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# SULFHYDRYL AND DISULFIDE GROUPS OF PLATELET MEMBRANES

## II. DETERMINATION OF DISULFIDE GROUPS

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SUMMARY

Two methods are described and evaluated for the quantitative determination of disulfide (S–S) bonds of platelet membrane proteins. In one method cleavage of S–S bonds by sulfite was followed by reaction of the resultant monothiols with $^{203}$Hg-labeled *p*-chloromercuribenzoate. The mercurial adducts of the membrane proteins were measured by a nitrocellulose-filter assay. The other method was based on the reduction of disulfide bonds by dithiothreitol and the spectrophotometric determination of the sulfhydryl groups with dithiobisnitrobenzoic acid. Several variables of these assay systems including pH, urea and sulfite concentration were examined. Both methods yielded a similar number of S–S groups in membrane proteins.

## INTRODUCTION

Disulfide bonds are the major determinants of the tertiary structure of proteins and have been found to be essential for the catalytic function of a number of enzymes[1]. Although according to the fluid mosaic model of biomembranes proposed by Singer and Nicolson[2] membrane proteins are embedded in a lipid matrix and thus may be subject to greater conformational restriction than their soluble counterparts it is very probable that S–S bonds play an important part in the maintenance of their structure as well. Methods for the assay of disulfide bonds in membrane proteins can be expected to differ somewhat from those applicable to soluble proteins. We have investigated two possible techniques for the quantitative determination of S–S bonds in membrane proteins. One measures the thiols resulting from sulfitolysis of the available S–S bonds as mercaptides by a filtration method which we found to be rapid and quantitative for the determination of *p*-chloromercuribenzoate (PCMB) protein adducts[3]. The other technique utilizes a spectrophotometric assay of the product of the reaction of dithiobisnitrobenzoic acid (DTNB) with the thiols resulting

Abbreviations: PCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); CNTP, 3-carboxylate-4-nitrothiophenolate.

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from reversible reduction of accessible disulfide bonds. These investigations were performed on membranes of human thrombocytes. Besides performing the usual physiologic functions of maintenance of structural integrity and transport, platelet membranes also play an essential role in hemostasis[4]. Although as yet no conclusive evidence has been presented directly implicating disulfide bonds in any of these membrane functions, by analogy to soluble proteins one might expect this to be the case.

## METHODS AND MATERIALS

*Isolation and preparations of platelet membranes*

Blood from normal male volunteers was collected into plastic bags containing acid–citrate–dextrose anticoagulant (ACD, U.S.P. formula "A"). Platelets were harvested and their membranes isolated as described before[3]. Membrane suspensions containing 0.5–1 mg protein in 0.154 M NaCl were stored at −20 °C for up to 3 weeks prior to use.

*Quantification of disulfide bonds*

(*a*) *Filtration method utilizing $^{203}$Hg-labeled PCMB and sodium sulfite.* Disulfide groups of platelet membrane proteins were measured according to the reaction scheme shown below. After cleavage by sodium sulfite in the presence of urea the resultant monothiols were assayed as [$^{203}$Hg]mercaptides.

$$RS\text{–}SR + SO_3^{2-} \rightleftharpoons RS^- + RS\text{–}SO_3^- \quad (1)$$

$$RS^- + PCMB \rightarrow RS\text{–}PCMB \quad (2)$$

0.1 ml of membrane suspension (40–60 $\mu$g protein) was added to 1.9 ml of 0.2 M Tris–HCl buffer, pH 9.0, 8 M urea, 0.2 M sodium sulfite and $7.5 \cdot 10^{-5}$ M $^{203}$Hg-labeled PCMB. The reaction mixture was incubated for 60 min at 37 °C and was then filtered through nitrocellulose membranes with weak suction. The platelet membrane proteins adsorbed onto the filter were washed with a total of 10 ml of washing solution consisting of 0.05 M NaCl buffered with 0.02 M Tris–HCl, pH 7.8. The radioactivity of the $^{203}$Hg-labeled PCMB protein adducts adherent to the membrane filters was measured in a liquid scintillation spectrometer using 2,5-diphenyloxazole (PPO) as the primary and 1,4-bis(2-(4-methyl-5-phenyloxazolyl))-benzene (POPOP) as the secondary fluor both dissolved in toluene.

(*b*) *Spectrophotometric determination of disulfide bonds with dithiothreitol and DTNB.* This method adapted from that described by Zahler and Cleland[5] is based on the reduction of protein disulfide bonds with dithiothreitol (DTT) and determination of the resulting monothiols with DTNB in the presence of arsenite according to the reaction sequence:

$$DTT + RS\text{–}SR \rightleftharpoons DTTS\text{–}SR + RS^-$$

$$DTTS\text{–}SR \rightarrow \text{oxidized DTT} + RS^-$$

$$DTT + HAsO_2 \rightleftharpoons DTT\text{–}AsOH$$

0.2 ml of platelet membrane suspension containing 200–300 $\mu$g protein were

mixed with 0.2 ml of either 8 M urea or 1.0% sodium dodecyl sulfate, 0.1 ml of 0.05 M Tris–HCl buffer, pH 9.0, and 0.1 ml of 3 mM dithiothreitol. Reduction of the protein disulfides was allowed to proceed for 60 min at 22 °C. Then 1.0 ml of 0.2 M Tris–HCl buffer, pH 8.1 and 1.5 ml of 5 mM $NaAsO_2$ were added, and the mixture was left standing for another 5 min at 22 °C.

To determine the monothiols, 0.1 ml of 3 mM DTNB dissolved in 0.05 M acetate buffer, pH 5.0, was added to the reaction mixture and the absorbance at 412 nm recorded at intervals of 1 min for a period of at least 15 min. The absorbance resulting from free SH groups and reduced disulfides was determined by extrapolation of the linear portion of the curve to the time of addition of DTNB and subtraction of the value obtained for a sample containing no protein. The number of disulfide bonds was calculated from the difference between total monothiols (free + reduced disulfides) and free SH groups divided by 2. Protein was determined by the method of Lowry *et al.*[6].

*Materials*

$^{203}$Hg-labeled *p*-chloromercuribenzoate was obtained from Amersham/Searle Corp., Arlington Heights, Ill. Dithiothreitol and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Calbiochem, Los Angeles, Calif. Nitrocellulose membrane filters, No. B-6, pore size 0.45 $\mu$m, 24 mm diameter were obtained from Schleicher and Schuell Inc., Keene, N. H.

## RESULTS

As there are no direct assays for disulfide bonds their quantitative determination depends upon the evaluation of the difference in the number of reactive thiols in the presence or absence of agents that cleave disulfide bonds. The extent of the cleavage of S–S bonds by $Na_2SO_3$ was found to be a function of the concentration of the reducing agent (Fig. 1). Increasing sulfite in the medium up to 50 mM resulted in a rapid rise of the number of mercaptides formed by the reaction of $^{203}$Hg-labeled PCMB with membrane protein thiols. Although there was relatively little change in the total number of $^{203}$Hg-labeled PCMB adducts in the range from 50 to 300 mM sulfite, even at the latter concentration of cleaving agent the reduction of disulfide bonds in membrane proteins had not yet gone to completion. In an effort to unmask disulfide bonds which were inaccessible to reduction with sulfite or to reaction with PCMB we added urea to the membrane suspensions. This protein denaturing agent when used in high concentration was able to solubilize platelet membranes, manifested grossly by partial or complete clearing of the turbid membrane suspensions. A small but significant increase in the number of mercaptides was observed when the urea concentration was raised to 4 M (Fig. 2). No further change, however, resulted from elevating the urea concentration to 8 M. The use of nitrocellulose filters for the quantitative reduction of PCMB–membrane protein adducts precluded solubilization of membranes with sodium dodecyl sulfate[3]. The $H^+$ concentration was found to be critical for the total number of mercaptides formed from free thiols and reduced disulfite groups. In the pH range from 5.0 to 9.0 a definite minimum could be observed at pH 6.0 while the largest number of PCMB–protein adducts was formed and retained at pH 9.0 (Fig. 3).

As the measurement of membrane protein S–S bonds with the disulfide reagent DTNB depended upon a spectrophotometric assay of the carboxylato-nitrothiophenolate (CNTP) anion released in the reaction it was desirable to obtain a clear

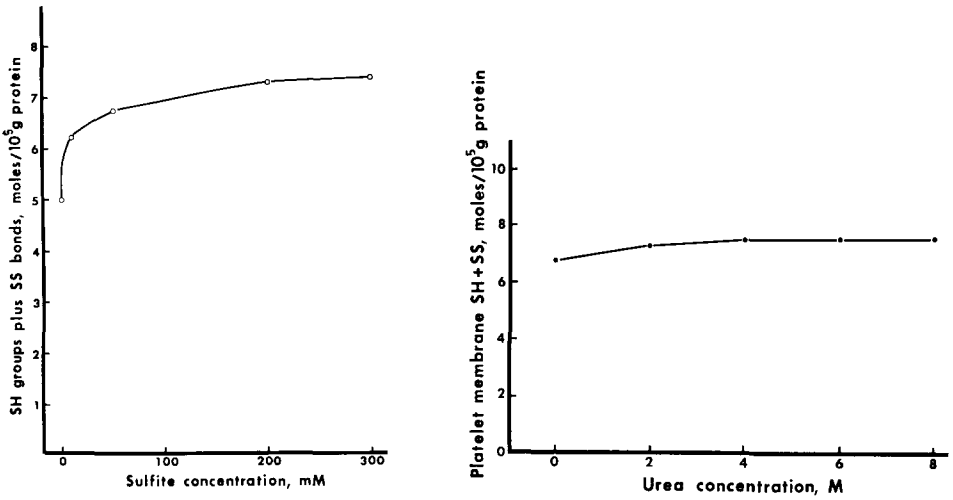


Fig. 1. Effect of sulfite concentration on the total number of mercaptides formed from free SH groups and reduced disulfide bonds of platelet membranes in the presence of 8 M urea. Detailed procedures are described in Methods and Materials.

Fig. 2. Titration of platelet membrane SH and S–S groups with $^{203}$Hg-labeled PCMB in the presence of 0.2 M sulfite and varying concentrations of urea. Methods are described in the text.

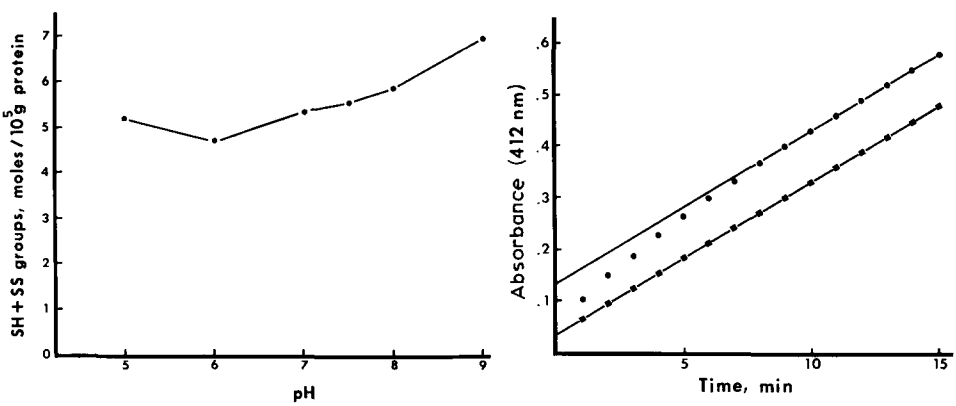


Fig. 3. Titration of SH and S–S groups of membrane proteins with $^{203}$Hg-labeled PCMB in the presence of 0.2 M sulfite and 8 M urea at different pH values. Three buffer systems were used in the pH range from 5.0 to 9.0; for pH 5.0, 0.2 M acetate; from pH 6.0 to 7.0, 0.2 M phosphate; and from pH 7.5 to 9.0, 0.2 M Tris–HCl. Incubation and filtration procedures are described in Methods and Materials. Washing of adsorbed membrane proteins was performed with the respective buffer solutions used for adjusting the pH.

Fig. 4. Spectrophotometric determination of membrane protein disulfides with dithiothreitol and DTNB. Methods are described in the text. The absorbance resulting from free SH groups and reduced disulfides was determined by extrapolation of the linear portion of the curve to the time of addition of DTNB (●) and subtraction of the value obtained for a sample containing no protein (■).

transparent solution of membranes. This could be achieved by the use of sodium dodecyl sulfate. The change in absorbance with time for the first 10 min of incubation following the addition of DTNB to solubilized membranes in which the accessible disulfide bonds were initially reduced by dithiothreitol is shown in Fig. 4. The rate of release of CNTP resulting from the reaction of DTNB with protein monothiols was considerably faster than that resulting from the reaction of dithiothreitol released from the dithiothreitol–arsenite complex, with the disulfide reagent. After 10 min

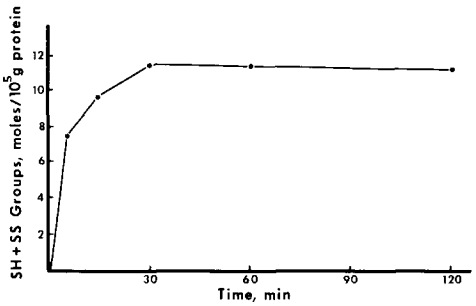


Fig. 5. Time course for the reduction of platelet membrane protein disulfides by dithiothreitol at pH 9.0. Methods were standard, but with the time of reduction varied as shown.

TABLE I

NUMBER OF SH AND S–S GROUPS OF PLATELET MEMBRANE PROTEINS

| *Reactive groups* | *Reagent for SH determination* | |
|---|---|---|
| | *$^{203}$Hg-labeled PCMB (10)** | *DTNB (4)** |
| SH groups | | |
| Native membrane proteins | 4.7 ± 0.5 | 3.1 ± 0.9 |
| Denatured membrane proteins | 4.7 ± 0.5 | 6.1 ± 1.1 |
| SH + S–S groups | 7.6 ± 1.1 | 11.3 ± 1.5 |
| S–S groups | 2.9 | 2.6 |

* Number in parentheses indicates the number of experiments performed.

of incubation blank and protein-containing sample had linear and parallel increases in absorbance thus allowing extrapolation to zero time of the curve representing the experimental sample. The time allowed for reduction with dithiothreitol of accessible protein disulfide bonds was critical for their quantitative assay. Incubation times of less than 30 min were found insufficient for complete reduction of S–S groups (Fig. 5). The total number of free thiols including those already reactive in non-denatured membranes and those reactive only after denaturation and solubilization of membrane proteins differed markedly with the two sulfhydryl reagents used (Table I). The number of disulfide groups, however, was very similar with the two methods employed.

## DISCUSSION

Our studies were mainly directed towards the development of methods for the quantitative determination of membrane disulfide bonds. Based on the results reported we feel that this goal has been realized. Sulfite as a cleaving agent for disulfide groups has been studied extensively both with simple disulfides such as cystine or oxidized glutathione and with protein disulfides[7]. The presence of 8 M urea has been found essential for allowing sulfite to react with all disulfide bonds in a soluble protein[8]. In bovine serum albumin which contains 17 disulfide bonds, none reacted with sulfite in the native protein[9]. When albumin was denatured with urea or guanidine, however, the extent of the reaction with sulfite was found to depend solely on the pH of the reaction mixture. The pH of our system was far in the alkaline range. At such pH the reaction between sulfite and S–S bonds is essentially a bimolecular one in that no or very little bisulfite anion is present in the incubation mixture[10]. The latter has been found to have very limited reactivity with disulfide bonds and only at pH less than 6.5. As the cleavage of S–S bonds by sulfite is reversible the concentration of sulfite must be high and that of the thiol anion low. By keeping the pH down to a value where the thiols are essentially unionized or by removing the SH groups in the form of heavy metal mercaptides one can make sulfitolysis go to completion. The second method which was found to be applicable to quantitative assay of membrane disulfide bonds was based on the technique of Zahler and Cleland[5]. Its sensitivity is double that of sulfitolysis followed by mercaptide formation by virtue of the fact that twice as many monothiols are produced after reductive cleavage by dithiothreitol which are then available for reaction with SH reagents. Adaptation of this disulfide assay, initially described for dithiol compounds, for the determination of protein S–S bonds depends upon linearity of the reaction of DTNB with protein sulfhydryl groups. Bovine serum albumin was found to react in a nonlinear manner both in the presence and absence of urea thus extrapolation to zero time of the release of thiol anions from DTNB was not possible[5]. In our studies of platelet membranes solubilized with sodium dodecyl sulfate the reaction rate of DTNB with protein-thiols paralleled that with dithiothreitol resulting from the dissociation of the dithiothreitol–arsenite complex after 10 min of incubation. Deduction of the free SH groups reactive in the absence of dithiothreitol from the values obtained after reductive cleavage with this agent allowed calculation of the number of disulfide bonds present. The time course of the reduction of membrane protein disulfides by dithiothreitol was similar to that observed with dithiol compounds although the time interval at which complete reduction of all available S–S bonds was achieved was somewhat longer in solubilized membranes. It has been reported that the thiol anion produced by the reaction of free sulfhydryl groups with DTNB is capable of cleaving disulfide bonds[11]. This claim supported by experiments with three soluble proteins could not be confirmed by our studies with solubilized membrane proteins. Further evidence against it has recently been published[12].

The importance of disulfide bonds in maintaining the catalytic function of certain enzymes and hormones has been well documented[13]. A recent report implicated disulfide bonds in preserving the serologic activity of Australia antigen[14]. It is thus apparent that intact S–S bonds are responsible for the maintenance of a variety of structural and functional aspects of protein molecules.

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