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Solubilization and denaturation of monomeric actin from erythrocyte membranes by *p*-mercuribenzenesulfonate

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Solutions of *p*-mercuribenzenesulfonate extract the peripheral proteins from the red cell membrane in a water-soluble form. Low concentrations of the reagent selectively solubilize actin, while at higher concentrations, spectrin, ankyrin and bands 4.2 and 4.1 are extracted. After brief exposure to the reagent, followed by displacement of the mercurial with dithiothreitol or 2-mercaptoethanol, the soluble actin is capable of inhibiting DNAse I activity. With prolonged exposure or with higher concentrations of the reagent, the ability to inhibit DNAse is gradually lost. The kinetics of both the release of actin capable of DNAse inhibition and the subsequent loss of that capability are pseudo-first-order with respect to time, but show second-order dependence on the concentration of mercurial. These data suggest that dissociation of the actin from protofilaments in the cytoskeleton requires exposure of more than one sulfhydryl group to the reagent. Subsequent inactivation also appears to be dependent on the reaction of further multiple sulfhydryl groups, possibly in buried regions of the actin molecule.

Introduction

The organic mercurial *p*-mercuribenzenesulfonate brings about the disruption of erythrocyte membranes, and the solubilization of the peripheral proteins spectrin, actin, ankyrin, glyceraldehyde-3-phosphate dehydrogenase (band 6), and bands 4.1 and 4.2 [1–4]. There appear to be several loci for this effect. Low concentrations of the reagent solubilize spectrin and actin [5] and have been shown to cause depolymerization of oligomeric spectrin and filamentous actin in vitro [6]. At higher concentrations of the reagent, band 3 protein is apparently modified, leading to the dissociation from band 3 of ankyrin and band 4.2 protein; release of both ankyrin and band 4.2 protein is inhibited by pretreatment with 1 mM *N*-ethylmaleimide at 37°C [7]. Reaction of the mercurial with band 3 protein also appears to lead to solubilization of glyceraldehyde-3-phosphate dehydrogenase, but this effect is not inhibited by pretreatment with *N*-ethylmaleimide [7]. At higher reagent concentrations, band 4.1 is solubilized [2,3].

While low concentrations of *p*-mercuribenzenesulfonate disrupt isolated erythrocyte cytoskeletons [5], the mechanism of this effect is unknown; in particular, it is not yet known in what form the solubilized proteins are released from the membrane: whether as hetero-oligomeric aggregates or as individual polypeptides.

In the present report we use the ability of native monomeric actin to inhibit the activity of deoxyribonuclease I (DNAse I) as a means of quantifying the release of native, monomeric actin from erythrocyte membranes by the mercurial [8].

Materials and Methods

Materials. Fresh, packed human red cells were obtained from the Red Cross Transfusion Service, Sydney. ATP (disodium salt), dithiothreitol, *N*-ethylmaleimide, 2-mercaptoethanol, *p*-mercuribenzenesulfonate, Trizma base, DNAse I (type II from bovine pancreas) and DNA (type I, from calf thymus, 'highly polymerized') were all obtained from Sigma. All other reagents were of analytical reagent grade.

Preparation of erythrocyte membranes. Erythrocyte membranes were prepared by hemolysis and washing with cold 5 mM sodium phosphate (pH 8.0), as described previously [9]. The membranes were then given a final wash by suspension in 5 mM sodium phosphate

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(pH 8.0), containing 0.2 mM ATP and 0.05 mM calcium chloride (the reaction buffer), followed by centrifugation at 35000 $\times g$ for 30 min at 2–4 °C. The inclusion of ATP and calcium chloride in this buffer was to stabilize against denaturation any monomeric actin that might be dissociated from protofilaments by the mercurial [10]. The final membrane suspensions had a protein concentration of approx. 6 mg/mL.

Incubation of membranes with p-mercuribenzenesulfonate. Stock solutions of the reagent were prepared with the reaction buffer, and were adjusted to pH 8.0, where necessary, with 0.1 M NaOH. Membrane suspensions (0.2 ml) were mixed rapidly with 0.8 ml of the appropriate dilution of the reagent in the reaction buffer on ice, to yield the desired final reagent concentration. A sample was taken immediately for the DNAse assay, and the remainder was held on ice, samples being taken for assays over the following 2 h.

Isolation of muscle actin. Muscle actin was isolated from rabbit (*Oryctolagus cuniculus*) skeletal muscle and was purified according to the method of Pardee and Spudich [11]. To increase the purity of the actin, the sample was subjected to a second polymerization–depolymerization cycle. The G-actin was generally used within 5 h of the final centrifugation step. If the G-actin was not to be used within this time, it was stored at 80 °C after freeze-drying [9].

DNAse I assays. DNAse I assays were performed, with minor modifications, according to the procedure of Blikstad et al. [8]. Samples of the incubation mixtures (20 μL) were removed at intervals and were added to 5 μL 0.25 M dithiothreitol in a quartz cuvette in order to sequester the free mercurial and to displace bound mercurial from actin. After 10 s, 50 μL DNAse solution containing 1 μg of the enzyme was added. DNA solution (1 ml 0.04 mg/mL) at 23°C was added immediately and the hypochromicity was followed at 260 nm. Muscle G-actin solutions were used to construct a standard curve for the assay.

Analysis of protein extracts. The protein concentrations of membrane samples were determined using the assay of Lowry et al. [12] and with either bovine serum albumin or muscle actin as the standard. The total amount of actin in a given membrane preparation was calculated assuming that actin represents 4.5% of the total membrane protein [13,14]. The validity of this assumption was checked by subjecting membrane samples and standard muscle actin samples to identical electrophoretic conditions [13], and comparing the stained zones directly with the aid of a Transidyne scanning densitometer.

Quantification of total extracted actin. Samples of erythrocyte membranes (0.2 mL) were treated with various concentrations of *p*-mercuribenzenesulfonate (0.8 mL) and were held on ice for periods up to 2 h. At appropriate times, the samples were mixed with 5 μL mercaptoethanol to sequester the mercurial, and were then centrifuged at 10000 rev/min for 5 min in an Eppendorf centrifuge model 5412 to pellet the membranes and membrane fragments. Samples (20 μL) were taken from the supernatants for electrophoresis in acrylamide slab gels containing 0.2% dodecyl sulfate. On each gel slab, 20 μL samples of unfractionated erythrocyte membranes were also examined. The gels were stained with Coomassie blue R250, and the stained patterns were quantified with a Transidyne scanning densitometer. The actin bands in each of the supernatant samples were expressed as a fraction of the actin in the unfractionated, control membrane samples.

Analysis of kinetics. The kinetics of DNAse I inhibition was determined by fitting the time course with a model comprising two consecutive pseudo-first-order steps. The parameters were determined by means of non-linear regression using the Marquardt algorithm [15].

Electron microscopy. Samples of erythrocyte membranes in 5 mM sodium phosphate buffer (pH 8.0), were treated with various concentrations of *p*-mercuribenzenesulfonate on ice for 30 min, after which time the membrane fragments were removed by centrifugation at 35000 $\times g$ for 30 min at 0–4 °C. Samples of the supernatant solution were either examined directly, or after being made to contain 1 mM dithiothreitol and 1 mM $MgCl_2$. The samples were then placed on parlodion-coated copper grids, negatively stained with either 0.2% (w/v) ammonium molybdate or 1% (w/v) uranyl acetate, and examined in a Philips model 201 electron microscope at 60 kV.

Results

After treatment of erythrocyte membranes for up to 2 h with *p*-mercuribenzenesulfonate at concentrations up to 1 mM, spectrin and actin were the major proteins solubilized, as revealed by gel electrophoresis. In the absence of mercurial, spectrin and actin were not released in detectable amounts over this time. Quantitative densitometry showed that, up to 0.4 mM reagent, the release of both spectrin and actin were approximately first-order with respect to time, and that the pseudo-first-order rate constant for, as well as the extent of, the release of actin was greater than that for spectrin. At concentrations greater than 0.4 mM reagent, the rates were too rapid for a kinetic analysis.

On treatment with 2-mercaptoethanol or dithiothreitol to sequester residual mercurial, the supernatant protein solution was capable of inhibiting DNAse I activity, and of polymerizing into characteristic actin filaments that were visible in the electron microscope. With prolonged exposure to the mercurial, however, these properties were gradually lost.

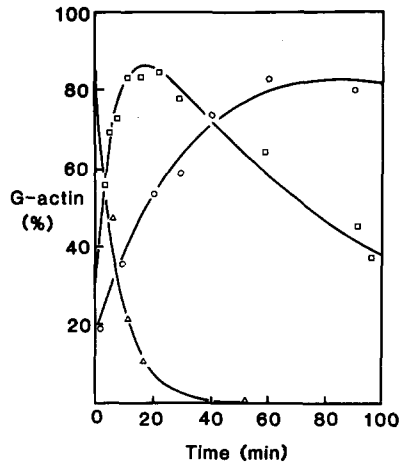

Fig. 1. Representative time courses for the inhibition of DNAse I activity by extracts of red cell membranes after exposure to the following concentrations of *p*-mercuribenzenesulfonate: ○, 0.2 mM; □, 0.4 mM; and △, 0.9 mM. The ordinate represents the concentration of putative G-actin in the extracts, expressed as a percentage of total actin originally present in the membranes. The curves, described by Eqn. 2, were fitted to the data by unweighted non-linear regression.

Fig. 1 shows the time course for the inhibition of DNAse I activity brought about by suspensions of erythrocyte membranes that had been treated with with various concentrations of *p*-mercuribenzenesulfonate. At low concentrations of the reagent, the ability of the suspensions to inhibit DNAse I activity increases with time. However, at higher reagent concentrations and at longer times of exposure to the reagent, the ability to inhibit DNAse I again decreased. With some concentrations of reagent (e.g., 0.4 mM), a maximum was seen in the time course for the ability to inhibit DNAse I. Incubation of membranes on ice in the buffer in the absence of mercurial showed that less than 10% of the available actin was capable of inhibiting DNAse, and this proportion did not change significantly over a period of 4 h.

Since it is known that monomeric actin is capable of inhibiting DNAse I [8], these data suggested that the mercurial was acting by solubilizing monomeric actin from the actin protofilaments within the cytoskeleton. Modification of buried thiol groups in monomeric actin has been reported to result in the denaturation and loss of polymerizability of G-actin [16,17]. The experimental data were therefore fitted by a model in which monomeric actin was released from the cytoskeleton by a pseudo-first-order process, followed by a second pseudo-first-order process leading to actin denaturation:

$$\text{Cytoskeleton} \xrightarrow{k_1} \text{G-actin} \xrightarrow{k_2} \text{denatured actin} \qquad (1)$$

Since some DNAse inhibition was detected with erythrocyte membranes that had not been treated with mercurial, the model incorporated a parameter representing the concentration of actin capable of inhibiting DNAse at zero time.

The concentration of free G-actin monomers is given by:

$$a = \exp(-k_2 t)\{a_0 + (k_1 b_0)/(k_2 - k_1)(\exp[(k_2 - k_1)t] - 1)\} \qquad (2)$$

where a, is the concentration of G-actin at time t; a_0 is the value of a at time zero; and b_0 is the concentration at time zero of actin protomers present within filaments. The pseudo-first-order rate constant k_1 is that describing the appearance of G-actin capable of DNAse inhibition, and k_2 is that for the subsequent inactivation of the G-actin.

The model was fitted to the time course data by means of unweighted non-linear regression, and the curves fitted to the data are shown by the solid lines in Fig. 1.

Both pseudo-first-order rate constants k_1 and k_2 of equation (2) were found to increase with mercurial concentration. However, the fit to a linear dependence of k_1 and k_2 on the concentration of *p*-mercuribenzenesulfonate was poor, with gross nonrandom distribution of residuals. Graphical analysis of the logarithm of inhibition versus the logarithm of reagent concentration yielded slopes of between 1.88 and 2.67 for four separate sets of experiments, suggesting that a quadratic function may better describe the dependence of both k_1 and k_2 on reagent concentration, although the slopes of the logarithmic plots tended to decrease towards unity at lower reagent concentrations. Accordingly, the values of k_1 and k_2 were each fitted to a quadratic in reagent concentration, and the data are shown in Figs. 2 and 3. Note that in Fig. 2, data are not shown beyond 0.4 mM, since the regression at the higher concentrations is relatively insensitive to the returned values of k_1, and accordingly the standard error exceeds 20% of the parameter values themselves. Similarly, the values of k_2

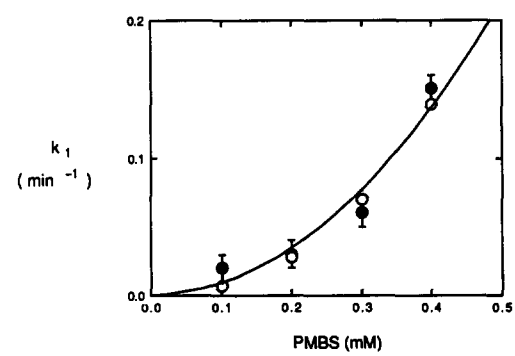

Fig. 2. Dependence of k_2, the rate constant for actin solubilization, on the concentration of *p*-mercuribenzenesulfonate. The line represents a quadratic function in mercurial concentration, fitted to the data by unweighted linear regression. (●) Data from DNAse inhibition studies. The error bars represent the standard errors of the estimates of k_1 approximated from the regression analysis. (○) Data from quantitative gel electrophoresis of the soluble extracts.

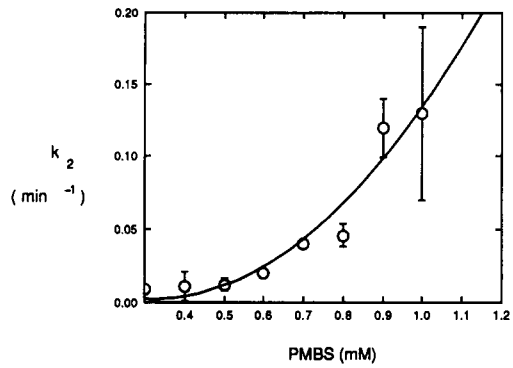

Fig. 3. Dependence of k_2, the rate constant for actin denaturation, on the concentration of *p*-mercuribenzenesulfonate. The line represents a quadratic function in mercurial concentration, fitted to the data by unweighted linear regression.

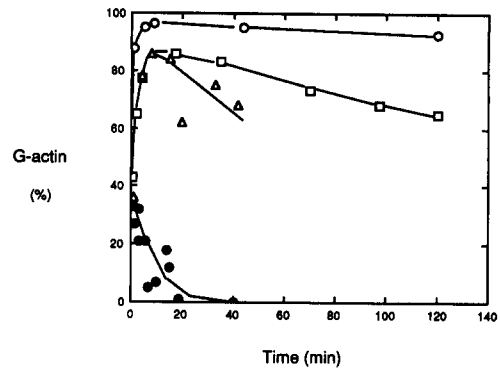

Fig. 4.Time courses for the inhibition of DNAse I activity by solutions of isolated rabbit muscle F-actin after exposure to the following concentrations of *p*-mercuribenzenesulfonate: ○, 0 mM; □, 0.4 mM; △, 0.5 mM; and ●, 1.0 mM. The ordinate represents the concentration of putative G-actin in the extracts, expressed as a percentage of the total actin originally present in the membranes.

cannot be determined precisely at mercurial concentrations below 0.3 mM.

The pseudo-first-order rate constants for the solubilization of actin, as determined by quantitative densitometry of electrophoresis gels, was in good agreement with that obtained for the dissociation step on fitting the DNAse inhibition kinetics. These data are included in Fig. 2.

Experiments performed with erythrocyte membranes that had been treated with 1 mM *N*-ethylmaleimide for 1 h on ice before the mercurial treatment gave results very similar to those shown in Fig. 1. However, the absolute rates for the reactions with the mercurial (both release of actin and denaturation) were significantly less after treatment with *N*-ethylmaleimide. Nevertheless, both processes also showed second order dependence on *p*-mercuribenzenesulfonate concentration after treatment with *N*-ethylmaleimide; the slopes of the logarithmic plots were 2.0 ± 0.1 and 1.6 ± 0.2, respectively.

Experiments with 1 and 5 mM phosphate buffer, without the inclusion of the ATP or calcium, yielded data very similar to that described above. However, inclusion of 0.1 M NaCl in the reaction solutions resulted in a decrease in the rates of actin solubilization by an order of magnitude.

The loss of DNAse inhibition was also seen after incubating isolated rabbit muscle actin (in both F- and G- forms) with various concentrations of *p*-mercuribenzenesulfonate. The kinetics of this process were similar to those for the denaturation stage obtained from fitting the inhibition kinetics. Dilution of F-actin into the low ionic strength buffer resulted in a rapid dissociation of the F-actin, even in the absence of the mercurial (Fig. 4). This effect tended to mask any specific effects the mercurial may have had in promoting actin depolymerization. At mercurial concentrations up to 0.5 mM, a pseudo-first-order rate constant between 0.4 and 0.6 min^{-1} was estimated for the initial increase in DNase inhibition. Although these rates were difficult to estimate with precision, there was no correlation of the value of k_1 with mercurial concentration.

Discussion

The results of the present study confirm that treatment of erythrocyte membranes with low concentrations of *p*-mercuribenzenesulfonate brings about the selective dissociation of actin from the erythrocyte membrane cytoskeleton. The rate of actin release at low concentrations of the reagent is markedly greater than that of spectrin, which implies that the actin is not being released as intact junctional complexes.

The monomeric nature of the solubilized actin is suggested by the ability of the membrane extracts to inhibit DNAse I [8]. However, it has been shown convincingly that the pointed end of actin filaments is also capable of inhibiting DNAse activity [18], and it might be argued that the increase in DNAse inhibition in erythrocyte membrane suspensions brought about by the mercurial may be the result of the severing of actin protofilaments to increasingly-shorter lengths. This alternative would view the kinetics as the increase in the concentration of free ends of actin filaments. However, under the conditions of the reaction and assay, short actin filaments would depolymerise rapidly from the free ends, so that short filaments with free ends are unlikely to contribute to the overall population of soluble actin. Furthermore, the inability to detect actin filaments or protofilaments in electron micrographs of the extracts (after centrifugation to remove membrane fragments) and the ability of such extracts to polymerize after addition of $MgCl_2$, supports the interpretation that the solubilised actin is in the form of monomers. Our conclusion is reinforced by the finding that the kinetics of the solubilization of total actin, as determined by means of quantitative gel densitometry, closely reflected that of the inhibition of DNAse I activity.

Our experiments are not able to distinguish between direct release of monomeric actin from disrupted protofilaments, and the alternative possibility of initial release of short sections of filament, followed by their subsequent rapid depolymerization. However, we believe the former possibility is the more likely.

The solubilization of actin appears to be linked to reaction of the mercurial with one or more thiol groups on the cytoskeleton. The dependence of this step on the second power of reagent concentration suggests that two thiol groups may be required to be modified before dissociation of the actin can occur. This nonlinear dependence was not simply a consequence of partial depletion of the reagent at low concentrations, brought about by the relatively high concentration of thiol groups in spectrin; even the lowest reagent concentrations used were in vast excess over the protein thiol concentration. Furthermore, second order dependence on *p*-mercuribenzenesulfonate concentration was also seen after pretreatment of the membranes with *N*-ethylmaleimide. Both of the thiol groups involved may reside on the same protomer, one each may reside in each of two adjacent actin protomers, or they may reside in the actin/spectrin junction. Since *N*-ethylmaleimide had no effect on this process, cysteine 373 of actin is not implicated in this step.

This initial stage of the reaction with mercurial appears to be reversible, in that treatment with dithiothreitol or 2-mercaptoethanol restored the ability of the actin both to inhibit DNAse and to polymerize into filaments in the presence of 1 mM $MgCl_2$. Prolonged treatment with the mercurial, however, led to additional changes that could not be reversed by treatment with dithiothreitol. This effect is presumably an irreversible denaturation enhanced by subsequent reaction of 'buried', less reactive thiol groups in the actin monomer [17]. Even in the absence of mercurial, some decay of the solubilized actin was observed over a period of 120 min in the present study, consistent with earlier observations [10]. Again, the second order dependence of this process on reagent concentration implicates modification of two thiol groups; since this process occurs in presumably monomeric actin, both thiols appear to reside in the same actin monomer. This step was also insensitive to treatment with *N*-ethylmaleimide.

While actin filaments are inherently unstable in the reaction buffer used in the present study, and would be expected to depolymerize rapidly, the actin protofilaments of the red cell membrane cytoskeleton are refractory to dissociation, and show an anomalously low critical concentration, due to their sequestration with other cytoskeletal proteins [19]. Erosion of the ends of the protofilaments seems to occur during washing of the membranes, down to the spectrin-actin junctions, and the resulting network is then stable to further depolymerization at low temperatures in the absence of mercurials [10,19]. At low temperatures and in the reaction buffer used in the present study, DNAse I does not bring about any increased dissociation of actin from washed ghosts [10].

The kinetics of actin solubilization are consistent with a mechanism in which the protofilaments are severed – either internally between adjacent actin protomers, or at the spectrin/actin junction – to produce free ends capable of dissociating freely. Provided that the rate constant for the severing process is substantially less than that for subsequent dissociation from the filament ends, the kinetics of the overall process will still be described by Eq. 2 and the two rate constants k_1 and k_2, where k_1 is the pseudo-first-order rate constant for filament severing. When the rate constant for severing the protofilaments approaches that of filament depolymerization, the kinetics will become more complex. The data obtained with rabbit muscle actin show that with up to 0.5 mM reagent, the rate of dissociation of actin monomers is independent of reagent concentration, and is greater than that seen with erythrocyte membranes. The relative magnitudes of the observed values of k_1 and the rate constant for depolymerization of actin filaments also supports the view that the inhibition of DNAse reflects the concentration of native, monomeric actin; any small section of F-actin could have only a fleeting existence.

A second order dependence of the pseudo-first-order rate constant for actin solubilization on the mercurial concentration implies a large degree of cooperativity. Since *N*-ethylmaleimide does not react with the thiols in question, they appear to be relatively unreactive, and may be inaccessible to the reagent. Reaction with mercurial may therefore require a prior transient conformation change or partial dissociation at the interface either between two adjacent actin protomers, or at a spectrin/actin junction, exposing two previously hidden groups to the reagent. Alternatively, though less likely, both groups may reside on the same protein molecule. Reaction of both exposed groups with mercurial would be rapid, and would prevent a return to the initial state, and lead to subsequent depolymerization from the free end or ends.

Studies on the rates of disruption of isolated erythrocyte cytoskeletons at 22°C showed that both spectrin and actin were capable of binding radiolabelled *p*-mercuribenzenesulfonate [5]. Although the first-order rate constants of the labelling process could not be determined with precision, and the temperature used in that study differed from that in the present report, the rates of radiolabelling of spectrin and actin are nevertheless consistent with that of actin solubilization found in the present study. Approx. 4 mol mercurial per mol spectrin and 1 mol mercurial per mol actin were incorporated during the process of cytoskeleton disruption brought about by *p*-mercuribenzenesulfonate [5]. This

stoichiometry supports the view that k_1 represents the rate constant for disruption of the actin/spectrin interface, a process that leads to the exposure of a previously hidden sulfhydryl group on each of the two proteins.

Although the dissociation of monomeric actin from the cytoskeleton would contribute substantially to cytoskeletal disruption and membrane fragmentation, this process appears to be slower than the initial step in disruption of the erythrocyte cytoskeleton, which appears to be the dissociation of the spectrin heterodimer [6].

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