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THE SOLUBILIZATION OF CYTOSKELETONS OF HUMAN ERYTHROCYTE MEMBRANES BY *p*-MERCURIBENZENE SULPHONATE

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The disruption of erythyrocyte membrane cytoskeletons brought about by treatment with *p*-mercuribenzene sulphonate (PMBS) has been followed by measurements of turbidity and the binding of ^{203}Hg-labelled PMBS. After pretreatment with *N*-ethylmaleimide to block readily reactive sulphydryl groups, incubation with [^{203}Hg]PMBS showed incorporation of approximately 4 moles radiolabel per mole of spectrin and one per mole of actin. The incorporation of radiolabel paralleled the decrease in turbidity, and the labelling of spectrin paralleled that of actin. The kinetics were pseudo first order, and the pH dependence of the observed rate constant indicated a normal pK_a value for the sulphydryl group involved. The calculated second-order rate constant for the reaction of the sulphydryl anion with PMBS, however, was several orders of magnitude less than expected from model compound studies. The results suggest that association between spectrin and actin may result in the steric hindrance of reactivity of a limited number of sulphydryl groups in each protein. Disruption of the spectrin-actin association may then be linked to the modification of the sulphydryl groups.

Introduction

The shape and deformability of red cell membranes and the distribution of intramembrane particles and glycoproteins are controlled by peripheral proteins which are arranged in a network on the cytoplasmic surface of the membrane [1,2]. The major proteins of the cytoskeleton are components 1 and 2 (nomenclature of Steck [1]) which are subunits of spectrin, component 5 which is now known to be erythrocyte actin [3] and components 4.1, 4.2 and 4.9 which as yet have unknown function.

The details of the protein interactions in the cytoskeletal network are not fully understood. One way of clarifying the organisation of the membrane components is to analyse the way in which selectively disruptive agents liberate various membrane constituents. This present report is a study of the mechanism of the solubilization of the cytoskeleton by *p*-mercuribenzene sulphonate (PMBS).

Charged organic mercurials such as PMBS cause solubilization of the cytoskeleton proteins under conditions of ionic strength at which solubilization does not occur in the absence of the mercurial [4,6]. Non-mercurial sulphydryl reagents such as *N*-ethylmaleimide and iodoacetic acid do not have this effect and they do not block the effect of PMBS [4–6]. Uncharged organic mercurials such as phenylmercury acetate and chloromercuri-2,4-dinitrophenol [6] block the actions of PMBS but they do not extract the proteins. Solubilization therefore appears to be dependent upon the introduction of the negative charge. It is likely that

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Abbreviations: PMBS, *p*-mercuribenzene sulphonate; [^{203}Hg]PMBS, ^{203}Hg-labelled PMBS.

PMBS reacts at weakly reactive thiol groups in particular proteins, resulting in the disruption of the cytoskeleton and dissociation of the peripheral proteins of the erythrocyte membrane [4–6].

The present report examines the ability of PMBS to disrupt intact cytoskeletons.

Materials and Methods

Fresh human packed cells were obtained from the Red Cross Transfusion Service, Sydney. Triton X-100 was obtained from Ajax Chemicals, and *p*-chloromercuribenzene sulphonic acid (monosodium salt) and *N*-ethylmaleimide were purchased from Sigma. *p*-Chloro[^{203}Hg]mercuribenzenesulphonic acid was purchased from Amersham Australia.

Preparation of erythrocyte ghosts

Red cells were washed three times in cold (4°C) 5 mM sodium phosphate buffer, pH 7.5, containing 0.95% NaCl, by repeated centrifugation at $800 \times g$ for 15 min. The 'buffy coat' was removed after each wash. Membranes were prepared by haemolysis at 0 to 4°C in 5 mM sodium phosphate buffer, pH 8, followed by repeated washing in the haemolysis buffer by centrifugation at $35\,000 \times g$ for 30 min until the ghosts were pale cream coloured [6]. After each centrifugation step the supernatant and a tightly packed pink pellet containing the remaining leucocytes and platelets underlying the membranes were removed. Ghost preparations were used as soon as possible after preparation.

Preparation of PMBS solutions

In all cases the sulphydryl reagent solutions were made up to the appropriate concentrations by dissolving the solid reagent in 5 mM phosphate buffer, pH 8.0. The pH was adjusted, where necessary, to pH 8.0 using either 0.1 M KOH or 0.1 M NaOH.

Preparation of cytoskeletons

The non-ionic detergent Triton X-100 solubilizes lipids and integral proteins from the membranes leaving an intact cytoskeletal network, the 'Triton shell', of peripheral proteins [7,8]. 50 ml ghost suspension was incubated for 30 min on ice with 250 ml of 2% Triton X-100 in 1 mM sodium phosphate buffer, pH 8.0. The cytoskeletons were collected by centrifugation for 30 min at $17\,000 \times g$, and washed by centrifugation under the same conditions in 5 mM sodium phosphate, pH 8.0. The washed cytoskeletons were then suspended in the appropriate buffer.

Disruption of cytoskeletons

The disruption of the cytoskeletal network caused by PMBS was followed spectrophotometrically [4] by observing the decrease in turbidity of the cytoskeleton suspensions as a change in apparent absorbance at 650 nm with time, in a Varian Superscan recording spectrophotometer. The spontaneous disruption of the cytoskeletons was followed before addition of PMBS, and at pH values below 9.4, the rate of this spontaneous disruption was negligible in comparison with the changes brought about by PMBS. For kinetic analyses, cytoskeleton suspensions were used at concentrations at which the Beer-Lambert relationship was obeyed with respect to apparent absorbance at 650 nm. This concentration corresponded to protein concentrations below 1 mg/ml. Although we are operationally defining 'disruption' in terms of turbidity, fragmentation of the cytoskeletons is expected to be a complex phenomenon.

Radiolabelling of cytoskeleton components

Cytoskeleton suspensions were first treated with 5 mM *N*-ethylmaleimide for 1 h on ice in order to decrease the number of sulphydryl groups available for reaction with radiolabelled PMBS so that sulphydryl groups involved in the disruption would be more selectively labelled.

The cytoskeleton suspensions were then treated with [^{203}Hg]PMBS. Excess PMBS was removed by applying the reaction mixture to a Sephadex G-25 column. In all kinetic experiments the unreacted PMBS concentration was always considerably in excess of the bound PMBS concentration. The protein-containing fractions were then pooled and treated with an excess of *N*-ethylmaleimide (20 mM) before addition of SDS. The *N*-ethylmaleimide was added to cause rapid blocking of newly exposed sulphydryl groups so that exchange of PMBS could not occur. Proteins were separated by gel filtration or gel electrophoresis in the presence

of SDS, and the separated proteins were analysed for radioactivity using a Searle automatic 1197 gamma counter.

Gel electrophoresis in 0.2% SDS was performed according to the method of Fairbanks et al. [9]. Gel filtration was conducted by applying a 1 ml sample to a 6% agarose column (50 cm × 1.5 cm). The protein was eluted in 1 ml fractions using a buffer comprising 10 mM sodium phosphate, pH 8.0, 50 mM NaCl and 1% SDS (w/v). Spectrin emerged at the void volume, but gel electrophoresis showed no proteins other than spectrin detectable in this fraction.

Results

The disruption of cytoskeletons

Treatment of the cytoskeletons with PMBS leads to their disruption as shown by a decrease in suspension turbidity. Independent evidence of shell fragmentation was also obtained from negatively stained preparations in the electron microscope.

The time course of reaction of PMBS with the cytoskeletons was reproducible and indicated an apparent pseudo-first-order process of disruption with respect to time in the presence of excess PMBS (Fig. 1), although, almost certainly, the events leading up to disruption are complex.

Pretreatment of cytoskeletons with 5 mM *N*-ethylmaleimide on ice for 30 min prior to addition of PMBS did not bring about shell disruption, and caused no observable change in the rate of disruption brought about by PMBS.

PMBS concentration dependence

The PMBS concentration dependence was studied by adding constant volumes of varying PMBS concentration to constant volumes of Triton shells suspended in 5 mM phosphate buffer, pH 8.0, to give a final PMBS concentration over the range 0 to 0.5 mM.

The pseudo-first-order rate constant determined by the method of Guggenheim [10], increased linearly with PMBS concentration (Fig. 2). The second-order rate constant (k_2) for the reaction of PMBS may be determined from the slope of this plot. This provided a value of $k_2 = 140\ M^{-1} \cdot s^{-2}$.

Ionic strength dependence

The ionic strength dependence of the disruption was examined by suspending cytoskeletons from human erythrocytes in separate solutions of 5 mM phosphate buffer, pH 8.0, with varying NaCl concentration. The rate of disruption was followed after addition of a constant volume of 10 mM PMBS to a final concentration of 0.45 mM PMBS.

The rate of disruption decreased with increasing salt concentration from 5 mM to 105 mM. The initial apparent absorbance of the cytoskeleton suspension also increased with increasing ionic strength. This would seem to be due to the increasing ionic strength causing a contraction of the cytoskeletons [11].

pH dependence

From the pH dependence of the PMBS reaction it is possible to determine the apparent pK_a of the reactive thiol group, which may allow an assess-

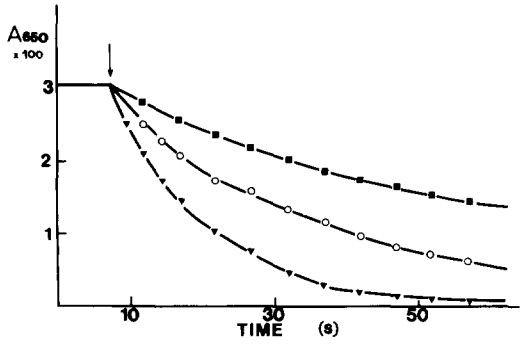

Fig. 1. Change in apparent absorbance of Triton shells with time after addition of varying concentrations of PMBS (arrowed). The concentrations of PMBS on the plot shown in this figure were 0.5 mM (▼), 0.25 mM (○) and 0.05 mM (■).

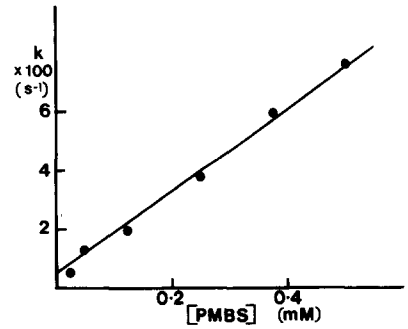

Fig. 2. The PMBS-dependence of the observed pseudo-first-order rate constant. The rate constants were found by the use of the method of Guggenheim. The line was fitted to the data using an unweighted least squares procedure.

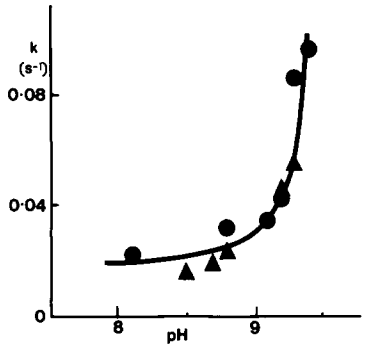

Fig. 3. pH-dependence of the observed pseudo-first-order rate constant for the reaction of PMBS with human Triton shells. The final concentration of PMBS used was 2 mM. The two different symbols represent pH-dependence studies carried out using different preparations of Triton shells.

ment of the extent of burial of the reactive group in the protein.

Cytoskeletons were suspended in buffers of various pH and ionic strength 0.05. Tris buffers were used for pH range 7.2 to 9.0, and glycine buffers for pH range 8.6 to 10.0. An overlapping set of pH ranges of the two types of buffer was used in order that any differences in rate due to the buffer compounds themselves may be detected. The pH dependence of the disruption was followed by adding a constant volume of 10 mM PMBS to a final concentration of 2.0 mM. This concentration of PMBS was used in order to provide an appreciable disruption rate under the conditions of relatively high ionic strength due to the pH buffers. The pH of the solution was measured after completion of the reaction.

The disruption of the cytoskeletons showed a marked pH dependence. The rate of disruption increased only slightly from pH 7.2 to pH 8.8 with a dramatic increase in rate with increasing pH above 8.8 (Fig. 3). Above pH 9.4 the rate became too fast to measure using the present techniques and there appeared to be increased spontaneous breakdown, presumably due to a loss of stability of the cytoskeletons in the alkaline buffers. Moreover, at pH values above 9.4 the shells appeared to be so greatly expanded that the total apparent absorbance change on reaction with PMBS was very small. For example, the total apparent absorbance change at 650 nm of cytoskeletons suspended in a buffer at pH 9.8 was 0.01 whereas at pH 9.0 it was 0.03.

From a plot of $1/k_{obs}$ versus $[H^+]$, where k_{obs} is the observed pseudo-first-order rate constant, the second-order rate constant for the reaction of the thiol anion with PMBS can be obtained from the intercept, and K_a, the acid dissociation constant for the thiol from the slope.

The apparent pK_a value for the reactive group in the cytoskeletons was found to be approx. 9.3, which is within the range of values determined for completely exposed thiol groups of model compounds [12,13].

On the other hand, the calculated second-order rate constant for reaction of the thiol anion form of cytoskeletons with PMBS was found to be only 56 $M^{-1} \cdot s^{-2}$.

Radiolabelling of cytoskeletal protein components with [^{203}Hg]PMBS

A study of the incorporation of radiolabel into cytoskeleton proteins during cytoskeleton disruption with [^{203}Hg]PMBS was conducted in order to determine which proteins are specifically involved in the disruption process.

Incorporation of label into cytoskeletons as a function of time after PMBS addition parallels the time course for disruption (Fig. 4). Gel filtration on 6% agarose in the presence of SDS showed that the radioactivity eluted at the positions of spectrin and actin (Fig. 5). Although some PMBS is incorporated before the first measurement can be made,

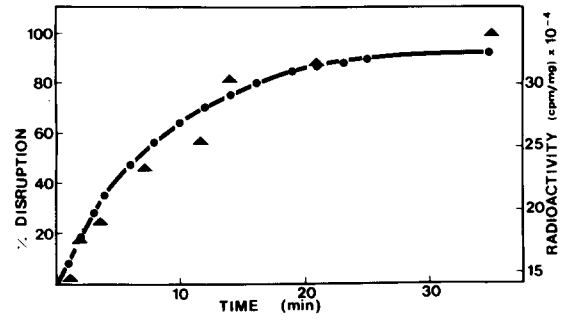

Fig. 4. Time course of PMBS-dependent cytoskeleton disruption followed by turbidity change (●) and incorporation of [^{203}Hg]PMBS (▲). Cytoskeleton suspensions were treated for varying times with [^{203}Hg]PMBS and the reaction was stopped by applying the samples to Sephadex G-25 columns. The protein was eluted with 5 mM sodium phosphate, pH 8.0 and the protein-containing fractions were analysed for radioactivity.

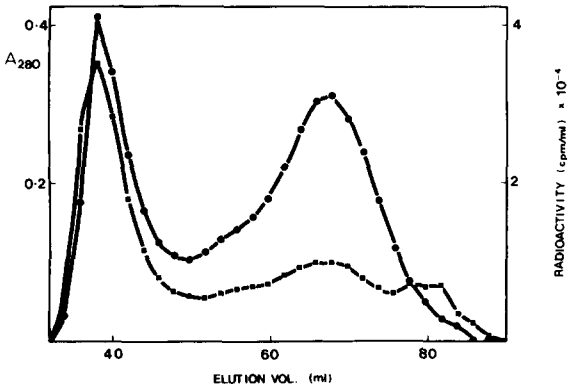

Fig. 5. Elution profile of protein (■) and radioactivity (●) from a 6% agarose column for [^{203}Hg]PMBS disrupted cytoskeletons. After reaction with PMBS, proteins were dissociated by addition of 2% SDS in the presence of 20 mM *N*-ethylmaleimide, and gel filtration was performed in a buffer comprising 10 mM sodium phosphate, pH 8.0, 50 mM NaCl and 1% SDS.

the incorporation during the shell disruption stage approaches the limiting value of 4.4 + 2.1 moles PMBS per mole of spectrin dimer and 1.4 + 0.4 moles per mole of actin (four experiments). The labelling of spectrin and actin was confirmed by polyacrylamide gel electrophoresis and gamma counting of individual protein bands. The ratio of labelling of spectrin to that of actin was constant with time suggesting that both proteins may be involved in the disruption effect. With prolonged incubation, the number of labelled groups in spectrin increased slowly towards eight, suggesting that further, even less reactive, sulphydryl groups may exist in spectrin.

Some labelling of band 4.1 as well as traces on residual bands 3 and 4.2 was also seen (the shoulder on the actin peak in Fig. 5). However, this labelling did not follow the kinetics of shell disruption.

Discussion

The disruption of the cytoskeleton

The disruption of red cell membrane cytoskeletons by PMBS as measured by turbidity changes, as well as the incorporation of [^{203}Hg]PMBS, was shown to be a pseudo-first-order process and the PMBS concentration dependence study showed that the pseudo-first-order rate constants for the reaction have a linear dependence on PMBS concentration.

The reaction of PMBS with the Triton shells is several orders of magnitude slower than the reaction of PMBS with sulphydryl groups of model compounds [12]. This indicates that the sulphydryl group responsible for the effect is not readily available for reaction with PMBS, and thus it is either buried within the protein molecule or there is steric hindrance preventing access of PMBS.

The marked pH dependence presumably reflects the ionisation of the sulphydryl group to the more reactive sulphide anion. The pH-dependence study provided a measure of the apparent pK_a of the sulphydryl group and this was found to be approximately the same as the pK_a for the exposed groups of model compounds. Therefore it is unlikely that the protonated sulphydryl group is buried since the normal value of the apparent pK_a of this group suggests that it is at least exposed to solvent water. On the other hand, if the thiol was normally 'buried', and required a conformational change to allow titration, the apparent pK_a would be significantly greater than that for exposed groups of model compounds. In addition, charge interactions may be expected to alter the apparent pK_a value by stabilization or destabilization of the thiol anion.

It appears, therefore, that the low reaction rate of PMBS with the sulphydryl group may be due to a form of steric hindrance of the sulphydryl group other than burial within the protein. It may therefore be necessary for an unfolding or dissociation step to occur before the subsequent attack by these molecules is possible. If the equilibrium for unfolding or dissociation lies heavily in favour of the folded or associated state then the concentration of available sulphydryl groups will be very low. This could account for the low rate of reaction with PMBS in comparison with model compounds. Reaction with PMBS may then cause the dissociation or unfolding step to be irreversible since, on attachment of the charged mercurial, the reformation of the native state may be restricted.

High ionic strength, which caused a decrease in the reaction rate with PMBS, may stabilise the protein in the native or associated state preventing the sulphydryl group from reaction with PMBS. Charge interactions with PMBS may also modify

its affinity with the sulphydryl group.

Radiolabelling studies implicate spectrin and actin as the principal targets for PMBS during the disruption process, although band 4.1 at this stage cannot be completely excluded from consideration. In a previous study from this laboratory [6], we reported that band 4.9 might be the locus of the PMBS-dependent cytoskeleton disruption. That study, however, involved the radiolabelling of whole membranes, and was dominated by the labelling of band 3 and another integral protein, staining poorly with coomassie blue and migrating to the same position as band 4.9. The present work, with isolated Triton shells, avoids the complication of the integral proteins, and allows the locus to be ascribed more clearly to spectrin and actin.

Approximately 4 sulphydryl groups are implicated in the spectrin dimer, and one in actin. PMBS may disrupt the interaction between spectrin and actin in the cytoskeleton. Alternatively it may be the action of PMBS on only one of these proteins which results in the fragmentation of the cytoskeleton, with the other sulphydryl groups reacting as a result of the disruption. It is known that the reaction of mercurials with actin monomers prevents actin polymerisation [14]. It is possible that PMBS causes the dissociation of the actin oligomers present in the cytoskeleton resulting in a loss of cytoskeleton stability. On the other hand, PMBS does not bring about the dissociation of spectrin tetramers to dimers (Ralston, G.B., unpublished observations). Further work on the PMBS action on the isolated cytoskeletal components may clarify the events leading to disruption.

Acknowledgements

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