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Spectrin-4.1-Actin Complex of the Human Erythrocyte: Molecular Basis of Its Ability to Bind Cytochalasins With High-Affinity and to Accelerate Actin Polymerization In Vitro

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The spectrin-4.1-actin complex isolated from the cytoskeleton of human erythrocyte [3] was found to be similar to muscle F-actin in several aspects: Both the complex and F-actin nucleate cytochalasin-sensitive actin polymerization; both bind dihydrocytochalasin B with similar binding constants; both can be depolymerized by DNase I with loss of cytochalasin binding activity. From these results, we conclude that the actin in the complex is in an oligomeric form. However, the presence of spectrin and band 4.1 in the complex not only stabilized the actin in the complex as evidenced by its resistance to depolymerization in low-ionic-strength conditions and to DNase I as compared with F-actin, but also altered the characteristics of the binding site(s) for cytochalasins believed to be located at the "barbed" (polymerizing) end of the oligomeric actin.

Key words: spectrin, actin, erythrocyte, cytochalasin, DNase I, actin polymerization

The structure of the human erythrocyte cytoskeleton and its membrane association has been the subject of intensive study in the past few years. As a result, a comprehensible picutre is beginning to emerge. The cytoskeletal proteins can be easily extracted into buffer of low ionic strength [1]. In the search for the location of high-affinity cytochalasin B (CB) binding sites of the human erythrocyte membrane that are not involved in sugar transport, we found that the extracted cytoskeletal proteins bound cytochalasins B, D, and E (CB, CD, CE) and dihydrocytochalasin B (H_2CB) with high affinity [2]. Later, by means of sucrose gradient centrifugation [3] and Sepharose 4B column chromatography [4], we traced this cytochalasin binding activity to a high molecular weight complex comprising spectrin, actin, and band 4.1. In contrast to the suggestion by Pinder et al [5, 6], we found that this complex, and not pure spectrin, was able to accelerate actin polymerization under ionic conditions that allow only a minimal rate of actin polymerization. Moreover, low concentrations (submicromolar) of CB, CD, CE, and H_2CB inhibited

Abbreviations used: Buffer A: 5mM Tris-HCl, pH 8.0, 0.2mM ATP, 0.2mM $CaCl_2$, 0.5mM 2-mercaptoethanol; DTT: dithiothreitol; SDS: Sodium dodecylsulfate; Cytochalasin B: CB; dihydrocytochalasin B: H_2CB; cytochalasin D: CD; cytochalasin E: CE.

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the ability of this complex to induce actin polymerization; the relative potencies of the drugs in inhibiting this process corresponded to their affinities for the complex. A complex with similar ability to accelerate actin polymerization isolated from sheep erythrocyte has been reported by Brenner and Korn [7, 8]. In addition, these authors also showed that the spectrin dimer and tetramer, irrespective of their state of phosphorylation, did not accelerate actin polymerization. Furthermore, polymerization of actin in the presence of the spectrin-actin complex from sheep erythrocyte was also inhibited by cytochalasins. These initial discoveries generated a lot of interest in the study of the nature of the actin polymerization process in general, and the mechanism of inhibition by the cytochalasins in particular. From work done in several laboratories, it is presently clear that cytochalasins bind to the polymerizing end of actin filaments and inhibit actin polymerization by blocking monomer addition to that end [9–13].

The present report describes the results of a study on the human erythrocyte spectrin-4.1-actin complex with respect to the site of cytochalasin binding and the state of actin in the complex. The data presented here are consistent with previous suggestions that the complex contains actin in a short, oligomeric form and that it accelerates actin polymerization by a simple nucleation mechanism [8, 14, 15].

MATERIALS AND METHODS

CB, CD, and CE were purchased from Aldrich; ^{3}H-CB, prepared by the method of Lin et al [16], was obtained from New England Nuclear; and ^{3}H-labeled and unlabeled H_2CB were prepared from the corresponding forms of CB by reduction with $NaBH_4$ as described [2]. Deoxyribonuclease from pancreas (DNase I, code: D) was purchased from Worthington Biochemical Corporation. Human erythrocytes were from blood generously donated by the Baltimore Red Cross Blood Center (used within two weeks after the blood was drawn). Muscle actin was prepared from rabbit back and leg muscle according to the procedure of Spudich and Watt [17].

Low-ionic strength extract of human erythrocyte ghosts was prepared by extraction at 37°C as previously described [3]. Spectrin-4.1-actin complex was separated from spectrin dimer and other minor proteins by chromatography on a Sepharose 4B column (1.5 × 90 cm) in a buffer containing 0.1 M KCl, 1 mM dithiothreitol, and 5 mM sodium phosphate, pH 8.0. The pooled fractions were dialyzed against two changes of buffer A, stored on ice, and used within 48 h. Muscle actin, low ionic-strength extract of erythrocyte ghosts, and purified spectrin-4.1-actin complex were all quantified by the Hartree modification of the Lowry method [18]. Electrophoresis on SDS-acrylamide gels were performed as previously described [3].

Cytochalasin binding was measured with the isoelectric precipitation assay [19]. Binding data were presented as "bound/free concentration" since this parameter was found to give a better correlation with the number of binding sites than the parameter "bound" [19].

Viscosity was measured as described [3]. Samples were prepared by mixing aliquots of muscle G-actin (at 25°C) with aliquots of spectrin-4.1-actin complex in buffer A (also pre-equilibrated at 25°C) to give a total volume of 0.5 ml. At zero time, 10 μl of 20 mM $MgCl_2$ was introduced into the sample, and the mixture was immediately transferred into an Ostwald-type viscometer and the flow time measured. Data were presented as specific viscosity (η_{sp}), which is calculated as:

$$\frac{\text{flow time of sample} - \text{flow time of buffer}}{\text{flow time of buffer}}$$

RESULTS

Molecular Properties of the Complex

Proteins in the low-ionic-strength extract of erythrocyte ghost membrane can be conveniently fractionated into several aggregation states by means of gel filtration on agarose columns. Consistent with results reported earlier [3, 4], when the extract was chromatographed on a Sepharose 4B column, a high-molecular-weight complex consisting of spectrin, actin, and band 4.1 was eluted at the void volume, followed by a broad peak of spectrin with a trailing shoulder containing actin and other minor components (data not shown). Binding experiments showed that high-affinity binding sites for ^{3}H-H_2CB were located exclusively in the void volume. Scatchard plot analysis of the binding data obtained with the isoelectric precipitation assay at pH 5 [19] indicated that the dissociation constant for binding was about 2–4 nM, and that there were 0.3–0.6 nmol of binding sites per mg of protein. Binding measured with the equilibrium dialysis method [2] at pH 8.0 gave a dissociation constant of 40–60 nM. The cytochalasin binding fractions eluting at the void volume of the Sepharose 4B column were the only ones capable of accelerating actin polymerization in a low-ionic-strength medium containing 0.4 mM $MgCl_2$.

Because of the possibility of the presence of aggregated material other than spectrin-4.1-actin complex in the void-volume fraction from the Sepharose 4B column, the low-ionic-strength extract was chromatographed in a Sepharose 2B column. The complex, as indicated by cytochalasin binding activity, was eluted in the included volume, just behind the void and ahead of spectrin dimer (data not shown). Densitometric analysis of SDS acrylamide electrophoretic gels of the cytochalasin binding fractions and Scatchard analysis of the binding data indicated that there are approximately 1 mole of spectrin tetramer, 0.5 mole of band 4.1, and 4 moles of actin per mole of high-affinity cytochalasin binding site.

High-Affinity Binding of Cytochalasins to F-Actin and to the Complex

Recent reports have shown that purified muscle F-actin contains high-affinity binding sites for ^{3}H-CB [9, 10]. Available evidence suggests that these binding sites are on the actin molecules located at the polymerizing ends of the filaments. The dissociation constants for this binding (5 nM at pH 5 and 30 nM at pH 8) are similar to those determined with the spectrin-4.1-actin complex. G-actin, in a nonpolymerizing buffer (buffer A), on the other hand, was shown to contain negligible amounts of high-affinity binding sites. In a polymerizing buffer, eg, buffer A + 2 mM $MgCl_2$; however, G-actin may assume a different conformation [20], namely, one that is similar to that of actin molecules at the ends of filaments and would therefore bind cytochalasin with high affinity. To test this possibility, muscle actin was polymerized by the addition of 2 mM $MgCl_2$ at various protein concentrations until equilibrium has been reached (20 h at 25°C). Cytochalasin binding was measured at each actin concentration, and the number of binding sites estimated from Scatchard plots. As shown in Figure 1, the number of high-affinity cytochalasin binding sites was a linear function of actin concentration as expected. However, the line drawn through the data points did not go through zero, but rather intercepted the abscissa at actin concentration of ~ 0.05 mg/ml.

The critical monomer concentration below which muscle actin remains in the monomeric form has previously been determined to be ~ 0.05 mg/ml (eg, [7]). These results, therefore, suggest that G-actin, under the same set of ionic conditions, ie, 2 mM $MgCl_2$, but below critical concentration for polymerization does not bind cytochalasin with high affinity.

The substrate specificity of the high-affinity cytochalasin binding sites associated with muscle F-actin has previously been determined in competitive displacement experiments; the relative affinity for this type of site is: CD > CE ≃ H_2CB [9]. The relative affinity of cytochalasins for binding sites located in erythrocyte complex was found to be similar, but not identical to that of sites associated with F-actin, ie, CE > CD >> H_2CB (data not shown). This difference in substrate specificity is a real one; similar displacement curves have been obtained with many different preparations of the complex, using the isoelectric precipitation assay at pH 5 as well as the equilibrium dialysis assay at pH 8.0.

Purified spectrin has been shown not to contain cytochalasin binding activity [3]. Band 4.1 does not appear to bind cytochalasin since membrane residue extracted with low-ionic-strength medium contains little H_2CB binding activity [2], but retains most of the band 4.1 of the ghost membrane. The results described in this section suggest that the site of cytochalasin binding in the erythrocyte complex is, in all likelihood, located in the actin molecules of these structures and that such molecules are present in an oligomeric rather than monomeric form.

Effect of DNase I on the Complex

DNase I has previously been shown to depolymerize actin filaments by binding with high affinity to G-actin to form a 1:1 molar complex [21]. Sheetz [22] has shown that

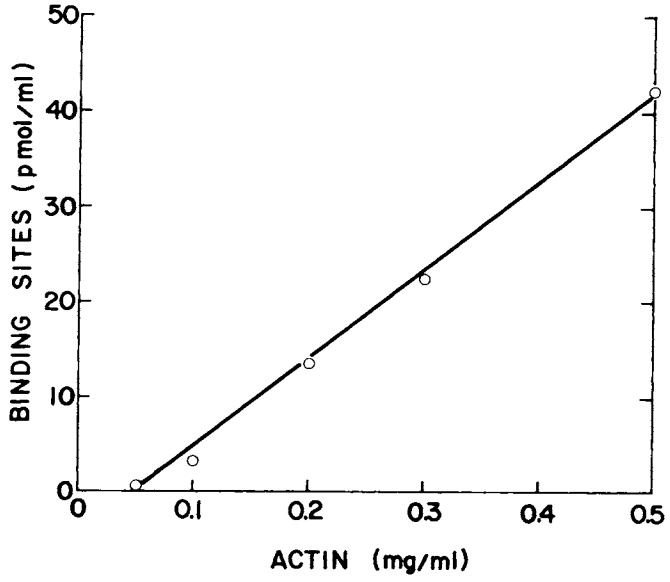

Fig. 1. Correlation between the number of cytochalasin binding sites and the amount of F-actin. Muscle G-actin, at the indicated concentrations, was polymerized by bringing the salt concentration to 0.1 M KCl and 2 mM $MgCl_2$ in buffer A and incubating for 20 h at 25°C. Cytochalasin binding was measured in 2–60 nM of ^{3}H-H_2CB. The number of binding sites was estimated from Scatchard plots of the binding data with each point on the graph representing one Scatchard plot.

addition of DNase I to the triton skeleton of erythrocyte membranes released a portion of erythrocyte actin and caused dissociation of the skeleton. In the following experiments, DNase I was used as a diagnostic tool to test the possibility that actin in the erythrocyte complex is in an oligomeric form as suggested by the cytochalasin binding studies described above.

When the low-ionic-strength extract of erythrocyte ghost was treated with DNase I, it gradually lost its cytochalasin binding activity. Fractionation of the DNase I-treated extract in a Sepharose 4B column showed that the cytochalasin binding complex that normally elutes at the void volume is no longer present in the extract (Fig. 2). The amount of DNase I required to inactivate cytochalasin binding in the extract showed approximate stoichiometry with the amount of actin in the extract (Fig. 3). SDS acrylamide gel electrophoresis of DNase I-treated extract did not reveal extra bands, indicating that the inhibitory action of the enzyme is not due to proteolytic activity contaminating the DNase I preparation.

The disappearance of the cytochalasin binding activity in the extract that resulted from DNase I treatment was also accompanied by the loss of its ability to accelerate actin polymerization in 0.4 mM $MgCl_2$. As shown in Figure 4, both types of activity in the extract showed corresponding decreases with time of exposure to stoichiometric levels of DNase I (with respect to the amount of actin in the complex). After 2.5 h of incubation with the enzyme, the extract had little effect on actin polymerization (Fig. 4a) and had few cytochalasin binding sites remaining (Fig. 4b). The effect of DNase I on actin polymerization

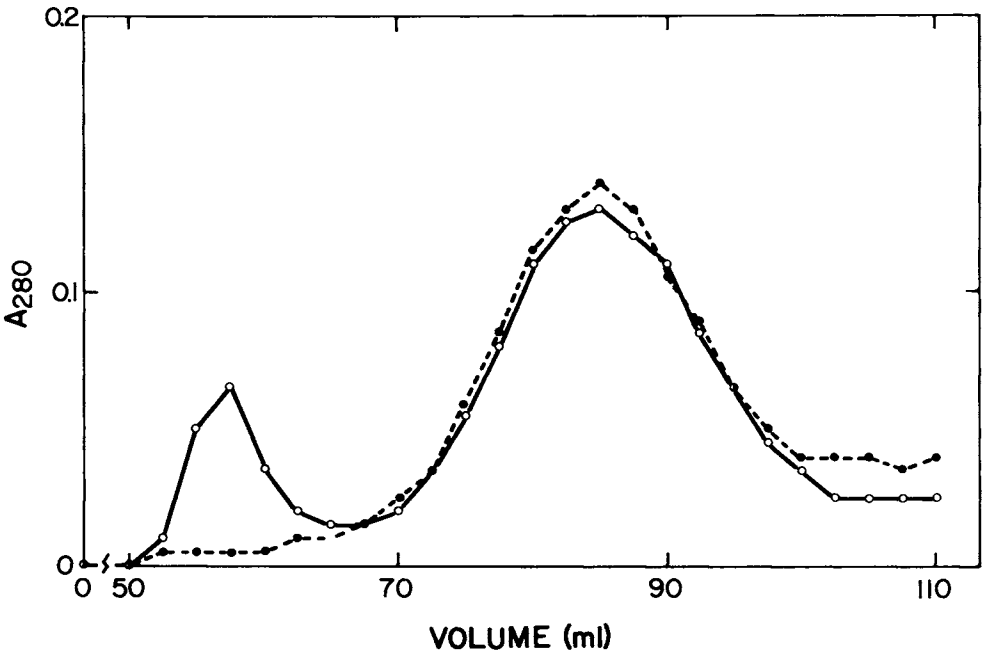

Fig. 2. Fractionation of DNase I-treated erythrocyte extract on Sepharose 4B. A 2.5 ml sample of the low-ionic-strength erythrocyte membrane extract containing 3.8 mg of protein was incubated with (•- - -•) or without (o——o) 1.1 mg of DNase I (molar ratio of DNase I to actin in the complex about 3.5:1) in 1 mM $MgCl_2$, 0.3 mM sodium phosphate, pH 8, and 1 mM DTT, at 4° C for 6 h. Both samples were fractionated on Sepharose 4B column as described in Materials and Methods.

was directed at the complex rather than G-actin since the level of DNase I used was sub-stoichiometric with respect to the latter. Furthermore, increasing the $MgCl_2$ concentration in the reaction mixture to 2 mM led to rapid polymerization of the actin, indicating that the G-actin had not formed complexes with DNase I and was free to undergo polymerization.

Stoichiometric levels of DNase I also caused a decrease in cytochalasin binding activity of F-actin in parallel with the decrease in the viscosity of the sample. This process, however, was much faster than the dissociation of the complex, being approximately 80% complete in 15 min (Fig. 5).

DISCUSSION

Recently, a number of laboratories have shown that actin filaments contain high-affinity binding sites for cytochalasin [9, 10, 12, 14, 23]. The experiments on purified muscle actin described in this paper have led us to conclude that the ability to bind cytochalasin with high affinity can be regarded as a distinctive feature of polymeric actin, a property not found in monomeric actin.

The search for the location of ^3H-H_2CB binding in the human red cell has led to the identification of a supramolecular complex containing actin as one of its major components [3]. Since such a complex contains high-affinity cytochalasin binding activity, it would be a logical conclusion that the actin in this structure is in a polymeric state and that the site of drug binding is associated with this protein. That the actin in the spectrin-

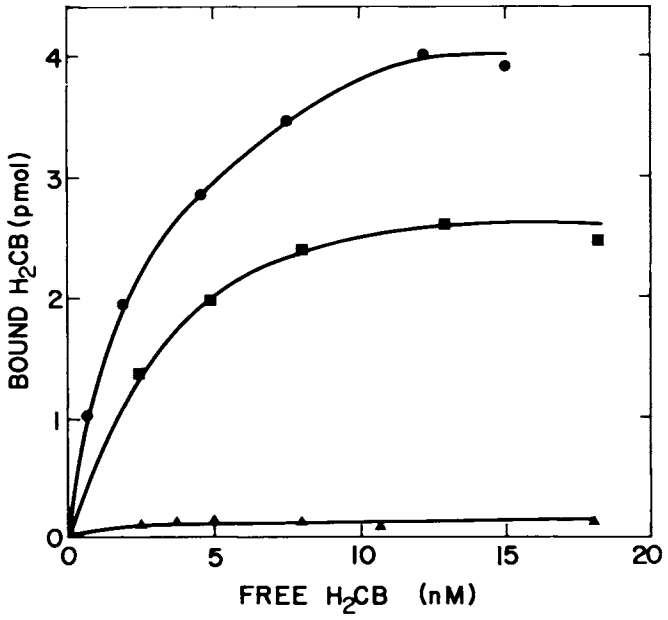

Fig. 3. Effect of DNase I on the binding of H_2CB to erythrocyte extract. Aliquots of the low-ionic-strength erythrocyte extract were incubated with DNase I at 0.033 mg/mg extract protein (a molar ratio of DNase I to actin of 0.4:1) (■), or 0.33 mg/mg extract protein (a molar ratio of DNase I to actin of 4:1) (▲), or without DNase I (●) for 20 h under the same conditions as described in Figure 2. H_2CB binding activity was assayed as described in Figure 1 using 42 μg of extract protein for each sample.

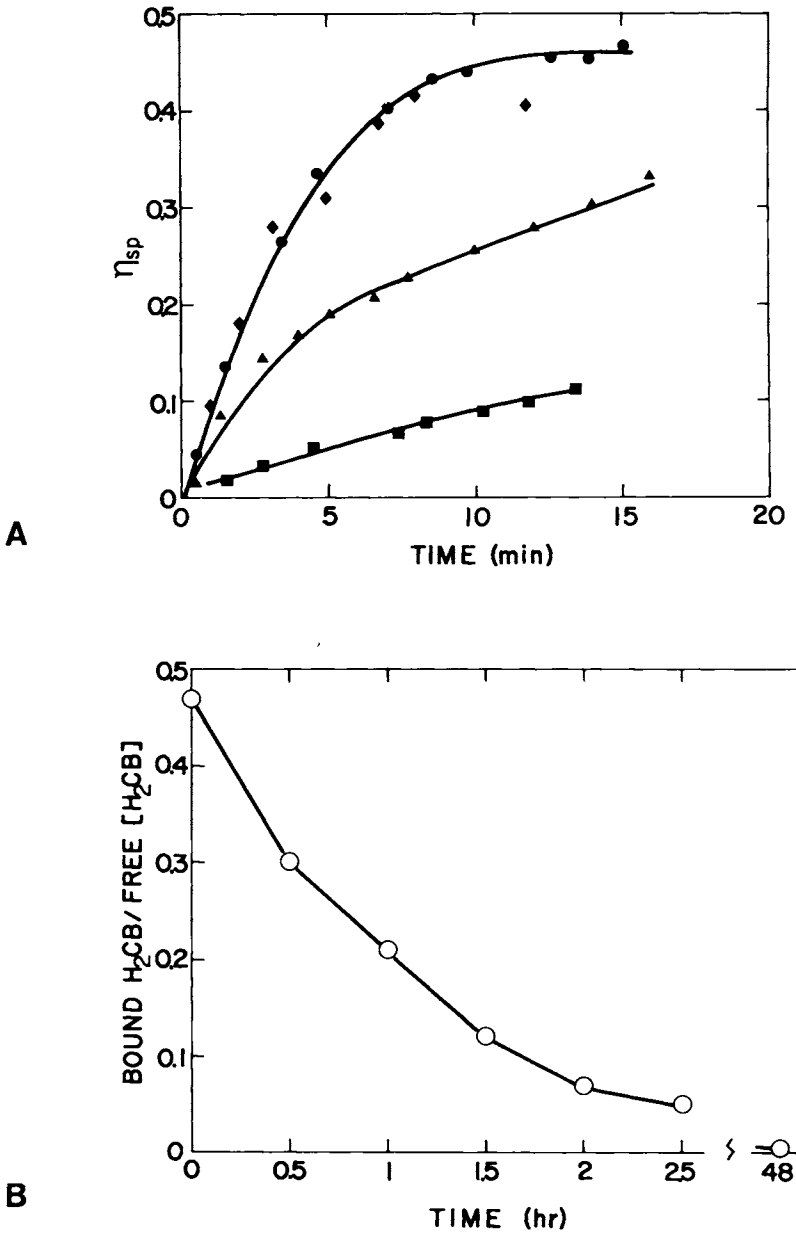

Fig. 4. Time course of the effect of DNase I on the ability to accelerate actin polymerization and to bind H_2CB by erythrocyte extract. A 0.3 mg sample of DNase I was added to 1 ml of extract containing 3.4 mg of protein (molar ratio of DNase I to actin about 1:1) in 1 mM $MgCl_2$, 0.3 mM sodium phosphate, pH 8, and 1 mM DTT and incubated at 25°C. (a) After incubation for 0 h (•), 0.5 h (♦), 1.5 h (▲), or 2.5 h (■), 50 μl of the mixture were added to 0.5 ml of muscle G-actin in buffer A with 0.4 mM $MgCl_2$ to initiate polymerization as described in Materials and Methods. The concentration of the muscle actin was 0.68 mg/ml. (b) Aliquots of 20 μl of the mixture were removed at the indicated times and added to 0.5 ml of muscle G-actin as in (a) except that it also contained 10 nM of ^3H-H_2CB. The binding was measured immediately as described in Figure 1.

4.1-actin complex is in an oligomeric rather than monomeric form has been suggested by several research groups previously. In particular, Ungewickell et al [24] was able to reconstitute the complex from purified tetrameric spectrin, 4.1, and F-actin. When G-actin was used in place of F, the complex did not form. Several lines of evidence presented here indicate that this actin is in close association with the spectrin and/or the band 4.1 protein in the complex. First, the complex is stable in low-ionic-strength medium, without divalent cation, a condition that would lead to rapid depolymerization of F-actin. Secondly, the actin in the complex is considerably more resistant to depolymerization induced by DNase I than is muscle F-actin. Finally, the cytochalasin binding site in the complex has a binding specificity that is similar but not identical to that of muscle F-actin. Therefore, it appears that the actin in the complex is in an oligomeric form, stabilized as well as influenced by association with spectrin and/or band 4.1 in a rather specific way.

The conclusion that the erythrocyte complex contains oligomeric actin provides a simple explanation for the previous observation that the complex can accelerate polymerization of actin under conditions where the reaction normally proceeds at a relatively slow rate [3]. The oligomeric actin in the complex simply provides a site for rapid addition of G-actin for filament formation. A similar conclusion has been drawn by Brenner and Korn [8]. The demonstration that the loss of cytochalasin binding activity in DNase I-

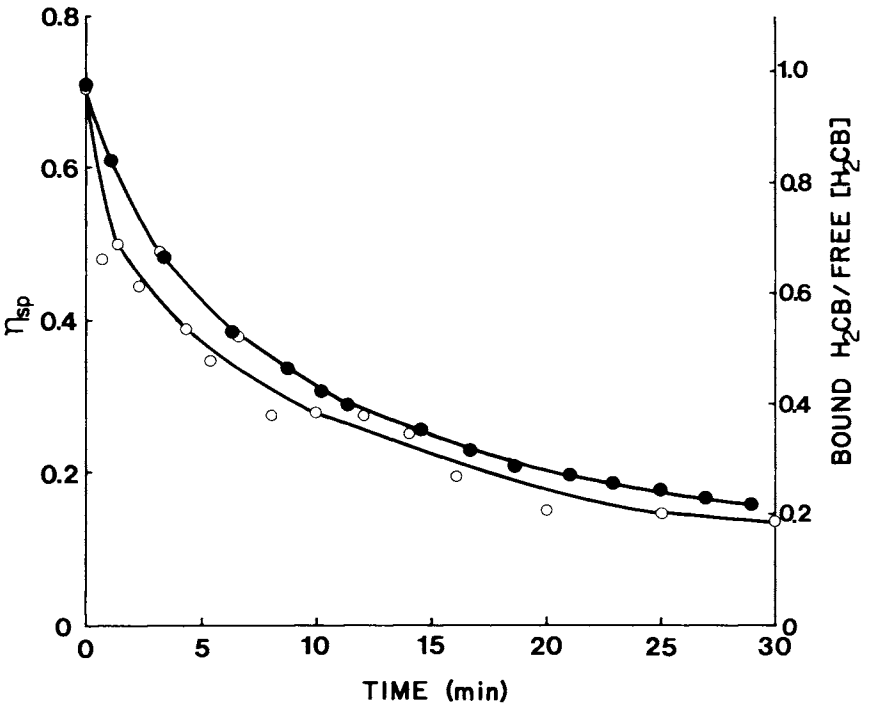

Fig. 5. Effect of DNase I on cytochalasin binding activity and viscosity of F-actin. DNase I was added to muscle F-actin (1 mg/ml, polymerized by addition of 2 mM $MgCl_2$) at 1:1 molar ratio. Both the viscosity (•) and the cytochalasin binding activity (○) of the sample were monitored as a function of time after the addition of DNase I. For cytochalasin binding, 0.12 ml aliquots of the mixture were removed at the indicated time and added to 0.2 ml of buffer A with 2 mM $MgCl_2$ containing ^3H-H_2CB and binding was measured as described in Figure 1.

treated extract is also accompanied by a decrease in ability to stimulate actin polymerization in 0.4 mM $MgCl_2$ provides further support for this view.

It is currently thought that actin monomers are preferentially added to one end and preferentially subtracted from the opposite end of an actin filament [11, 25]. Using heavy-meromyosin-decoration as a marker, the "barbed" end of the filament has been implicated as the preferred end for monomer addition [26]. Recently, MacLean-Fletcher and Pollard [13] showed by electron microscopy that cytochalasin B inhibited monomer addition to the "barbed" end without affecting monomer addition at the "pointed" end of actin filaments. Cohen and Branton [15] have previously reported that actin filaments emanating from inside-out vesicles of erythrocyte ghosts when decorated with heavy meromyosin have the arrowheads pointing toward the membrane. These filaments have apparently grown from the oligomeric actin in the spectrin-4.1-actin complex in the low-ionic-strength extract they used to reconstitute the inside-out vesicles. More specifically, the filaments were formed by monomer addition to the "barbed" end of the oligomeric actin in the complex. Furthermore, experiments done in this laboratory (Lin and Lin, unpublished results) and by Brenner and Korn [8, 11] have shown that filaments polymerized in the presence of the spectrin-actin complex do not depolymerize upon addition of cytochalasins, whereas filaments polymerized in the absence of the complex depolymerized rapidly to a very low level of viscosity. Taken together, it can be concluded that in the complex, the preferred depolymerizing end (the "pointed" end) of the oligomeric actin is blocked. The preferred polymerizing end, on the other hand, is free to bind cytochalasin and for monomer addition.

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