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# TOPOLOGY OF MEMBRANE SULFHYDRYL GROUPS IN THE HUMAN ERYTHROCYTE

## DEMONSTRATION OF A NON-REACTIVE POPULATION IN INTRINSIC PROTEINS

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## Summary

A major fraction of the protein sulfhydryl groups of human erythrocyte membranes can be oxidized to disulfide bonds by the lipid soluble reagent, diamide, and the hydrophilic reagent, tetrathionate. Furthermore, the same fraction also reacts with the monofunctional reagent, *N*-ethylmaleimide. About 20% of the SH groups, however, do not react with any of these agents even upon prolonged treatment and increased concentrations.

These 'non-reacting' SH groups were now localized by a procedure involving blockage of the accessible SH groups by non-labelled *N*-ethylmaleimide or by diamide, subsequent isolation and solubilization of the membranes in SDS and labelling of the now accessible, residual SH groups with *N*-[*ethyl*-2-$^3$H]ethylmaleimide.

The distribution of the radioactivity over the peptide fractions shows that the non-reacting SH groups are mainly localized in the intrinsic proteins, while essentially all of the SH groups of the extrinsic protein, spectrin, are reactive.

After solubilization of the membranes with Triton X-100 the non-reacting SH groups became reactive towards *N*-ethylmaleimide. It is proposed that lack of reaction of SH groups in the native membranes is due to their localization within the hydrophobic core of the membrane.

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Abbreviation: SDS, sodium dodecyl sulfate.

## Introduction

SH groups of soluble proteins readily react with a great variety of alkylating and oxidizing agents [1—6]. The same is true for membrane proteins, e.g. of the erythrocyte. Permeable monofunctional as well as bifunctional SH reagents, were shown to react with a large number of membrane SH groups in intact erythrocytes as well as in ghosts [1—3,5,6], while impermeable SH reagents have access to a small number of SH groups, located on the external membrane surface [4].

In previous studies we could demonstrate that up to 80% of the membrane SH groups are readily oxidized, e.g. by diamide, to disulfide bonds in the intact cell, indicating that pairs of SH groups are permanently or temporarily very close to each other [5,6]. Lack of reactivity of the remaining 20% of the membrane SH groups towards oxidizing agents might simply be due to a non-pairwise arrangement of these SH groups. Alternatively, non-reactivity might be due to factors such as an unfavorable microenvironment or localization of the SH groups deep within the protein matrix. In an attempt to clarify this problem, we now quantitatively determined the reaction of SH groups with monofunctional SH reagents. In agreement with the previous results for oxidizing agents, the alkylating SH reagent *N*-ethylmaleimide did not react with about 20% of membrane SH groups. Moreover, a major fraction of these non-reactive SH groups could be shown to be located in intrinsic membrane proteins.

## Methods

*Incubation procedure*

Erythrocytes from freshly collected heparinized human blood were washed three times with 154 mmol · $l^{-1}$ NaCl and ghosts prepared according to Dodge et al. [7]. 1 vol. of erythrocytes was suspended in 10 vol. of a medium containing (mmol · $l^{-1}$): KCl (90), NaCl (45), sodium phosphate (10) and sucrose (44) (medium A). Ghosts were suspended in 5 mmol · $l^{-1}$ sodium phosphate buffer (medium B).

Subsequently, one of the following SH reagents: iodoacetamide, 4,4′-dithiodipyridine, *N*-ethylmaleimide, tetrathionate (Fluka), cystine dimethyl ester (Sigma), ethyleneimine (ICN Pharmaceuticals) or diamide (diazinedicarboxylic acid bisdimethylamide, Calbiochem) was added and the suspension incubated (pH 8.0, 37°C) for different times. Concentrations of the reagents are indicated in the text. After the incubation erythrocytes and ghosts were washed three times with medium A and B, respectively, and ghosts prepared from the cells. In the case of incubations of ghosts with iodoacetamide and high concentrations of *N*-ethylmaleimide, removal of the reagents by this washing procedure appeared to be incomplete, resulting in a reaction of SH groups with residual reagent after solubilization of the membranes with SDS for the quantification of membrane SH groups (see below). In these cases ghosts were incubated with dithioerythritol (10 mmol · $l^{-1}$, 15 min, 37°C pH 8.0) subsequent to the treatment with the reagents, in order to bind the remaining free SH reagent, and then washed four times with medium B. Treatment of ghosts with dithioery-

thritol in this way does not cleave native disulfide bonds since the number of SH groups did not increase.

*Localization and quantification of non-reacting membrane SH groups*

Ghosts prepared from erythrocytes treated with SH reagents as described above were solubilized by the addition of 0.1 vol. of 20% (w/v) SDS in $H_2O$. This solubilization in SDS is generally accepted to expose buried SH groups [8—10]. SH groups were quantified by 5,5'-dithiobis(2-nitrobenzoic acid) as described before [12] or by labelling with *N*-[*ethyl*-2-$^3$H]ethylmaleimide. To this latter end 1 ml of solubilized ghosts were treated with 1 ml of medium B (pH 8.0) containing 1 mmol · $l^{-1}$ *N*-[*ethyl*-2-$^3$H]ethylmaleimide (specific radioactivity 92.5 · $10^{10}$ Bq · $mol^{-1}$) for 15 min at 37°C. Under these conditions all residual SH groups react with *N*-ethylmaleimide, as shown by the lack of reaction with 5,5'-dithiobis(2-nitrobenzoic acid). The labelled proteins were subjected to electrophoresis on gels containing 5.0% acrylamide, 0.1% *N,N'*-methylenebisacrylamide and 1.0% SDS according to Fairbanks et al. [11], but omitting disulfide-reducing agents. Gels were stained with Coomassie blue, destained and sliced according to the staining profile [12]. From the amount of protein applied to the gel, the total radioactivity on the gel and the specific radioactivity of *N*-ethylmaleimide, the number of SH groups per mg of protein was calculated [12].

The distribution of free SH groups over the different peptide fractions was calculated from the radioactivity of complete defined bands on the gel as described before [12].

*Reactivity of SH groups after membrane solubilization with Triton X-100*

1 vol of ghosts [7] was mixed with 1 vol of medium B (pH 8.0) containing 2% Triton X-100 and 5 mmol · $l^{-1}$ dithioerythritol. This thiol was added to block oxidants present as contaminants in Triton X-100. After 5 min one sample was added to 8 vol. of medium B containing 1% Triton and 15 mmol · $l^{-1}$ in dithioerythritol and incubated for 30 min at 37°C. After concentration (7X) by ultrafiltration (Amicon PM 10), Triton X-100 and the excess of dithioerythritol were removed by column chromatography on Sephadex G-200 using Tris-HCl buffer (pH 8.0) containing 1% SDS (column 1 X 50 cm). The peptide fraction was concentrated by ultrafiltration and protein SH groups quantified [12].

## Results and Discussion

In previous studies we obtained evidence that about 20% of the membrane SH groups of erythrocytes and ghosts cannot be oxidized by diamide. In order to investigate the possibility of a more complete reaction of membrane SH groups with other SH reagents, we extended our studies to monofunctional SH reagents, such as *N*-ethylmaleimide, iodoacetamide and cystine dimethyl ester. As becomes evident from Fig. 1, the total number of SH groups which can be modified in ghosts greatly depends on the type of reagent used. Iodoacetamide, which reacts by a substitution mechanism, combines with 50% of the membrane SH groups within 1 h. This percentage does not increase upon prolonged incubation. Cystine dimethyl ester, a disulfide agent reacting with SH groups

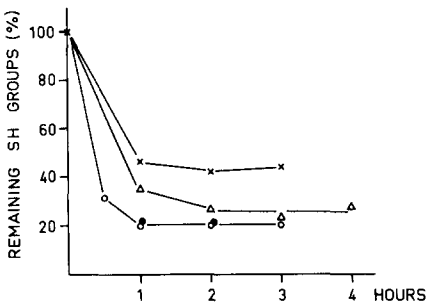


Fig. 1. Time dependency of the reaction of membrane SH groups of ghosts with SH reagents. Ghosts [7] were treated with 40 mmol · $l^{-1}$ iodoacetamide (X), 20 mmol · $l^{-1}$ cystine dimethyl ester (△), 20 mmol · $l^{-1}$ diamide (●) or 10 mmol · $l^{-1}$ *N*-ethylmaleimide (○), then washed and solubilized with SDS. Non-reactive SH groups were then quantified as described in Methods.

via a disulfide-sulfhydryl interchange mechanism, binds to a maximum of about 75% of the membrane SH groups. The most effective reagents were *N*-ethylmaleimide, which acts via an addition mechanism and, as previously shown, diamide. Both reagents react with 80% of the membrane SH groups within 1 h.

A treatment of ghosts with *N*-ethylmaleimide after a diamide treatment, or with diamide after a treatment with *N*-ethylmaleimide did not diminish the number of SH groups below the 17—20% resistant to treatment with only one of the two reagents. This indicates that it is the same fraction of about 20% of the SH groups that does not react with either of the two reagents.

An increase of the exposure time (Fig. 1) or of the concentration of *N*-ethylmaleimide (Table I) did not increase the number of reactive SH groups. Moreover, as shown in Table II for intact erythrocytes, the same percentage of SH groups is reactive to lipid soluble agents (diamide, *N*-ethylmaleimide, 4,4′-dithiodipyridine) and to the hydrophilic anionic reagent, tetrathionate ($S_4O_6^{2-}$), whereas the positively charged reagent ethyleneimine only reacts with about 30% of the SH groups. A comparison of the data for ghosts (Fig. 1, Table I) and erythrocytes (Table II) shows a slower rate of reaction in the intact erythrocyte compared to that in ghosts. This difference is probably due to a limiting role of the penetration rate of the reagent into the cells and to competition

TABLE I

CONCENTRATION DEPENDENCY OF THE REACTION OF *N*-ETHYLMALEIMIDE WITH MEMBRANE SH GROUPS

Ghosts [7] were exposed (37° C, 1 h, pH 8.0) to 10 or 40 mmol · $l^{-1}$ *N*-ethylmaleimide and membrane SH groups quantified as described in Methods.

| *N*-Ethylmaleimide (mmol · $l^{-1}$) | SH groups remaining | |
|---|---|---|
| | nmol/mg protein (mean value ± S.E.) | % |
| 0 | 89.6 ± 0.9 ($n$ = 35) | 100 |
| 10 | 17.3 ± 1.5 ($n$ = 4) | 19.3 |
| 40 | 18.1 ± 2.0 ($n$ = 4) | 20.2 |

TABLE II

DECREASE OF MEMBRANE SH GROUPS OF ERYTHROCYTES INCUBATED WITH VARIOUS REAGENTS

1 vol. of erythrocytes was treated (pH 8.0, 37°C), with 10 vol. of medium A containing one of the reagents listed below. After various time intervals, cells were washed three times, ghosts prepared and membrane SH groups quantified [12].

| Reagent | Incubation conditions | | % SH groups remaining (±S.E.) | Number of experiments |
|---|---|---|---|---|
| | Concentration (mmol · $l^{-1}$) | Time (min) | | |
| None | — | 0 | 100 | 12 |
| Ethyleneimine | 20 | 90 | 67.9 | 2 |
| | 20 | 150 | 68.8 ± 4.8 | 3 |
| Dithiodipyridine | 2 | 60 | 36.0 ± 3.9 | 5 |
| | 2 | 150 | 21.4 | 2 |
| *N*-Ethylmaleimide | 10 | 60 | 27.0 ± 0.3 | 3 |
| | 10 | 120 | 18.7 | 1 |
| Diamide | 5 | 60 | 37.0 ± 1.5 | 6 |
| | 5 | 120 | 25.8 ± 2.1 | 3 |
| Tetrathionate | 20 | 60 | 48.5 ± 3.3 | 6 |
| | 20 | 120 | 29.8 ± 2.4 | 3 |
| | 20 | 180 | 20.9 ± 0.9 | 3 |

between membrane SH groups and SH groups of glutathione and hemoglobin for the reagent.

The amounts of reactive SH groups are obviously very similar in ghosts and in intact cells. The same extent of reaction suggests that membrane SH groups are equally accessible to SH reagents in erythrocytes and ghosts and that thus no additional shielding of SH groups seems to exist in the membrane of intact cells as compared to ghosts.

In order to establish possible reasons for the lack of reaction of 20% of the SH groups we studied the distribution of this fraction over the different membrane proteins. For this purpose reactive SH groups were first blocked by a pretreatment of ghosts with *N*-ethylmaleimide or diamide. After removal of the excess of reagents by washing, the remaining unreacted SH groups were exposed by solubilization of the membranes in SDS and labelled with *N*-[*ethyl*-2-$^3$H]ethylmaleimide. After gel electrophoresis, the total activity on the gel and activities of complete bands were determined. Calculation gives a number of 13 ± 4 nmol of non-reactive SH groups per mg of membrane proteins (±S.D. $n = 4$). This amount is about equal to that quantified by 5,5′-dithiobis(2-nitro benzoic acid) (Table I).

The agreement between the number of SH groups determined by a specific [9] SH reagent, 5,5′-dithiobis(2-nitrobenzoic acid), and by the less specific reagent [13], *N*-ethylmaleimide makes a possible reaction of *N*-[*ethyl*-2-$^3$H]-ethylmaleimide with amino groups rather unlikely. Evidence against such a side reaction also comes from the lack of reaction of labelled *N*-ethylmaleimide with spectrin (Table II) under our conditions. As a final argument against the reaction of *N*-ethylmaleimide with $NH_2$ groups under our labelling conditions

(low concentrations of *N*-ethylmaleimide, short exposition times) no decrease of amino groups, as determined by 2,4,6-trinitrobenzenesulfonate [14], could be detected.

As may be derived from Table III, the extrinsic proteins, band 1, 2, 4.1, 5 and 6 (nomenclature according to Steck [15]) are labelled only to a low extent (25% of the total), whereas intrinsic proteins, band 3 and 4.5, contain most (75%) of the label in the 'non-reactive' fraction. This result was obtained for ghosts pretreated with both, the monofunctional SH reagent *N*-ethylmaleimide and the SH-oxidizing agent diamide. According to the data of Table III essentially all SH groups of the major extrinsic protein, spectrin (bands 1 and 2), react. This means that the 20 SH groups of band 1 and the 18 of band 2 [16] are accessible to *N*-ethylmaleimide.

Even more interesting than the reaction of all the SH groups of spectrin with *N*-ethylmaleimide is the complete oxidation by diamide of its SH groups to disulfide bonds, which demonstrates that SH groups of spectrin are arranged pairwise or come temporarily very close to each other. Previously, we have shown [5] that SH groups of spectrin can be oxidized to intermolecular disulfide bonds between spectrin monomers. The number of such intermolecular disulfide bridges, however, could not be determined. Furthermore, we found [6] that in intact erythrocytes six SH groups of spectrin can form mixed disulfide bonds with the intracellular glutathione. Our present data show, that in ghosts, i.e. in the absence of glutathione, these six SH groups can also be oxidized to either inter- or intramolecular disulfide bonds. This observation supports our previous proposal that an arrangement of SH groups in pairs may be important to trap free radicals during oxidative stress of erythrocytes, preventing irreversible damage of sensitive membrane structures.

For other extrinsic membrane protein fractions, band 5 and band 6, the number of non-reactive SH groups per protein molecule is also very low, namely 0.4 out of 5 [18] and 3 [17], respectively. This lack of reaction may be

TABLE III

DISTRIBUTION OF 'NON-REACTIVE' SH GROUPS OF GHOSTS AMONG MEMBRANE PROTEIN FRACTIONS

Ghosts were treated with 10 mmol · $l^{-1}$ *N*-ethylmaleimide or 20 mmol · $l^{-1}$ diamide, washed and solubilised in SDS. Non-reactive SH groups were then labeled with *N*-[*ethyl*-2-$^3$H]ethylmaleimide, peptides separated by gel electrophoresis and radioactivity of defined bands determined (see Methods for details).

| Band number | % of radioactivity (mean value ± S.E.) | | Number of SH groups remaining per protein molecule after reaction of ghosts with *N*-ethylmaleimide (mean value ± S.E.; $n = 4$) |
|---|---|---|---|
| | Diamide ($n = 2$) | *N*-Ethylmaleimide ($n = 4$) | |
| 1 + 2 | 5.5 | 3.4 ± 1.6 | <0.2 |
| 3 | 36.7 | 34.5 ± 2.7 | 1.2 ± 0.1 |
| 4.1 | 6.5 | 4.0 ± 2.5 | 0.5 ± 0.2 |
| 4.2 | 6.5 | 4.2 ± 1.2 | 0.7 ± 0.2 |
| 4.5 | 37.0 | 44.1 ± 2.8 | 3.6 ± 0.7 |
| 5 | 3.7 | 3.4 ± 0.7 | 0.4 ± 0.1 |
| 6 | 3.0 | 4.8 ± 1.2 | 0.3 ± 0.1 |
| 7 | 1.7 | 2.1 ± 0.2 | <0.2 |

due to a slow reaction of one SH group of these proteins. Indeed for glyceraldehyde-3-phosphate dehydrogenase (band 6) it has been shown that a major fraction of its SH groups is very reactive, whereas a minor fraction is only slowly reacting (Ref. 19, p. 16).

In contrast to the almost complete reaction of SH groups of extrinsic proteins, SH groups of intrinsic proteins are obviously less reactive. For the major intrinsic protein, band 3, 1.2 SH groups out of 6 do not react with *N*-ethylmaleimide. This single SH group is most probably located in the hydrophobic membrane-spanning chymotryptic 17 000 dalton fragment [20] of band 3. This claim rests on the following two pieces of evidence. Firstly, the 17 000 dalton fragment has been shown to contain one of the six SH groups of band 3 [20]. Secondly, only five of the six SH groups of band 3 were found to react with *N*-ethylmaleimide [21], all of them located in proteolytic fragments other than the 17 000 dalton fragment.

Surprisingly, the band 4.5 fraction, which is constituted by numerous peptides [22], even contains a mean number of 3.6 non-reactive SH groups per molecule on the basis of a molecular weight of 55 000. Accepting that, in analogy to other membrane proteins, 1 mol% of the amino acids of the band 4.5 proteins are cysteine residues, proteins of this fraction would have 4—5 SH groups per molecule, almost all of them non-reactive.

The lack of reactivity of 20% of the membrane SH groups might be due to the fact that these groups are deeply buried within the protein matrix or that they are shielded by the electrostatic properties or the hydrophobicity of the environment of these SH groups (Ref. 19, pp. 311—332). In order to investigate to what extent shielding of SH groups by adjacent lipids is responsible for the non-reactivity observed, reaction of SH groups was studied after solubilization of the membrane by Triton X-100, a detergent known not to denaturate erythrocyte membrane proteins such as the inorganic anion-exchange system located in the band 3 fraction and the monosaccharide carrier located in the 4.5 fraction [23,24]. Only 3.9 ± 0.9 nmol SH groups/mg protein (±S.E.; $n = 4$) were found to remain in ghosts solubilized with Triton X-100 in the presence of *N*-ethylmaleimide as compared to 96 ± 16 nmol in ghosts solubilized in the absence of the reagent. The exposure of SH groups by Triton, therefore, may indicate that the segments of the band 3 and 4.5 protein bearing these groups are localized in a domain of the membrane, which is perturbed by the detergent. It seems rather unlikely that this domain consists of the lipids surrounding the intrinsic proteins, since solubilization by the detergent merely replaces native lipid by detergent without altering the hydrophobic environment. As a more likely alternative, the enhanced reactivity of the SH groups after solubilization by Triton could be explained by the disruption of protein-protein interactions shielding SH groups in the native membrane.

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