADP.

Later evidence, however, suggested strongly that the inference was not correct, and that the actin was in fact polymeric. Thus in vitro rapidly sedimenting complexes were formed between actin, spectrin, and protein 4.1 only when the actin was in the polymerized state (9). Moreover, the membrane was shown to contain binding sites of high affinity for cytochalasins (10), a class of ligands known to attach to the preferentially growing (or "barbed") ends of actin filaments and not to G-actin (11, 12, 13). Further, there are nuclei in the membrane that will initiate the polymerization of G-actin (14), and low-ionic strength extracts contain high-molecular weight complexes comprising spectrin, actin, and 4.1, which are active with

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respect to both cytochalasin binding (10) and initiation of actin polymerization (10, 15, 16). Next, the appearance of covalent dimers and trimers of actin when the membrane is exposed to bifunctional reagents (17, 18) indicates the proximity of actin subunits to one another, and finally Atkinson et al. (19) have isolated short actin filaments containing ~30 subunits from membranes, after addition of phalloidin.

The balance of the evidence then suggests that the membrane-associated actin is polymeric. This actin is surmised to have the form of short filaments, or "protofilaments" as they have been termed (16). Such an arrangement, however, raises new questions. In known biological systems actin appears to occur predominantly as monomers, or as long filaments. The monomers may be free at or below the critical concentration for polymerization, or sequestered as a complex with another protein, and long filaments will in general be in equilibrium with monomers. Because of the high cooperativity of the polymerization process (20), oligomers are expected to be thermodynamically unstable. If therefore the "protofilaments" are not to disproportionate to form long filaments, it must be supposed that some mechanism operates to suppress depolymerization. One possibility is clearly that interaction of the actin subunits with the other proteins in the complex prevents dissociation. In addition, one end of each "protofilament," the slowly-growing, or "pointed" end, could be blocked. In other cells the long actin filaments that form part of the cytoskeleton seem commonly to be attached at one end, which is probably therefore inert with respect to the monomer-polymer equilibrium. At the same time it seems probable that actin filaments are capable of generating new ends by reason of a finite probability of spontaneous scission (21).

Whether the actin in the erythrocyte plays anything more than a passive structural role is not yet clear. The shape and mechanical properties of the cell depend for their maintenance on the presence of ATP in the cytosol (22, 23). The basis of this effect is not understood. Recent work suggests that the phosphorylation of spectrin, which had been thought to be implicated (24, 25), is not correlated with the macroscopic character of the cell (26, 27). The possibility that the actin "protofilaments" may be the seat of action of the ATP must be considered. One effect of ATP is to reduce the critical monomer concentration (28); the disappearance of the intracellular ATP might thus result in an increased tendency for short filaments to dissociate, with at least a partial redistribution of filament lengths. Changes in the arrangement of the network might, on the other hand, come about by way of ATP-dependent perturbation of the interaction between actin and the other cytoskeletal constituents (29, 30, 31). We have attempted in the first place to resolve the residual conflicting evidence regarding the state of the actin, and further to establish whether the "protofilaments," if they exist, give rise to an equilibrium concentration of monomers, whether they contain blocked ends, and to what extent they are capable of dissociation; we have sought also to place their stoichiometry on a more satisfactory quantitative basis, so that a more accurate model of the cytoskeletal complex can be constructed. To study the dynamics of the cytoskeletal actin we have had recourse to the DNAase I assay technique of Blikstad et al. (32), and we have developed procedures for applying it directly to the analysis of the total erythrocyte actin. We have also studied the membrane-associated actin in terms of its capacity to nucleate the polymerization of added G-actin, making use of a fluorescent derivative of G-actin (33), which undergoes a large increase in quantum yield when it enters the polymerized state.

MATERIALS AND METHODS

Membrane and Protein Preparations: Spectrin was prepared from fresh human erythrocytes by standard procedures and purified by chromatography on Sepharose 4B as described elsewhere (34). Actin from rabbit skeletal muscle was prepared by the method of Spudich and Watt (35). Protein 4.1 was extracted from spectrin-depleted membrane vesicles and purified as described previously (9). The oligomeric fraction containing spectrin, actin, and 4.1 (15), which is extracted together with free spectrin from the membrane at low-ionic strength, was collected from the void volume of the Sepharose 4B column or was prepared by sucrose gradient centrifugation (15); 1 mM magnesium chloride and 0.2 mM ATP were present throughout to protect the actin. Purified cytoskeletons were prepared from erythrocytes by extraction with high concentrations of Triton X-100, followed by treatment with a medium of high ionic strength, using a modified procedure based on that of Sheetz (5): washed, packed cells were treated with fresh 0.05% phenylmethylsulphonyl fluoride (Sigma Chemical Co., St. Louis, MO) for 5 min, diluted with an equal volume of isotonic PBS, and then stirred gently on ice with an equal volume of 0.15 M sodium chloride, 24 mM HEPES, 0.5 mM EDTA, 0.5 mM DTT, 150 mg/ml Triton X-100, pH 7.0. The resulting cytoskeletons were washed and stripped of peripheral proteins by pelleting through an equal volume of a solution, containing 30% sucrose, 0.6 M potassium chloride, 24 mM HEPES, 0.5 mM ATP, 0.5 mM DTT, 0.5 mM EDTA, pH 7.0. The cytoskeleton pellet was washed with high salt buffer and analyzed for protein composition by PAGE in the presence of SDS in a discontinuous buffer system (36), followed by staining with Coomassie Brilliant Blue R.

Quantitative Analysis of Protein Compositions: For quantitative determinations, freshly washed cells were lysed either in 5 mM phosphate, pH 8, or isotonically by suspension in saline (0.15 M sodium chloride, 5 mM sodium phosphate, pH 8.0), followed by addition of an equal volume of 5% saponin (Gurr-BDH Chemicals Ltd., Poole, Dorset, U. K.) in saline. Following lysis, the membranes were pelleted (5 min in bench-top Eppendorf centrifuge, model 3200), dissolved, either directly or after four washes with lysis buffer, in 3% SDS, and diluted threefold with stock sample buffer for electrophoresis (36, 37).

Samples were heated for 5 min in a boiling water-bath, and aliquots were applied to a 20 cm^2 flat-bed vertical 10% polyacrylamide gel and subjected to electrophoresis using either a discontinuous (36) or continuous (37) buffer system. Two-dimensional isoelectric focussing and electrophoresis analyses were performed according to O'Farrell (38). For quantitation, a series of concentrations of spectrin, actin, and 4.1, standardized by colorimetric micro-Kjeldahl nitrogen analysis (36), were run on the same gel as the ghosts, the loads being kept within the range of linear densitometric response. After staining and densitometry, calibration curves for the gel were set up in terms of the initial standards, and the quantities of spectrin and actin determined by interpolation. The limiting factor in the accuracy of these determinations was the presence of a finite staining background in the region of the actin and 4.1 zones and, in the case of the latter, the possibility of unresolved minor bands. The proportions of spectrin, actin and 4.1 in isolated cytoskeletons were determined in similar manner.

Nucleotide Analysis: The identity of the actin-bound nucleotide was determined on purified salt-extracted cytoskeletons, which contain essentially only spectrin, actin, 4.1, and 4.9. For this purpose, exposure of the cytoskeletal actin to EDTA was avoided, and 1 mM EGTA with 0.2 mM magnesium chloride was substituted for EDTA in the buffer described above, together with 0.1 mM ATP or ADP when required. The method employed was the luciferin-luciferase assay (40), using an LKB photometer (Luminometer 1250, LKB, Bromma, Sweden) and standard reagent kit. The ADP was determined, using an ATP-regenerating enzyme system (40). To remove unbound nucleotides, we centrifuged the cytoskeletons through a column of 30% sucrose containing hypertonic salt, as described above, with or without 0.1 mM ATP or ADP. Care was exercised in removing the red supernatant, the walls of the tube being carefully washed down onto the sucrose cushion several times. The nucleotide was extracted quantitatively from the pellet derived from a known volume of packed cells by vortex-mixing for 1 min with 0.1 ml 1 M Tris buffer and 0.5 ml of 1 N sodium hydroxide to denature the protein. The suspension was rapidly neutralized with hydrochloric acid, centrifuged, and the ATP and ADP concentration of the supernatant assayed. Over the period of the extraction, no hydrolysis of ATP, either in the assay or in the buffer, could be detected.

Additional experiments were performed without maintaining the ATP level after lysis, in case the cytoskeletal actin should, in contrast with free F-actin (20), permit exchange of bound ADP for ATP on the time scale of the experiment. The cytoskeletons were prepared as described above and assayed for ATP and ADP, as was the supernatant.

Deoxyribonuclease Assay for Actin: For estimations of G-actin, the spectrophotometric DNAase I inhibition assay (32) was used with calf thymus DNA (Sigma Chemical Co.) as substrate. The DNAase I (Sigma type D-0751) was freed of proteases and other impurities by chromatography on hydroxyapatite

(41). The method was modified by incorporation of 1 mM calcium chloride, 0.1 M sodium chloride, and 0.01% PMSF throughout. Purified DNAase I was calibrated for inhibition, using G-actin, standardized spectrophotometrically (42) and corrected for any denatured fraction in terms of the change in intrinsic fluorescence on denaturation with EDTA, following Lehrer and Kerwar (43).

The DNAase assay procedure was adapted to take account of the large absorbance background generated by lysed cells, due mainly to hemoglobin and in part also to scattering. Accordingly, 1-mm path spectrophotometer cells were used. Packed erythrocytes were diluted with an equal volume of lysis buffer, containing Triton X-100 (32); 30 μl of the resulting lysate were pipetted, together with 10 μl of DNAase at 80 μg/ml to form a mixed drop, onto the wall of a plastic centrifuge tube, containing 3 ml of DNA, equilibrated at 30°C. The reaction was initiated by rapidly vortex-mixing the contents of the tube, which were immediately transferred to a 1-mm cell, equilibrated at 30°C in the thermostatted cell-housing of the spectrophotometer (Perkin Elmer Coleman 575). With DNA solution in the reference channel, the absorbance increase was observed at 260 nm, with an absorbance range of 0.02 full scale. In our conditions a total of 0.5 μg of actin brought about some 50% inhibition of activity, in good accord with expectation. Membrane-free hemolysates were prepared by centrifugation of the lysed cells, prepared as described above, for 15 min at 80,000 *g*, and analyzed for DNAase inhibition as before. Gel electrophoresis was used to demonstrate that the lysates contained no membrane material. With the use of the expanded absorbance scale and recorder zero suppression in the assay, it proved possible to measure slopes (dA/dT) of less than 10^{-3} s^{-1} with a precision of some 5%. Actin concentrations were referred to hemoglobin, determined spectrophotometrically in the lysate after assay.

To determine the concentration of dissociated subunits of F-actin, we used the procedure of Blikstad et al. (32); the ghosts were incubated for 5 min on ice in a medium containing 0.75 M guanidine hydrochloride, 0.5 M sodium acetate, 10 mM Tris, 0.5 mM calcium chloride, 0.5 mM ATP, pH 7.0, and then diluted into the assay mixture as before. To liberate additional tightly bound actin, tryptic digestions were performed; trypsin was added to a concentration of 2.5 μg/ml to cells dispersed in the lysis medium containing Triton X-100, in the presence of the DNAase. After 5 min at 20°C the reaction was stopped by addition of soyabean trypsin inhibitor to 25 μg/ml. The incubation with guanidine hydrochloride was then carried out as before. To ensure that spectrin and 4.1 but not the actin had been degraded, we made aliquots 1% in SDS and examined them by gel electrophoresis. The actin is relatively resistant to trypsin under the above conditions even without DNase, but in its presence it is completely protected. The DNase activity is not affected by trypsin under the above conditions. DNase assays were referred in all cases to controls with no actin.

To examine the effect of blocking filament ends, we added cytochalasin E (Sigma Chemical Co.) in dimethylsulphoxide to the erythrocytes to a concentration of 0.1 μM and allowed them to equilibrate for some hours. The cells were then washed in phosphate-buffered isotonic saline and examined as before.

Nucleation of Actin Polymerization: Pyrene-labeled G-actin was prepared following Kouyama and Mihashi (33). Nucleation was observed by adding the fluorescent actin to ghosts in a fluorescence micro-cell, and following the rise in fluorescence intensity at 408 nm (excitation, 366 nm) with time, using a Perkin Elmer Hitachi MPF 3L spectrofluorometer. Absorbances at the excitation wavelength were well below 0.05. The ionic conditions were selected to give infinitesimally slow polymerization in the absence of the pre-existing nuclei, *viz.* 15 μg/ml actin diluted from a stock solution into a medium designed to give a final composition of 75 mM potassium chloride, 20 mM Tris, 0.2 mM ATP, 0.2 mM dithiothreitol, 5 mM magnesium chloride, 0.2 mM calcium chloride, 1 mM sodium azide, pH 8.2 at 30°C. The critical monomer concentration under these circumstances is 10–15 μg/ml. Cytochalasin E was added in the form of a concentrated stock solution in dimethylsulphoxide. An identical volume of the solvent (1% vol/vol) was added to controls. Measurements of nucleated polymerization were made after allowing the system to equilibrate for at least 2 h.

RESULTS

Actin Content of the Erythrocyte

Calibrations of staining intensity with Coomassie Brilliant Blue R as a function of the mass of protein applied to the electrophoretic gel were set up. Estimations of unknowns were confined to the linear range of dye uptake. The color response for actin was found to be greater than that of the spectrin subunits by factor of ~1.8 (Fig. 1). The tacit assumption in earlier work that color values for all proteins were effectively the same would lead to a large underestimate in the actin: spectrin ratio. On the other hand, washing ghosts after lysis with hypotonic buffer causes a small loss of both actin and

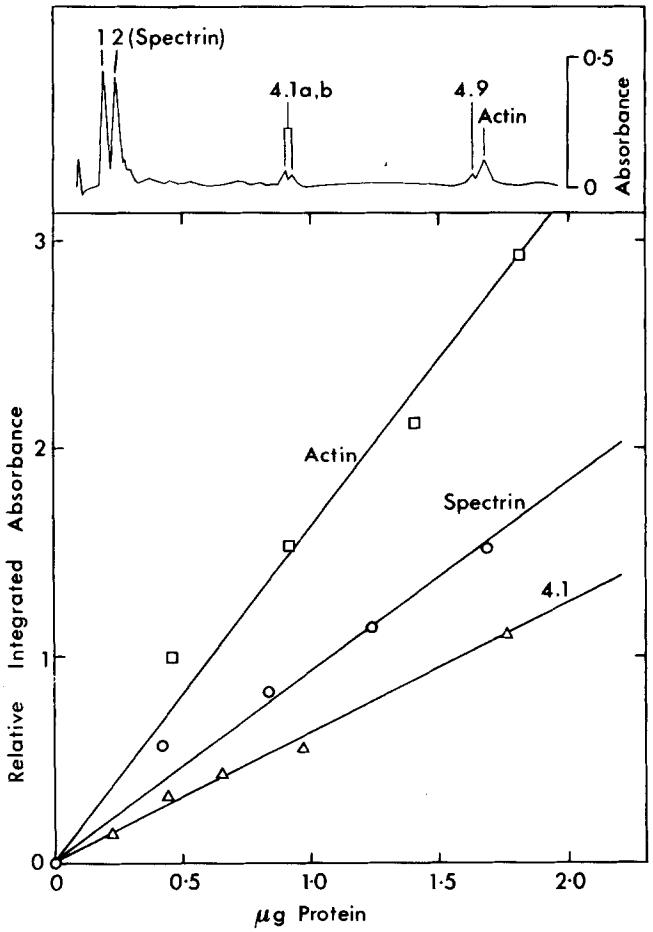

FIGURE 1 Determination of relative concentrations of spectrin, actin, and 4.1. The plots represent typical data for the color response of the three purified proteins after gel electrophoresis in the presence of SDS, staining with Coomassie Brilliant Blue, densitometry and integration. Concentrations of the protein standards were determined by nitrogen analysis. A series of such standards was run on the same gel as the unknown for each determination. The upper panel shows a densitometer trace of the electrophoretic pattern derived from cytoskeletons isolated by extraction of erythrocyte membranes with Triton X-100 and high ionic strength (5), as described in the text. In analyses of whole ghosts the 4.1 cannot be accurately determined because of incomplete resolution from the leading edge of the broad band 3 zone.

spectrin, but that of actin is greater. Elimination of the bulk of the hemoglobin is inescapable in the quantitation of actin, for otherwise at the concentration of ghosts required for a satisfactory analysis by densitometry there is gross distortion of the electrophoretic patterns and trailing of globin in the actin region. Based on a hemoglobin content of 33.5 pg per cell (44), the amount of spectrin dimer emerged as 2.0×10^5 molecules per cell. This is in good agreement with earlier estimations (45, 46). The corresponding number of molecules of actin per cell was found to be 5.0×10^5 (270 μg/ml of packed cells). After four washes with ice-cold 5 mM Tris, pH 7.8, the actin:spectrin dimer ratio fell from 2.5 to 2.0. No reliable estimations of 4.1 in intact membranes could be made because of incomplete resolution from the leading region of the broad band 3 zone. In isolated cytoskeletons (5), which give a simple electrophoretic pattern with much lower background staining, so that the areas under the peaks in densitometer traces can be integrated with improved precision, the ratio of actin to spectrin dimer is

also 2.0:1. In this complex the 4.1 can be estimated, and was found to be roughly equimolar with the spectrin dimer. It may be remarked that the color response of 4.1 under our conditions of staining was invariably lower than that of spectrin by a factor of >2 (Fig. 1). The differences in color response between the different proteins is not in fact exceptional: considerable differences have, for example, been reported between the major myofibrillar proteins (47).

The validity of the estimations additionally depends on the absence of contaminants co-migrating with the main components in the electrophoresis. Two-dimensional SDS-electrophoresis and isoelectric focusing of the total membrane protein revealed the presence of a single minor component with the same electrophoretic mobility as actin. This persisted in the purified cytoskeletons, from which all other proteins but the major structural constituents had been removed. It seems likely that it represents a modified form of actin. If it is unrelated protein it would lead to a small overestimate in the densitometric value for total actin concentration.

Identity of the Bound Nucleotide

To determine the identity of the actin-associated nucleotide, we prepared cytoskeletons, containing only actin, spectrin, 4.1, and a small amount of 4.9, by a procedure similar to that of Sheetz (5). After separation of unbound nucleotide by centrifugation through the sucrose-containing barrier, as described, the nucleotide released on adding alkali to the pellets was determined by the luciferin-luciferase assay. The simultaneous determination of ATP and ADP showed vanishingly low ATP levels in the cytoskeleton pellet. The recovery of ADP in the pellet was 35–40%; thus, more than half of the nucleotide had been dissociated from the actin in the wash. We cannot formally exclude that an appreciable part of the actin had been associated with ATP; however, analysis of the sucrose-containing supernatant, through which the cytoskeletons had been centrifuged, established that it contained an amount of nucleotide almost equal to the deficit in the cytoskeletal actin. This was at least 80% ADP. In the original hemolysate, by contrast, the nucleotide was ~90% ATP. It thus seems likely that the ADP in the supernatant was indeed the nucleotide lost by the actin. The results disagree with the inference from earlier work (8), in which the nucleotide in the high-molecular weight complex extracted from the membrane was examined chromatographically and reported to be ATP. Our conclusion that the cytoskeleton-associated nucleotide is predominantly ADP is in accord with the otherwise unanimous evidence that the actin is in a polymerized state. Unlike F-actin, however, (2), at least a sizable fraction of the cytoskeletal actin appears readily to lose its nucleotide.

Examination of the State of the Actin by Deoxyribonuclease Assay

To determine whether monomeric actin is present in the cell (that is to say, whether by implication the actin-containing complexes are in equilibrium with monomers), we performed DNAase assays on lysates generated by addition of washed cells to the detergent-containing medium (32). Notwithstanding the very exacting photometric requirements, (absorbance changes in the linear phase of <0.01 against a background of considerable absorption and scattering), it proved possible to attain surprisingly good reproducibility and precision. A typical time course is shown in Fig. 2. That there is no effect of hemoglobin or other solutes present in the lysate on DNAase activity or on the availability for binding of free monomeric actin was shown by adding comparable amounts of extraneous (muscle) actin to the hemolysate; the DNAase assay recorded the correct value for the incremental monomeric actin concentration. Moreover, after incubation of the lysate with EDTA to denature the G-actin (43) no inhibition was recorded. The apparent concentration of endogenous monomer was found to be 19(±6) μg/ml of packed cells. This determination is suspect, however, for the following reason: it has been found (48) that DNAase binds with high affinity and no inhibition of activity to the fast-growing ends of F-actin filaments. This becomes important only in relation to systems in which filament ends are abundant compared with monomer; the erythrocyte cytoskeleton exemplifies such a case. To avoid sequestration of an important part of the DNAase by the protofilaments in the membrane, the latter was removed by centrifugation before assay. (This was not done in the experiments that were the subject of a brief preliminary report (49)). The G-actin concentration in the membrane-free cytosol was determined to be 16(±7) μg/ml. This value was independent of the lysis procedure used, including hypotonic buffer, freezing and thawing, saponin, and exposure to Triton X-100 as already described. When the erythrocytes were equilibrated overnight at 4°C with cytochalasin E, to block the fast-growing ends of the filaments, the concentration of monomeric actin in the membrane-free cytosol remained essentially unchanged at 15(±5) μg/ml.

To determine whether the equilibrium with free actin monomer was re-established after removal of the cytosol, we resuspended washed ghosts, equilibrated for 4 h at room temperature with a volume of buffer equal to that of the original packed cells, which were then again centrifuged. The supernatant contained only a small amount of monomeric actin, typically 3 μg/ml, by DNAase assay, indicating that the protofilaments were incapable of restoring the original equilibrium monomer pool.

After exposing the ghosts to guanidine hydrochloride at a concentration (0.75 M) known to dissociate but not denature F-actin (32), the amount of actin monomer rose to 176 (±47) μg/ml of packed cells. This did not change with time up to 30 min in the dissociating medium and evidently represents the total dissociable fraction of the erythrocyte actin. The value is still well below that corresponding to the stoichiometry estimated from gel electrophoresis. The data are summarized in Table I.

The inference from the actin assays following dissociation is that a proportion of the actin is refractory to dissociating media because it is strongly sequestered as a complex with the other cytoskeletal proteins. This is supported by assays on solutions of the column-purified oligomer fraction, comprising spectrin, actin, 4.1 and 4.9: no detectable monomeric actin whatever was present in this system. Moreover, the oligomer was not to any major extent dissociated by DNAase and it eluted from the Sepharose column in the same position after incubation with the enzyme; its composition was also unchanged as judged by gel electrophoresis, and the DNAase remained fully active even after prolonged incubation. This is contrary to the report by Lin (50). We have observed extensive degradation of the cytoskeletal proteins when exposed to DNAase that has not been purified with hydroxyapatite and, therefore, contains proteases.

To obtain further evidence for our conclusion that a major part of the cytoskeletal actin is sequestered in the form of a complex, resistant to dissociating conditions, we have taken advantage of the high lability of 4.1 and spectrin to proteolysis,

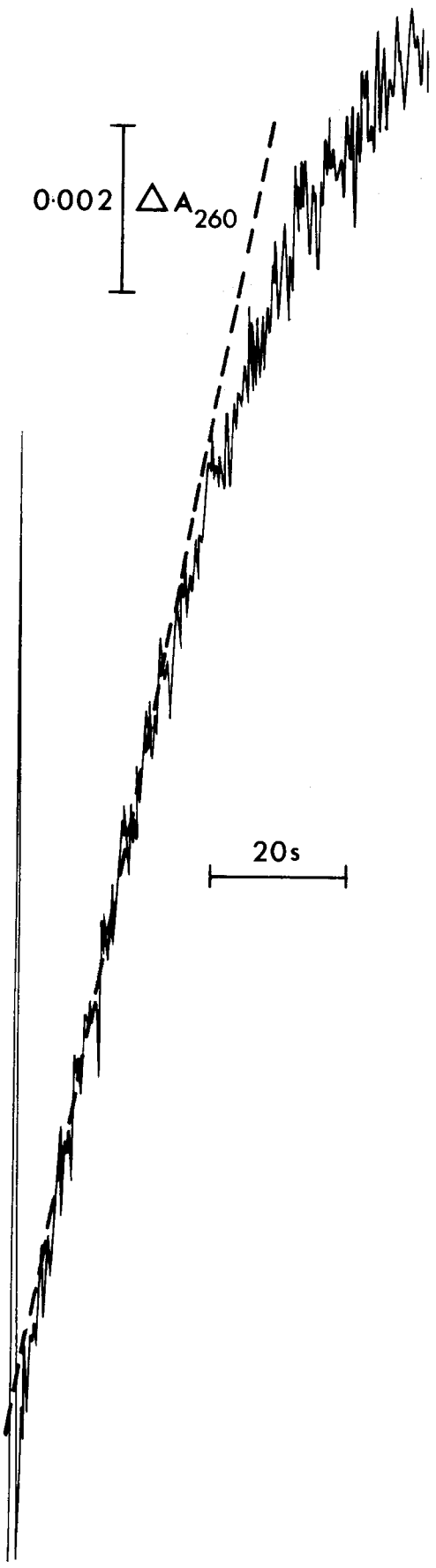

FIGURE 2 Typical assay for monomeric actin in whole erythrocytes. The cells were diluted, as described, with a lysis medium containing Triton X-100. The lysed cells were then mixed with deoxyribonuclease I and rapidly introduced into the assay medium containing DNA. The optical path was 1 mm and the ordinate corresponds to a full-scale absorbance of 0.02. The activity is obtained from the linear part of the curve, indicated by the broken line, and related to a corresponding rate of change of absorbance at 260 nm for the reaction in the absence of inhibitor.

TABLE I

States of Complexation of Actin in the Erythrocyte

	Actin (μg/ml packed cells)	Standard error	*n*	Number of subunits per protofilament§
Free monomer	16*	7	19	1
Free monomer after treatment with 0.2 μM cytochalasin E	15*	5	21	1
Dissociable with guanidine hydrochloride	176*	47	65	9
Dissociable with guanidine hydrochloride only after trypsin treatment	251*	66	16	13
Total actin	270‡	39	6	15 (±2)

* Determined by assay with DNAase I (32) at 30°C in lysis medium at physiological ionic strength

‡ Determined by gel electrophoresis, staining and densitometry (see text).

§ Number-average of subunits in given state.

compared with the considerable resistance of actin in both the F- and G-states. With trypsin, enzyme concentrations can readily be found that bring about complete degradation of 4.1 and fragmentation of spectrin and leave the actin totally unaffected, when DNAase is present to provide additional stabilization (Fig. 3). The treatment with guanidine hydrochloride, followed by DNAase assay, was repeated for ghosts exposed to trypsin under these conditions. The amount of actin detected rose to 250 μg/ml packed cells, which is much closer to the total actin content of the cell. If we can rely on the stoichiometry determined by gel electrophoresis, a minor part of the actin remains unavailable even after trypsin treatment. In the case of the purified oligomer it is striking that even after successive treatment with trypsin and 0.75 M guanidine hydrochloride no monomeric actin could be detected by the DNAase assay, even though, as gel electrophoresis showed, the actin remained intact.

Nucleation of G-Actin Polymerization by Ghosts and Isolated Spectrin-containing Oligomers

In accordance with earlier observations (14), Fig. 4 shows that the actin nuclei present in ghosts will stimulate the polymerization of G-actin. This process was followed by means of the increase in fluorescence intensity of pyrenyl G-actin (33) added to the ghost suspension at a concentration at which it undergoes no spontaneous polymerization on the time scale of the experiment. Cytochalasin E was used to investigate whether the slowly growing ends of the protofilaments in the membrane are free or blocked: at sufficiently high ionic strength and in the presence of magnesium ions, actin will undergo bidirectional growth, the ratio of rates of elongation at the fast and slowly growing ends being about 4:1 (51). Thus, F-actin alone should display a residual rate of extension of corresponding magnitude after incubation with excess cytochalasin E to block the fast-growing end. As Fig. 4 shows, this is indeed observed.

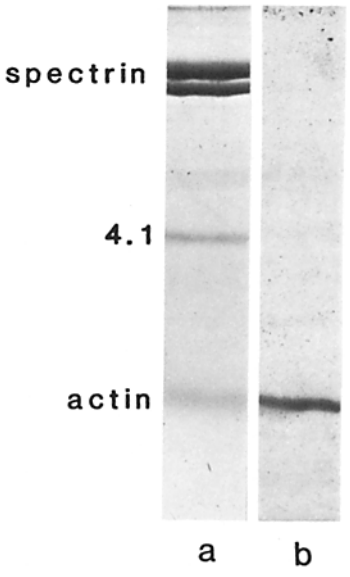

FIGURE 3 Gel electrophoresis in the presence of SDS of a typical cytoskeletal complex preparation, containing spectrin, actin, and 4.1, before (*a*) and after (*b*) treatment with trypsin in the presence of deoxyribonuclease I under conditions described in the text. Note that only actin survives intact.

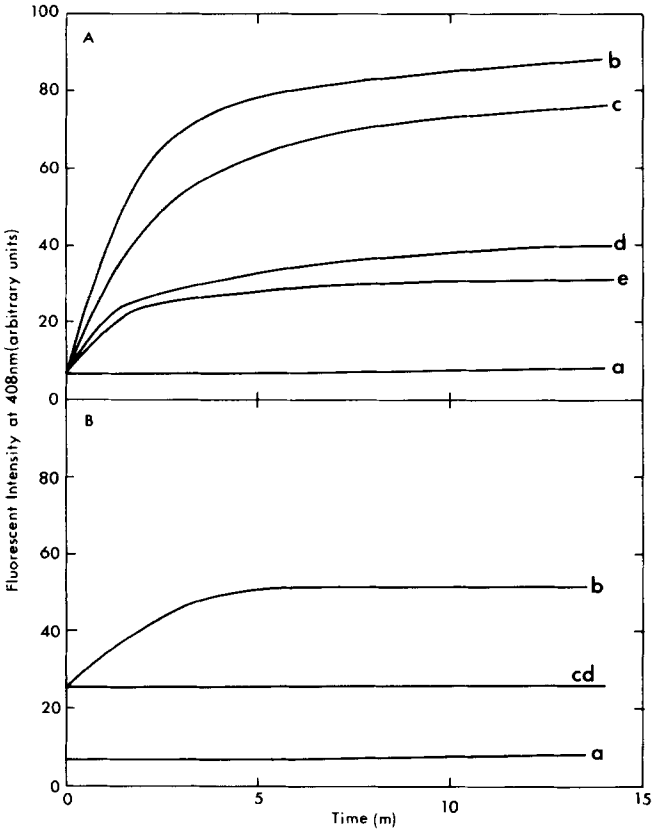

FIGURE 4 Nucleated polymerization of pyrene-labeled G-actin by F-actin (*A*) and erythrocyte ghosts (*B*). The fluorescent G-actin was added to samples equilibrated with the appropriate concentration of cytochalasin E in the fluorimeter cell at 30°C. Polymerization was observed in terms of the increase in fluorescence at 408 nm from the pyrene chromophore excited at 366 nm. (*A*) *a*, pyrene-labeled G-actin (15 μg/ml) alone; *b*, the same with added F-actin at 0.34 mg/ml; *c* as *b* but equilibrated with 0.02 μM cytochalasin E; *d* with 0.2 μM and *e* with 2 μM cytochalasin E. (*B*) *a*, pyrene-labeled G-actin (15 μg/ml) alone; *b*, the same containing 20 μl packed ghosts per ml; *c* as *b* but with 0.2, 2, or 20 μM added cytochalasin E. In *B* the ghosts gave rise to a background of Rayleigh scattering and a filter with a cut-off at 390 nm was introduced on the emission side. Note total inhibition of nucleated polymerization onto ghosts by the cytochalasin, and incomplete inhibition of nucleation with F-actin.

In these experiments the actin and cytochalasin were incubated together at 30°C for at least 2 h before addition of pyrenyl-G-actin for the nucleation assay; during this time a small degree of depolymerization occurs as the critical monomer concentration rises to the level characteristic of filaments blocked at their fast growing ends. When ghosts were similarly treated the nucleating activity was totally eliminated. That saturation of the fast-growing ends in the membranes had been attained was shown by the effect of cytochalasin concentration (Fig. 4); literature data (52) confirm that saturation is indeed to be expected in the cytochalasin concentration range used in these experiments. The results may be interpreted as showing that in the membrane the slowly-growing ends of the actin protofilaments are either blocked or sterically occluded.

DISCUSSION

The results presented above, taken together with earlier work, allow us to draw the following conclusions about the actin of the erythrocyte. (*a*) Since the earlier evidence (8), suggesting that the actin-associated nucleotide in extracted cytoskeletal complex was ATP, is not applicable to the cytoskeleton as a whole, in which the nucleotide is evidently wholly or predominantly ADP, all criteria now agree in indicating that the actin is filamentous. (*b*) The actin is of the β-type, characteristic of most cytoplasmic actins (53). This conclusion is based on two-dimensional gel electrophoretic analysis (7). There have been two reports that have asserted that a mixture of β and γ actins is present (54, 55). However, no resolution into two spots can be discerned in the electrophoretic separation shown in either case. When a comparison is made with autoradiograms of labeled β and γ actins from brain, the erythrocyte actin coincides precisely with the β zone (7). (*c*) The actin is capable of phosphorylation in vitro by an endogenous cAMP-dependent kinase (56), but the extent of phosphorylation in vivo and its functional consequences are not yet clear. (*d*) The filaments coexist with a monomer pool, but the monomer concentration at 0°C, determined by DNAase assay, is almost certainly less than the critical concentration at the same temperature, considering especially that nonmuscle actins appear to have higher critical concentration than skeletal actin (57). However, the critical concentration at physiological temperature is much lower. Data for the temperature-dependence of this concentration are available, though only for a low-salt high-magnesium solvent (57). They adhere to the form of a van't Hoff plot (58, 20), and, if we assume a similar temperature dependence under other solvent conditions, we may estimate the critical concentration at physiological ionic strength and temperature to be ~10 μg/ml. Thus, the monomer pool is probably within the range of concentration that the protofilament ends are capable of generating under these circumstances. (*e*) The unchanged monomer concentration and the total suppression of nucleating activity when cytochalasin E is added to ghosts strongly suggest that the slow-growth end is unavailable. This agrees with the conclusions of Brenner and Korn (16), working with sheep erythrocytes. The slow-growth end may be blocked by a capping protein, perhaps protein 4.9, or by an interaction with spectrin and 4.1, such as evidently readily occurs at the ends of actin filaments (12), or indeed it may merely be sterically obstructed by the proximity of the bilayer. (*f*) The monomer pool cannot be indefinitely replenished. This suggests that the ends of the "protofilaments" can be eroded only down to some point at which there is a stable complex with other proteins. (*g*) The dissociating agent, guanidine hydrochloride, renders

only a part of the actin available for assay. The cytoskeletal complexes, which we take to involve ternary association of spectrin, 4.1, and actin at or near the ends of the spectrin tetramers, are therefore more stable than F-actin alone. Most of the subunits become available when the spectrin and 4.1 are degraded with trypsin. It is possible that a fraction remains sequestered even after proteolysis and treatment with guanidine hydrochloride. This conclusion must remain tentative, for it rests on the evidence of a small difference between two rather large numbers, the actin concentrations determined by densitometry of electrophoretic gels and by DNAase assay. Some support for the existence of a small highly refractory fraction of actin comes from the properties of the stable oligomer fractions extracted with spectrin from the membrane, which nucleates actin polymerization, but retains its actin in unreactive form after proteolysis and exposure to dissociating agent.

If such elements indeed exist in the cytoskeleton, they presumably result from multiple, probably cooperative interactions (preferential association of spectrin and 4.1 with actin subunits adjacent to other spectrin-bound subunits) and are resistant to dissociation by guanidine hydrochloride and to access by trypsin. Cooperativity could come about from the apparently dimeric nature of 4.1 (17, 18, 59), which would favor clustering of spectrin molecules on the "protofilaments." Since the binding sites for 4.1 and actin are near the ends of the elongated spectrin molecules (60), they could remain functional after proteolytic truncation of the dimers. The distribution of the actin monomers between the different operationally defined populations is summarized in Table I.

We consider finally the implications of the above observations for the structure and stability of the cytoskeleton. We take it that the structural members of the network are spectrin tetramers and that the branch points consist of actin protofilaments and 4.1. The protofilaments are stabilized by the ternary complex (and possibly protein 4.9) and are prevented from disproportionating to fewer long filaments. That this would otherwise occur is indicated by the appearance of actin filaments in the membrane after exposure to a protease to destroy the associated proteins (2, 61). The number of branch points, assuming that each possesses one cytochalasin binding site, is 3–4 $\times$ 10^4 per cell (10). With our value for the total actin, this leads to a number-average degree of polymerization of the protofilaments of 12–17, (which differs from earlier estimates only by reason of our revised value for the total actin content of the cell). A constraint on the system is the attachment of the spectrin to effectively fixed binding sites in the membrane; these consist of protein 2.1 (ankyrin), which is present to the extent of one molecule per spectrin tetramer (45, 62). The distance between these receptors is apparently fixed, for the capacity of the membrane to bind spectrin dimers and tetramers is the same (63); two-point attachment of tetramers is therefore evidently precluded. The condensation of the cytoskeleton when it is removed from the membrane with elimination of this spatial constraint may allow cooperative clustering of spectrin molecules at some branch points at the expense of others. This may explain why a large part of the isolated complex fails to disperse in the cold at low ionic strength (7) (or a smaller part in the presence of high concentration of polyanions [30]), whereas spectrin together with actin and part of the 4.1 is liberated from intact membranes by low salt media.

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