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ON THE MECHANISM FOR INACTIVATION OF CYTOCHALASIN BINDING ACTIVITY ASSOCIATED WITH F-ACTIN AND SPECTRIN-BAND 4.1-ACTIN COMPLEX BY SULFHYDRYL REAGENTS

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The sulfhydryl group modifying reagent, p-hydroxymercuribenzoate, inhibited the cytochalasin binding activity of the actin nuclei in the spectrin-band 4.1-actin complex from the erythrocyte membrane and of muscle F-actin. Kinetic studies indicated that while the cytochalasin binding activity was immediately inhibited, the actin remained filamentous and depolymerized slowly over a period of 1 to 2 h. Scatchard analysis of the binding data revealed that initially only the K_D was affected. However, prolonged incubation led to depolymerization of the F-actin and dissociation of the spectrin-band 4.1-actin complex, resulting in loss of binding sites. It thus appears that certain actin sulfhydryl group(s) are important for cytochalasin binding. However, the most reactive sulfhydryl group (cys-374) on actin does not appear to be involved.

The cytochalasins are a group of fungal metabolites which are powerful inhibitors of cell motility. Results of binding studies with [^{3}H]cytochalasin B ([^{3}H]CB)[1], together with kinetic and electron micrographic data on actin polymerization, have led to the conclusion that cytochalasins bind with high affinity to the "barbed" end, the preferred polymerizing end, of actin filaments (F-actin) (1-3). In a previous study, we reported that high-affinity cytochalasin binding to the spectrin-band 4.1-actin complex from erythrocyte membrane was sensitive to the sulfhydryl modifying reagent, p-hydroxymercuribenzoate (pCMB) (4). Organomercurials such as pCMB and salyrgan have been shown to inhibit the polymerization of muscle actin (5,6). Since monomeric actin (G-actin) does not bind CB with high affinity (1), the observed inhibition of cytochalasin binding to the erythrocyte spectrin-band 4.1-actin complex could be an indirect result of depolymerization of the oligomeric actin in the complex due

[1] Abbreviations used: CB, cytochalasin B; DTT, dithiothreitol; F-actin, filamentous actin; G-actin, globular actin; NEM, N-ethylmaleimide; pCMB, p-hydroxymercuribenzoate; SDS, sodium dodecyl sulfate.

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to modifications by pCMB. On the other hand, it is also possible that actin sulfhydryl groups are directly or indirectly involved in the active site of cytochalasin binding. Modification of these sulfhydryl groups by pCMB could lower the binding affinity of actin for the cytochalasins.

To distinguish between these two possible mechanisms of inhibition and to learn more about the cytochalasin binding site on actin, we carried out kinetic studies with the spectrin-band 4.1-actin complex as well as muscle actin.

MATERIALS AND METHODS

pCMB and N-ethylmaleimide (NEM) were from Sigma Chemical Company. Unlabelled CB was from Aldrich Chemical Company. [^{3}H]CB was from New England Nuclear.

Human erythrocyte ghosts were prepared from freshly drawn blood donated by the Baltimore Red Cross Blood Center. The low ionic strength extract was prepared by incubating ghost membranes at 37°C as previously described (7). Spectrin-band 4.1-actin complex was purified by Sepharose 4B column chromatography in a buffer containing 0.1 M KCl and 5 mM sodium phosphate, pH 8.0 (8). Protein concentration was determined by the Hartree modification of the Lowry method (9).

Actin was prepared from acetone powder of rabbit skeletal muscle according to the procedure of Spudich and Watt (10). It was stored in polymerized form in 0.2 mM ATP, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 5 mM Tris, pH 8.0. Aliquots were dialyzed to remove 2-mercaptoethanol prior to use against the same buffer without 2-mercaptoethanol. Modification of actin by NEM was performed essentially as described by Lusty and Fasold (11). The modified actin was carried through one cycle of polymerization and depolymerization before use. Actin concentration was determined by the method of Bradford (12). Analysis of sulfhydryl groups remaining after modification indicated that 0.9 mole of sulfhydryl/mole actin had reacted with NEM.

Binding of [^{3}H]CB to erythrocyte spectrin-band 4.1-actin complex and to muscle F-actin was measured with the isoelectric precipitation assay (13). It has previously been shown that by lowering the pH to 4.8-4.9, F-actin is immediately and quantitatively recovered in the precipitate, and that the amount of CB bound does not change even if the centrifugation was not carried out until 1 h later (8). Therefore, this assay is suitable for kinetic studies of CB binding.

Actin viscosity was measured at 25°C with an Ostwald-type capillary viscometer.

RESULTS AND DISCUSSION

Effect of pCMB on CB binding to erythrocyte spectrin-band 4.1-actin complex.

The cytochalasin binding activity of the spectrin-band 4.1-actin complex from the erythrocyte membrane has previously been shown to be sensitive to pCMB (4). In the present study we further investigated the nature and mechanism of this inhibition. At the level of the complex used (48 μg/ml), we found that CB binding activity was progressively

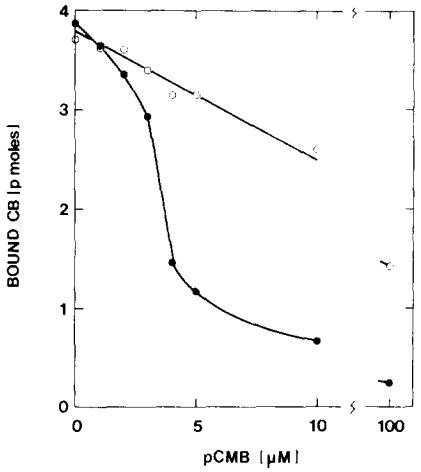
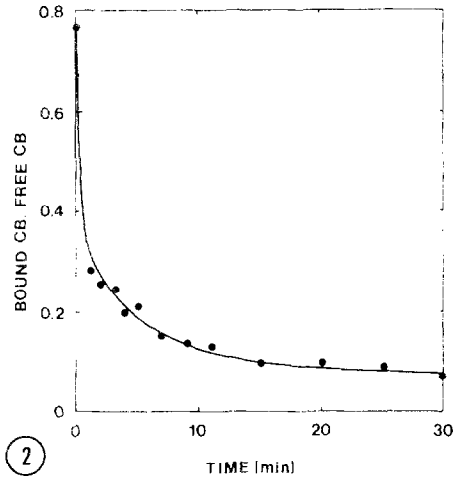


Fig. 1. Concentration dependence of the inhibition of CB binding to spectrin-band 4.1-actin complex by pCMB.

Column-purified spectrin-band 4.1-actin complex (24 μg) was incubated with pCMB at the indicated concentrations in a total volume of 0.5 ml in 0.1 M KCl, 5 mM sodium phosphate, pH 8.0, at 25°C. Samples were immediately assayed for CB binding (●), or after another 30 min of incubation following addition of 10 mM DTT (○). [^{3}H]CB was at a final concentration of 1 x 10^{-8} M. (See "Materials and Methods" and also Ref. 13 for details).

inhibited at pCMB concentrations between 1 and 10 μM (Fig. 1). At 100 μM of pCMB, inhibition reached 95%. The sulfhydryl reducing agent dithiothreitol (DTT) was only partially effective in reversing the inhibitory effect of pCMB. The total number of sulfhydryl groups in the complex can be estimated from the number of sulfhydryl groups in the major proteins, spectrin (23/dimer) and actin (5/monomer), and the mole ratio of these proteins in the complex (as estimated from Coommassie Blue-stained SDS-gels). Using these figures, the total concentration of sulfhydryl groups at the level of the complex used (48 μg/ml) was about 2.8 μM. Thus, it appears that for maximal inhibition the stoichiometry of pCMB to sulfhydryl groups in the complex must be higher than 1:1.

We next looked at the kinetics of the inhibitory action of pCMB on CB binding to the spectrin-band 4.1-actin complex. As shown in Figure 2, the inhibition appeared to have a biphasic time course. A rapid decrease in binding activity was observed in the first minute or two, followed by a slower decline in the next 30 min. To learn more about the mechanism of inhibition by pCMB, we measured CB binding to the complex after 30



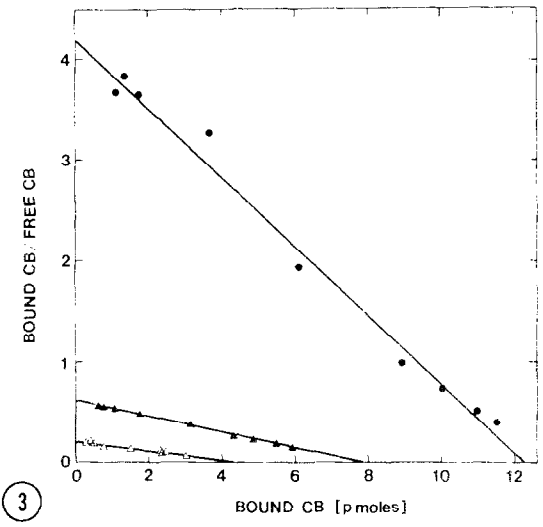


Fig. 2. Time course of the effect of pCMB on CB binding activity of erythrocyte spectrin-band 4.1-actin complex.

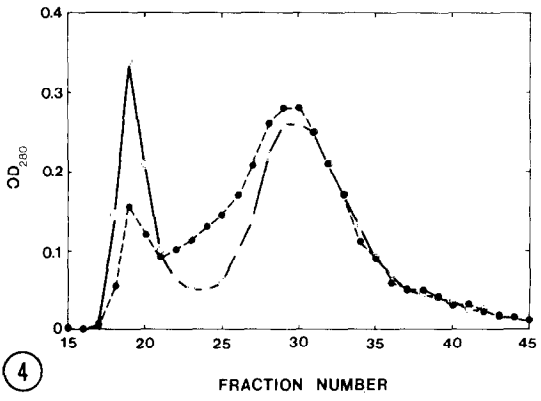
Column-purified spectrin-band 4.1-actin complex (40 μg/ml) was incubated with 30 μM pCMB in 0.1 M KCl, 5 mM sodium phosphate, pH 8.0, at 25°C. At the indicated times, 0.5 ml aliquots were assayed for CB binding by mixing with [^{3}H]CB (to give a final concentration of 1 x 10^{-8} M), and the proteins immediately precipitated by lowering the pH to 4.9. The CB binding activity is presented as bound CB/free CB (13).

Fig. 3 Scatchard plot analysis of the effect of pCMB on the CB binding activity of spectrin-band 4.1-actin complex.

Column-purified spectrin-band 4.1-actin complex (50 μg/ml) was incubated with 2 mM DTT (●), 10 μM pCMB (▲), or 400 μM pCMB (△) in 0.1 M KCl, 5 mM sodium phosphate, pH 8.0, at 25°C. After 30 min of incubation, 0.5 ml aliquots were assayed for [^{3}H]CB binding activity as in Fig. 1. The concentration range of [^{3}H]CB used was 0.3 to 6.0 x 10^{-8} M.

min of incubation with pCMB and analyzed the binding data by Scatchard plots (Fig. 3). From these plots, it can be seen that the number of CB binding sites decreased from 12 pmoles in the control to 8 and 4 pmoles, after treatment of the complex with 10 and 100 μM of pCMB, respectively. At the same time however, the dissociation constants of these remaining binding sites increased from 3 nM to 13 and 22 nM under these conditions.

The loss of CB binding sites could be a result of dissociation of the spectrin-band 4.1-actin complex. Using Sepharose 4B gel filtration chromatography, we found that the amount of material eluting at the void volume (where the complex normally elutes) was reduced more than 50% in the sample incubated with pCMB (Fig. 4). Therefore, it appears that pCMB modification did cause dissociation of the spectrin-band 4.1-actin complex, and this could explain the observed loss of CB binding sites.



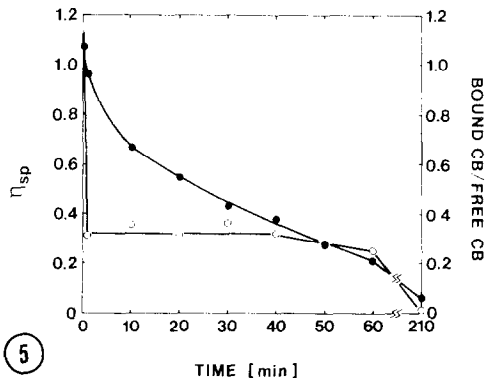


Fig. 4. Sepharose 4B column elution profile of the low ionic strength extract of human erythrocyte membrane.

Low ionic strength extract of ghosts (4 ml containing 4 mg of protein) prepared as described in "Methods", was chromatographed on a 1.5 x 90 cm Sepharose 4B column before (○—○) or after (●—●) incubation with 300 μM pCMB in 0.1 M KCl, 5 mM sodium phosphate, pH 8.0, at 4°C for 4 h. Fractions of 2.5 ml were collected and their relative protein content estimated from OD_{280}.

Figure 5. Time course of the effects of pCMB on CB binding and on the viscosity of muscle F-actin.

F-actin (1 mg/ml) was incubated with 300 μM pCMB in 0.2 mM ATP, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Tris, pH 8.0, at 25°C. At the indicated times, 0.5 ml aliquots were removed for viscosity measurements (●) and 0.1 ml aliquots for CB binding assays (○) as in Fig. 1.

Effect of pCMB on CB binding to F-actin

We next studied the effect of pCMB on the CB binding activity of muscle F-actin. As shown in Figure 5, upon the addition of a 2.6-fold excess of pCMB (over actin sulfhydryl groups), an immediate drop in CB binding was observed by the time the earliest point was taken (1/2 to 1 min). After the initial drop, CB binding activity then slowly declined and was inhibited by >99% after 2.5 h. The depolymerization of actin resulting from modification by pCMB, on the other hand, had a completely different time course. One minute after the addition of pCMB, the viscosity of the F-actin decreased by <10%; a 50% decrease in specific viscosity was observed after approximately 20 min. After 2.5 h, the viscosity dropped to a level similar to that of monomeric actin. Clearly, the initial drop in CB binding activity of the F-actin took place far in advance of its depolymerization. The effects of pCMB on CB binding activity and viscosity of F-actin could be fully reversed by the addition of DTT to the treated samples.

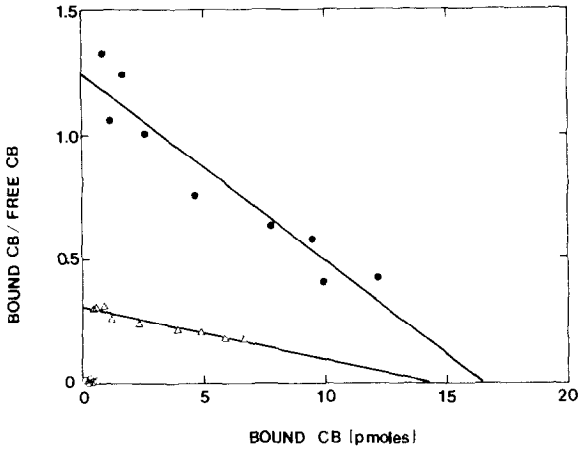


Figure 6. Scatchard plot analysis of the effect of pCMB on the CB binding activity of muscle F-actin.

F-actin (100 μg in 0.5 ml) was incubated with 0 (●) or 400 μM pCMB (△,○) in 0.2 mM ATP, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Tris, pH 8.0, at 25°C for 1 min (△) or for 3 h (○), and assayed for CB binding as in Fig. 1.

To further characterize the nature of the inhibition of CB binding to F-actin by pCMB, we again used Scatchard plots to analyze the binding data (Fig. 6). F-actin samples were preincubated with pCMB for 1 min before the addition of [^{3}H]CB for the binding assay. This time point was chosen because the initial rapid decrease in CB binding activity was maximal at 1 min (Fig. 5), at which time the F-actin had not appreciably depolymerized. As shown in Figure 6, the K_D for CB binding changed from 13 nM to 47 nM, while the number of binding sites remained essentially unchanged (14.3 vs. 16.4 pmoles). Two and one-half hours after incubation with pCMB, when the viscosity of the actin had decreased by >95%, the CB binding activity was also inhibited by >95%. These results suggest that upon mixing the F-actin with pCMB, the sulfhydryl groups at or near the CB binding sites at the ends of actin filaments were immediately modified, resulting in lowered affinity of these sites for CB. The further decrease in CB binding activity that came much later, however, probably reflects a decrease in the number of actin filaments as a result of depolymerization.

It is known that NEM modifies only one of the five sulfhydryl groups on actin, cys-374, and that this modification does not change the poly-

Table 1. Effect of NEM modification on the steady state viscosity and CB binding characteristics of F-actin. NEM-actin was prepared as described in "Materials and Methods". The viscosity was measured with 0.5 mg/ml of actin after polymerization with 2 mM $MgCl_2$ at 25°C. CB binding was measured as described in Fig. 6, and analyzed by Scatchard plots, except that 125 µg of actin (in 0.5 ml) was used.

	Control Actin	NEM-Actin
Specific Viscosity	0.65	0.59
K_D	9.6 nM	9.2 nM
Binding Sites	16.0 pmoles	11.8 pmoles

merizability of actin (11,14). To find out if the inhibition of CB binding to actin by pCMB was due to modification of cys-374, we first blocked this sulfhydryl group by reacting the actin with NEM. As shown in Table 1, measurements of CB binding to the NEM-actin indicated that the affinity for binding was the same as the control. The number of binding sites was somewhat lower, but this corresponded to the slightly lower level of steady state viscosity of the NEM-treated preparation. Moreover, NEM modification did not block the inhibitory effect of pCMB on actin polymerization or CB binding activity (data not shown). These results suggest that cys-374, the fastest reacting sulfhydryl group of actin, is not essential for actin polymerization or for CB binding.

The overall stoichiometry of pCMB needed for maximal effect on the affinity of CB binding at short incubation times (i.e., one minute) was less than 1 mole of pCMB per mole of actin (data not shown). However, since high-affinity CB binding involves only the actin molecule(s) at the end of each actin filament, the present study does not allow a calculation of the number of sulfhydryl groups involved at the CB binding site.

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