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DYNAMIC CHANGES OF RED CELL MEMBRANE THIOL GROUPS FOLLOWED BY BIMANE FLUORESCENT LABELING

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Summary

Monobromobimane labels red cell membrane protein thiol groups; bands exhibit fluorescence after sodium dodecyl sulfate acrylamide gel electrophoresis and correspond to almost all of those staining with Coomassie blue. The response of membrane protein thiol groups to oxidative challenge and the dynamics of recovery of the thiol groups may be followed. Diminished labeling is found after oxidation with diamide, with both intrachain and interchain disulfide bond formation demonstrated by sodium dodecyl sulfate acrylamide gel electrophoresis. Regeneration of thiol groups under physiological conditions (incubation with glucose) after a moderate degree of diamide oxidation is shown to be complete (with respect to thiol group content and degree and distribution of bimane label) in normal human red blood cell membranes. Even after oxidation of almost half of the membrane protein thiol groups (maximum degree of oxidation achieved), regeneration of thiol groups is almost complete; a minor fraction resides in the form of disulfide-linked high molecular weight proteins (demonstrated by the electrophoretic profile) which may be reduced completely with dithiothreitol.

Bimane fluorescent labeling provides a convenient and sensitive method for following membrane thiol group status under physiological conditions.

Abbreviations: SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid; diamide, diazene dicarboxylic acid bis(dimethylamide).

Introduction

We have recently described a new class of fluorescent labeling agents, the bromobimanes [1,2], and have shown that hemoglobin, glutathione (GSH), and membrane proteins of intact red cells were efficiently labeled under physiological conditions. Of the six thiol groups of hemoglobin, only the two reactive thiols were labeled, either in the intact cell or in solution [2,3]. Following SDS-acrylamide gel electrophoresis of the labeled membrane proteins, numerous bands were readily visible under ultraviolet illumination and easily photographed. The labeled materials (as lyophilized solids, in solution, or in gels) were stable to light and air, and resistant to fading under intense illumination (e.g., under examination with a fluorescence microscope). The identity of the groups labeled in the membrane was not established.

We have now found that all, or essentially all, of the labeled groups in the membrane are thiol groups. Furthermore, it is convenient and simple to follow the dynamics of changes in the content and distribution of membrane protein thiol groups after exposure of the cells under physiological conditions to various challenges (oxidation or alkylation) and to quantitate the recovery from oxidation. The appearance of high molecular weight intermolecular disulfides after oxidation and their disappearance after reduction are easily revealed by the bimane fluorescence labeling technique. Bimane fluorescent labels provide a new means for probing the thiol status of cell membranes, for following the dynamics of the changes in that status, and for relating the thiol status of cell membranes to functional properties of the cell.

Materials and Methods

Red cells. Human blood, anticoagulated with heparin, was obtained from normal individuals. After centrifugation, the buffy coat was removed, the cells washed twice with buffer (135 mM NaCl/10 mM phosphate, pH 7.4) and resuspended in the same buffer.

Reagents. Monobromobimane (available from Calbiochem-Behring, La Jolla, CA) was stored as a 60 mM solution in CH_3CN in the dark. Under these conditions, the solutions appear to be stable for at least 2 months. Diamide (available from Calbiochem-Behring and others) was made up in the pH 7.4 buffer; sterile 10—20 mM solutions are stable for days in the refrigerator. *N*-Ethylmaleimide and dithiothreitol were dissolved in the buffer shortly before use.

Treatment of cells with reagents. Cell suspensions (7%) were mixed with diamide (0.5—2.0 mM) as previously described [4,5] and incubated at 37°C for 15—60 min. A similar procedure was used for 1—5 mM *N*-ethylmaleimide. Following treatment, cells were centrifuged, washed and resuspended in buffer. Aliquots were mixed rapidly with monobromobimane solution (15—30 μl/ml 7% cell suspension), incubated for 15—60 min at 37°C, centrifuged, then washed with buffer. Other aliquots were carried through the procedure without addition of monobromobimane, then used for the analysis of SH groups (see below). In some experiments, diamide-treated cells were incubated in the presence of glucose (10 mM) for 2 h, or with dithiothreitol (5 mM) for 1 h, then washed and labeled with monobromobimane as noted above.

Preparation and analysis of cell components. Red blood cell ghosts were prepared by using the procedure of Steck and Kant [6]. Ghosts were solubilized, the solution boiled, then cooled, and samples analyzed by SDS-acrylamide gel electrophoresis according to established methods [7,8] in the absence of dithiothreitol. After electrophoresis, the gels were fixed in methanol/acetic acid/water and photographed under ultraviolet (360 nm) illumination. (Gels may be stored for 1 week or more prior to photography.) The gels were then stained with Coomassie blue, washed and photographed again. In some experiments, gels were stained through periodic acid cleavage-Schiff base formation (PAS) [7], in order to reveal the position of the glycoproteins. Densitometric measurements of fluorescence intensities were made on clear, enlarged photographs (Kodakolith), while similar measurements on the stained gels was made in the same way or directly on the gel, in all cases with a Gilford densitometry attachment. Aliquots of solubilized membrane were bleached (H_2O_2) and utilized for absorbance and fluorescence measurements (absorption maximum, 380—385 nm; fluorescence emission maximum 480—485 nm), using, respectively, a Beckman spectrophotometer and a Perkin-Elmer-Hitachi MPF-4 spectrofluorimeter. Thiol group analysis (membrane proteins) was carried out according to the method of Habeeb [9], using membranes isolated from cells which had not been treated with monobromobimane. The hemoglobin and non-protein thiol groups were analyzed as previously described [10]. Protein analysis [11] was carried out on samples of both labeled and unlabeled membranes.

Results

Relationship of membrane protein labeling to membrane thiol groups in intact cells. Red cells were treated with either diamide or *N*-ethylmaleimide, then both treated and untreated (control) cells were labeled with monobromobimane. The results are shown in Table I. Efficient labeling was achieved by incubation of the cells with 15 μmol monobromobimane/ml red cells for 15 min at 37°C, with maximum labeling being reached with 24—28 μmol monobromobimane/ml packed red cells for 45—60 min; under conditions in which maximum labeling was attained, the absorbance of the monobromobimane-labeled proteins was 0.30—0.35 absorbance units/mg protein per ml at 385 nm. Pretreatment of the cells with *N*-ethylmaleimide diminished both the extent of labeling by monobromobimane and the membrane thiol group content, the reduction in fluorescence intensity being 50—80% along with respective decreases in SH content of 50—80%. Pretreatment with the thiol-oxidizing agent, diamide (7—28 μmol/ml packed cells), diminished membrane fluorescence by 30—70% and membrane thiol content by 20—50%. The percent diminution in monobromobimane labeling of cells after diamide treatment was the same for cells labeled with small (12 μmol/ml cells) or large (28 μmol/ml cells) amounts of monobromobimane using incubation periods between 15 and 60 min.

Aliquots of diamide-treated cells were further incubated with glucose or dithiothreitol, the samples centrifuged, the cells washed, then labeled with monobromobimane. The results are summarized in Table I. Glucose treatment

TABLE I

MONOBROMOBIMANE LABELING OF INTACT HUMAN RED BLOOD CELLS: MONOBROMOBIMANE LABELING OF MEMBRANE PROTEINS IN RELATION TO MEMBRANE THIOL GROUP CONTENT

Expt. No.	Reagent	μmol/ml cells	Length of treatment (min)	Membrane SH groups remaining (by absorbance)		Groups labeled with monobromobimane (by fluorescence) (%) [c]
				% [a]	% [b]	
1A	diamide	7	45	80	68	70
B	diamide	14	15		54	59
C	diamide	14	45	64	43	52
2A	diamide	14	15		57	57
B	diamide	14	30	59	56	56
3A	diamide	14	15	68	64	57
B	diamide	28	15	60	43	50
C	diamide	28	60	52	30	34
1A	diamide/glucose [d]	(7)	(45)			
			120	98	98	97
2A	diamide/glucose	(14)	(15)			
			120	94	80	83
B	diamide/glucose	(14)	(30)			
			120		88	87
3B	diamide/glucose	(28)	(15)			
			120	86	79	76
2B	diamide/dithiothreitol (5 mM)	(14)	(30)			
			60	98	100	110 [e]
3B	diamide/dithiothreitol (5 mM)	(28)	(15)			
			60	102	106	110
4	*N*-ethylmaleimide	14	15	50	43	51
	N-ethylmaleimide	70	30	34	32	30
5	*N*-ethylmaleimide	70	60	22	19	25

[a] Membrane SH groups determined by DTNB analysis (Ref. 9) after the designated treatment divided by the number found for membrane SH groups from untreated cells × 100. The number measured for the control cells incubated with or without glucose for 60—120 min was 74—88 nmol/mg protein.

[b] Cells were treated as specified, then incubated at 37°C for 15—60 min with 12—28 μmol monobromobimane/ml red cells; membrane was isolated for the determination of optical absorption and fluorescence emission. Control cells exhibited optical absorptions of 0.18—0.35 absorbance units/mg protein per ml.

[c] Fluorescence intensities for treated membranes divided by fluorescence intensities for control cells from samples prepared as described in footnote b, then multiplied by 100.

[d] Incubations were carried out with 10 mM glucose. The parentheses are used to set off numbers recorded earlier in the table but repeated to identify the nature of the treated sample.

[e] Dithiothreitol leads to the appearance of SH groups not detected before treatment. Either such thiol groups were 'hidden' and did not react with the DTNB reagent used for the analysis, or they were generated from disulfide bonds present in the original preparation.

resulted in essentially full recovery (greater than 95%) of the capacity for monobromobimane labeling of samples to which the lower concentrations of diamide had been applied, accompanied by greater than 95% recovery of SH groups. For samples treated with higher concentrations of diamide, incubation with glucose led to a recovery in monobromobimane labeling capacity of about 80—90% of control values, with SH content also rising to 85—95% of that of the control. Complete recovery of monobromobimane labeling capacity and SH group content could be accomplished by incubation of the treated samples

with dithiotheirol.

It may be noted that intracellular glutathione, which was oxidized completely by diamide treatment, was almost fully regenerated in the course of the glucose incubation after treatment with the lower concentrations of diamide and about 70% regenerated after treatment with the higher concentrations of diamide. The reactive SH groups of human hemoglobin were not reactive towards diamide at any of the concentrations which were used: 6—6.4 mol SH/mol hemoglobin were found in hemoglobin isolated from both control cells and diamide-treated cells.

Distribution of the monobromobimane label among membrane proteins. An SDS-acrylamide gel electrophoresis pattern for membranes from monobromobimane-labeled cells is shown in Fig. 1A and densitometric measurements are plotted in Fig. 2A. All fluorescent bands in the gels have Coomassie blue-stained counterparts. However, not all stained bands exhibit fluorescence. As may be seen in Figs. 1A and 2A, no fluorescence is observable in band 4.1. (The classification of the bands is done according to the enumeration of Fairbanks et al. [7].) Band 4.1 is apparently not a glycoprotein, since it is not stained by the periodic acid-Schiff base procedure. Under the conditions used for the electrophoresis shown in Fig. 1, the major membrane glycoprotein, PAS-1 (glycophorin), is located within the higher molecular weight portion of the band 3 region (not illustrated).

After alkylation by *N*-ethylmaleimide, and then labeling with monobromobimane, it was found that the fluorescence of the bands in the SDS-acrylamide gel sample diminished markedly in comparison to that of the control, while there was no change in the distribution of the Coomassie blue-stained bands (not shown).

Following oxidation with diamide, electrophoretic analysis of membrane samples shows that the distribution of the label as well as the distribution of the proteins has been altered (Figs. 1B and 2B). Less labeling is noted in all bands, but particularly in the spectrin bands; Coomassie blue staining shows that many of the bands, but especially spectrin 1 and spectrin 2, as well as bands 4.2 and 7, have decreased in concentration and there has been a significant increase in the high molecular weight protein at the top of the gels. The high molecular weight proteins are weakly fluorescent.

After incubation of the diamide-treated cells with glucose, the recovery of the capacity for monobromobimane labeling is seen by means of the gel electrophoresis patterns (Figs. 1C and 2C). For cases in which there had been (after diamide treatment) a 30% decrease in monobromobimane labeling and a 20% decrease in SH content, full recovery of the monobromobimane-labeled protein pattern is noted (not illustrated). For cases in which the diamide treatment had diminished the membrane SH content by 30—50%, incubation with glucose led to partial but not complete recovery of the control pattern. Especially noteworthy is the fact that some high molecular weight proteins remained, and that a significant fluorescence was associated with the high molecular weight protein. The increase in fluorescence of the spectrin 1 and spectrin 2 bands, as well as that of all other bands, is evident, as is the increase in the relative intensity of the staining of these bands.

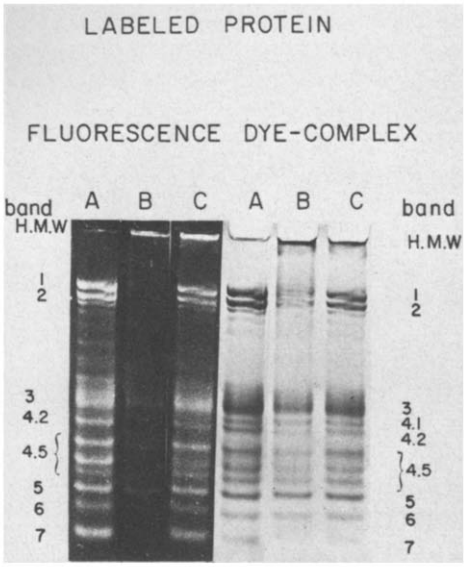


Fig. 1. SDS-acrylamide gel electrophoresis patterns of red blood cell membranes isolated from red blood cells after treatment with monobromobimane, diamide followed by monobromobimane, and diamide followed by glucose, then monobromobimane. Red cell suspensions (7%) were incubated without and with diamide at 37°C for 30 min, then washed. An aliquot of diamide-treated cells was further incubated in the presence of 10 mM glucose at 37°C for 120 min. All samples were incubated with monobromobimane (1.4 μmol/ml of 7% cell suspension) at 37°C for 15 min, washed and membranes isolated and processed as described in Materials and Methods. Samples were run on a slab gel, using 4% acrylamide. (Left) Fluorescence patterns. (Right) Patterns of dye-protein complexes. A, control cells; B, diamide-treated cells; C, diamide-treated cells, incubated with glucose. H.M.W., high molecular weight protein.

Incubation of diamide-treated cells with dithiothreitol results in the complete recovery of the capacity for monobromobimane labeling of the protein,

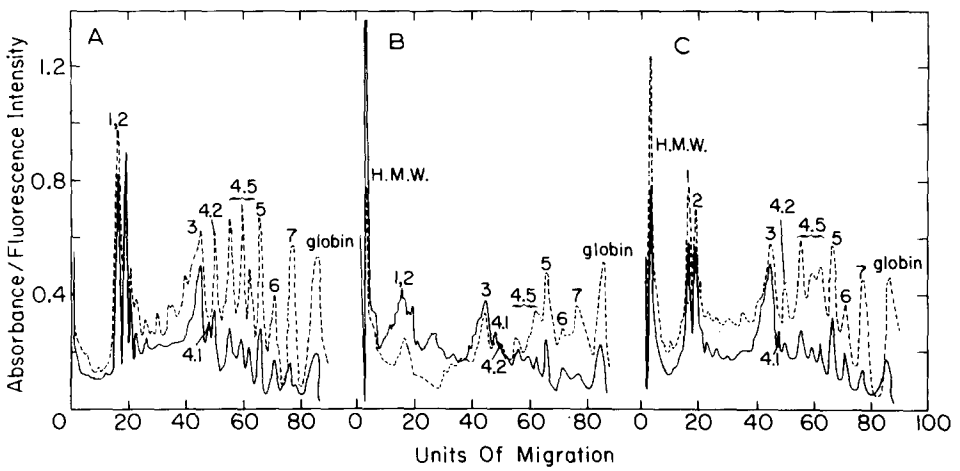


Fig. 2. Densitometric scans of protein distributions observed by either fluorescence or Coomassie blue staining on SDS-acrylamide gel electrophoresis. For experimental conditions, see Fig. 1. (A) Control; (B) diamide-treated cells; (C) diamide-treated cells, incubated with glucose. Fluorescence, - - - - -; protein-dye complex, ———. H.M.W., high molecular weight protein.

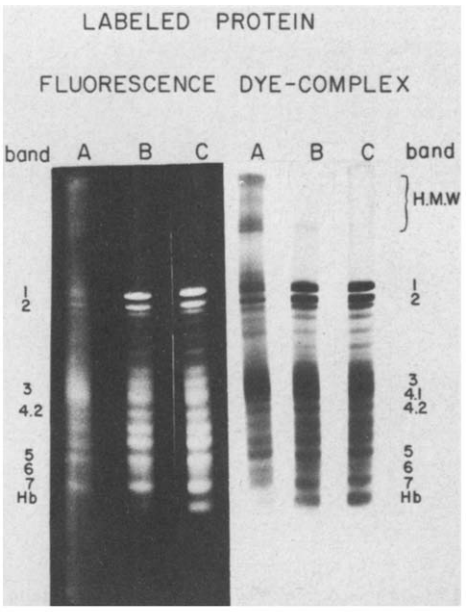


Fig. 3. Effect of dithiothreitol on the protein distribution produced by SDS-acrylamide gel electrophoresis samples of red cell membranes after diamide treatment, as observed by fluorescence or Coomassie blue staining. Red cell suspensions (7%) were incubated without and with 1 mM diamide at 37°C for 15 min, and washed. Aliquots of control and of diamide-treated cells were then incubated with 5 mM dithiothreitol at 37°C for 60 min, washed, resuspended and incubated with monobromobimane (1.4 μmol/ml of 7% cell suspension) at 37°C for 30 min. Cells were then washed and processed further as described in Materials and Methods. Samples were run on cylindrical gels, which contained 2.5% acrylamide and 0.3% agarose [8]. A, diamide-treated cells; B, diamide treatment followed by dithiothreitol; C, dithiothreitol-treated control cells. H.M.W., high molecular weight protein.

TABLE II

SUMMARY OF CHANGES IN MONOBROMOBIMANE LABELING OF RED CELL MEMBRANE PROTEINS AFTER OXIDATIVE OR OXIDATIVE AND REDUCTIVE TREATMENTS

HMW, high molecular weight protein.

Band No.	Diamide		Diamide/glucose	
	Protein [a]	Fluorescence [b]	Protein [a,c]	Fluorescence [b,c]
HMW	+++	+	+	+++
1,2	——	———	—	—
3	—	——	n	n
4.1 [d]	n	0 [d]	n	0 [d]
4.2	——	———	—	——
4.5 [e]	—	———	n	— [e]
5,6	—	——	n	n
7	—	——	n	—

[a] +++, significant increase (greater than 100%); +, modest increase; —, decrease by 10—30%; ——, decrease by 40—70%; n, close to normal.

[b] +++, significant increase (greater than 100%); +, modest increase; —, slight decrease; ——, decrease by 30—60%; ———, decrease by 70% or more.

[c] After dithiothreitol reduction of diamide-treated cells, the membrane protein distribution and fluorescence labeling pattern were identical to those of the control.

[d] No fluorescence was observed for this band in the control.

[e] A composite of several sub-bands; the middle sub-band showed much decreased labeling after diamide treatment and the labeling capacity was scarcely recovered after incubation of the cells with glucose.

and disappearance of the high molecular weight proteins; both the fluorescence and staining patterns of the proteins on the gels are similar to those of the control (Fig. 3). (The same result is obtained with diamide-treated cells after incubation with glucose followed by treatment with dithiothreitol.) The alterations in the protein profile and in the distribution of the monobromobimane label are summarized in Table II.

Discussion

The bimane fluorescent labeling of red cell membrane proteins under physiological conditions represents a convenient, new approach to various problems concerning membranes. At present, we will consider four aspects: (1) the nature of the labeled group; (2) the oxidation-induced changes in membrane proteins; (3) the reductive recovery from oxidative changes; and (4) the consequences of changes in the thiol status of red cell membranes.

Nature of labeled group. The results reported in the present article imply strongly that most or all of the groups labeled by monobromobimane are thiol groups. First, *N*-ethylmaleimide diminishes the labeling produced by monobromobimane to an extent parallel to the diminution of the thiol group content (as measured with DTNB). *N*-Ethylmaleimide leads only to alkylation of SH groups under the conditions used [12]. Second, the thiol-oxidizing agent, diamide [4], which oxidizes some membrane thiol groups in addition to intracellular GSH under the conditions used (i.e., very high concentrations, 10-times those necessary for the oxidation of red cell GSH) diminishes the extent of monobromobimane labeling in a fashion parallel to the extent of thiol oxidation. Third, dithiothreitol reduction of the disulfides produced by diamide oxidation (see below) results in complete recovery of the expected levels of monobromobimane labeling. Data are given in Table I.

The labeling agent, monobromobimane, apparently reacts with the same groups which are alkylated by *N*-ethylmaleimide, and with most of the SH groups remaining after treatment with diamide. The quantity of SH groups with which monobromobimane reacts is approx. 75—90 nmol/mg protein (assuming that the monobromobimane-labeled membrane protein has the same extinction coefficient, approx. 4000, as monobromobimane-labeled globin). The concentration of red cell membrane SH groups measured using DTNB titration on samples of membranes isolated under the conditions used in our experiments is 74—88 nmol/mg protein, in agreement with published values [13]. The concentration of SH groups in intact red cell membranes, obtained through measurement of the degree of labeling by *N*-[^{14}C]ethylmaleimide, has been reported as 84 ± 13 nmol/mg protein [12]. The quantitative agreement is sufficiently good to suggest that all, or almost all, of the groups labeled by monobromobimane are thiol groups and that these are the same groups alkylated by *N*-ethylmaleimide.

Oxidation-induced changes in membrane proteins. After oxidation of membrane thiol groups by diamide, the total amount of monobromobimane label introduced into the membrane protein is decreased. In addition, the distribu-

tion of fluorescent label among the membrane proteins changes, and the molecular weight distribution of the membrane proteins also changes. Bands 1 and 2 are the most seriously affected, decreasing markedly after diamide treatment as seen both by Coomassie blue staining and fluorescent labeling. New high molecular weight protein bands appear, and these are weakly fluorescent.

The changes in the distribution of proteins with respect to molecular weight can be ascribed to the formation of intermolecular disulfides, through cross-linking of SH groups via oxidation with diamide. The diminution in labeling without apparent change in molecular weight is probably due to the formation of intramolecular disulfide bonds. Both types of molecular change result from the diamide treatment. The mechanism of diamide oxidation of thiols [14] suggests the possibility that unstable sulfenylhydrazines could be formed within the membrane, but their stability is so low at pH 7.4 (estimated half-life, 10 s) that they should not be present at the time of analysis (after ghosts are produced), Such intermediates would only be important in the present context if no other thiol group were in the vicinity of the sulfenylhydrazine, a lack that would prevent the formation of a disulfide bond. Another possible consequence of diamide oxidation is that some thiol groups may become sterically inaccessible to the labeling agent because of conformational changes after disulfide formation. Thus, one may account for the fact that diamide apparently oxidizes fewer SH groups than is apparent from the diminution in monobromobimane labeling. Furthermore, we may have an indication of how many 'lonely but willing' (single and reactive) SH groups may be present in the membrane. The fact that there are some SH groups which are unreactive towards agents such as *N*-ethylmaleimide, although titratable with DTNB (in ghosts) [12], indicates the possibility of still more classes of SH group. We must regard the present study as suggestive of a number of classes of membrane SH group, and may expect that different SH reagents will react with different groups of membrane SH group, according to the dissociation constant and steric environment of the SH groups.

Reductive recovery from oxidative changes. After diamide-treated cells are incubated with glucose for 2 h, SDS-acrylamide gel electrophoresis reveals that the extent and distribution of the monobromobimane labeling depend upon the degree of the initial oxidation. For cell membranes in which a moderate fraction of the thiol groups had been converted to disulfides, recovery of the control pattern was complete, both with respect to extent of labeling and the distribution of that label among the various fractions separated by gel electrophoresis. For higher degrees of oxidation, recovery was less complete (less total label) and the distribution of the label appreciably different from that of the control. In particular, the high molecular weight protein remaining was appreciably labeled. That the appearance of a high molecular weight fraction was due to disulfide bond formation rather than other types of bonding was shown by the disappearance of the high molecular weight fraction on incubation of diamide-treated cells with dithiothreitol. (Diamide-treated cells first incubated with glucose, and then with dithiothreitol, yielded a similar sequence of SDS-acrylamide gel electrophoretic patterns.)

These experiments demonstrate that the normal human red cell membrane

can undergo considerable change in response to oxidative stress and still return to its original state, at least insofar as the thiol group population is concerned. That higher degrees of oxidative stress produce incompletely reversible changes is of considerable interest, and of especial importance in connection with regulating dosage levels of so-called 'oxidant drugs' (cf. Ref. 5) in various genetic diseases. Although there is no doubt a connection between membrane thiol status and intracellular thiol status, the enzymes and cofactors regulating this connection cannot now be specified.

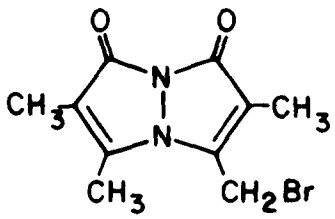
Consequences of changes in membrane thiol status. The alkylation and oxidation as well as the reactivity of thiol groups in several specific membrane proteins have been examined [12,13,15—22]; it is thought that an appropriate membrane thiol status is required for normal functioning of the human red cell. Fischer et al. [23] have found that the introduction of a small number of intermolecular disulfide links in red cell spectrin produces a decreased deformability of that cell. The great importance of high molecular weight proteins in affecting the shape and deformability of the red cell has become evident in the work of Lorand and coworkers [24—26] on Ca^{2+}-induced membrane protein γ-glutamyl-ϵ-lysine cross-linking promoted by transglutaminase. Whether there is any connection between high molecular weight proteins produced by protein cross-linking and those produced through disulfide bond formation is not known. The latter may contribute to shape changes under conditions of metabolic deprivation [27,28]. Oxidation of a certain proportion of membrane SH groups enhances the susceptibility of some of the membrane phospholipids to phospholipases [15].

We have shown elsewhere that certain types of oxidative challenge to red cell membranes, in the absence of intracellular GSH, can lead to alterations in membrane lipids and eventually to the destruction of the cell membrane [29,30]. Given the reasonable presumption that there is a close connection between intracellular thiol status and membrane thiol status, we can see that changes in the thiol status of the cell as a whole may affect the cell shape, deformability, and structural integrity as well as the enzymatic and transport functions of the cell membrane.

Implications of bimane labeling technique. The fact that monobromobimane is readily reacted with intact cells under physiological conditions along with the facility with which one may observe, measure and manipulate the labeled sample, makes the bimane labeling technique a method of choice for many problems in which labeling is used. Although a number of other reagent types have been used for the study of membrane thiol groups, including maleimides, iodoacetamides, disulfides and mercury derivatives (see Ref. 31 for review), no detailed application of these agents to intact cells has been published. A coumarinylmaleimide has been reported to give promising results in the labeling of protein thiol groups [32].

A number of other advantages of bimanes may also be mentioned. The bimane moiety is small (see formula for monobromobimane below; the Br is replaced by RS after reaction with the thiol) and should cause a relatively small perturbation in protein structure, and therefore in biological activity.

The bimane group is stable to many chemical and biochemical procedures, and peptide mapping has been successfully carried out on the β-chain (labeled at cysteine-β-93) isolated from the labeling of intact human red cells [3]. The bimane label is relatively stable under the conditions used for photography and fluorescence microscopy. Various chemical modifications which should make the bimane moiety more specific with respect to particular receptors are fairly easy to carry out. In the present work, we have shown how useful the bimane labeling technique is for revealing various aspects of reaction stoichiometry and dynamic changes for membrane thiol groups.



Monobromobimane

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