Biochimica et Biophysica Acta, 691 (1982) 345–352
Elsevier Biomedical Press

BBA 71368

MEMBRANE THIOL-DISULFIDE STATUS IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENT RED CELLS

RELATIONSHIP TO CELLULAR GLUTATHIONE

NECHAMA S. KOSOWER, YEHUDIT ZIPSER and ZEHAVA FALTIN

Department of Human Genetics, Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv, Tel-Aviv 69978 (Israel)

(Received March 10th, 1982)

Key words: Membrane protein; Thiol-disulfide status; Glutathione; Glucose-6-phosphate dehydrogenase; (Erythrocyte)

The behavior of glucose-6-phosphate dehydrogenase (G6PD)-deficient red cell membrane proteins upon treatment with diamide, the thiol-oxidizing agent (Kosower, N.S. et al. (1969) Biochem. Biophys. Res. Commun. 37, 593–596), was studied with the aid of monobromobimane, a fluorescent labeling agent (Kosower, N.S., Kosower, E.M., Newton, G.L. and Ranney, H.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3382–3386) convenient for following membrane thiol group status. In diamide-treated G6PD-deficient red cells (and in glucose deprived normal cells), glutathione (GSH) is oxidized to glutathione disulfide (GSSG). When cellular GSH is absent, membrane protein thiols are oxidized with the formation of intrachain and interchain disulfides. Differences in sensitivity to oxidation are found among membrane thiols. In diamide-treated normal red cells, GSH is regenerated in the presence of glucose and membrane disulfides reduced. In G6PD-deficient cells, GSSG is not reduced, and the oxidative damage (disulfide formation) in the membrane not repaired. Reduction of membrane disulfides does occur after the addition of GSH to these membranes. A direct link between the thiol status of the cell membrane and cellular GSH is thereby established. GSH serves as a reductant of membrane protein disulfides, in addition to averting membrane thiol oxidation.

Introduction

In individuals carrying one of the common varieties of glucose-6-phosphate dehydrogenase deficiency (G6PD deficient), no clinical effects are seen under normal conditions. Hemolytic crises are precipitated by certain drugs or infections. Under these conditions, red cell glutathione (GSH) is irreversibly oxidized to the disulfide (GSSG) due to the failure of NADPH regeneration in the deficient cells [1,2]. Similar perturbations in cellular GSH can be induced experimentally and the consequences studied [3].

A new class of fluorescent labeling reagents, the bromobimanes, derivatives of *syn*-9,10-dioxabimanes [4], has recently been described [5]. Reactive thiol groups of hemoglobin, GSH, and membrane proteins can be labeled readily under physiological conditions in the intact red cell [5–7]. The response of membrane proteins to oxidative challenge and the dynamics of recovery can be followed easily through labeling with monobromobimane [7]. Normal red cell membrane proteins form intrachain and interchain disulfides in response to oxidative stress but can recover to a large extent the original thiol status along with the reduction of GSSG [7].

We have now investigated the diamide oxidation of G6PD-deficient red cell membrane proteins. In these cells, GSSG is not reduced to GSH; we show here that oxidative damage (disulfide formation) in such G6PD-deficient red cell membranes is not repaired. The addition of GSH to ghosts prepared from diamide-treated cells results

in reduction of disulfides in the membrane proteins.

The results establish a direct link between the thiol status of the cell membrane and intracellular GSH, GSH functioning to repair oxidative damage (i.e., reduce disulfides) in addition to averting thiol oxidation in the membrane.

Materials and Methods

Red cells. Human blood, anticoagulated with heparin, was obtained from normal individuals and from healthy males with G6PD deficiency, Mediterranean type (glucose-6-phosphate dehydrogenase activity 2–7.5% of normal). After centrifugation, the buffy coat was removed, the cells washed twice with phosphate-buffered saline (135 mM NaCl/10 mM phosphate, pH 7.4), and resuspended in the same buffer.

Treatment of cells with reagents. Solutions of reagents were prepared as previously described [7]. Cell suspensions (8%) were mixed with diamide [3,7] and incubated at 37°C. Two types of experiments were carried out:(a) (−glucose) cells were incubated with diamide in the absence of glucose for 15–60 min. Following incubation, cells were centrifuged, washed and resuspended in buffer to 5–10% packed cell volume. Aliquots of diamide-treated cells were subsequently incubated in the presence of 10 mM glucose for 2 h, or with 5 mM dithiothreitol for 1 h, then washed. (b) (+glucose) cells were incubated with diamide in the presence of glucose, then washed.

Aliquots of the above samples were mixed rapidly with a monobromobimane solution (25 μl of a 60 mM monobromobimane/CH_3CN solution per ml cell suspension) [7]. Samples were then incubated for 15–30 min at 37°C, centrifuged, and washed with buffer. Other aliquots were carried through the procedure without monobromobimane, then used for the DTNB determination of SH groups [8].

Preparation and treatment of ghosts. Red cell ghosts were prepared from control and diamide-treated cells according to the procedure of Steck and Kant [9], and resuspended in phosphate-buffered saline. Aliquots were incubated at 37°C with or without various agents: 1–5 mM GSH or 1–5 mM NADPH or 1–5 mM dithiothreitol. Ghosts were then washed and labeled with monobromobimane.

Analysis of cell components. Thiol group analysis, absorbance and fluorescence measurements of monobromobimane-labeled cell components, electrophoresis of membrane proteins, photography of gels and densitometric measurements of protein profiles were carried out according to methods previously described [7].

Results

Quantitative aspects of cellular and membrane thiol oxidation and regeneration

G6PD-deficient and normal red cells responded in a similar way to diamide in the absence of glucose (Table I). GSH was completely oxidized and membrane SH groups were diminished to 50–70% of control values. Subsequent treatment of normal cells with glucose led to the recovery of membrane thiol groups, along with the regeneration of most of the GSH. In G6PD-deficient cells, neither membrane thiol groups, nor GSH, were

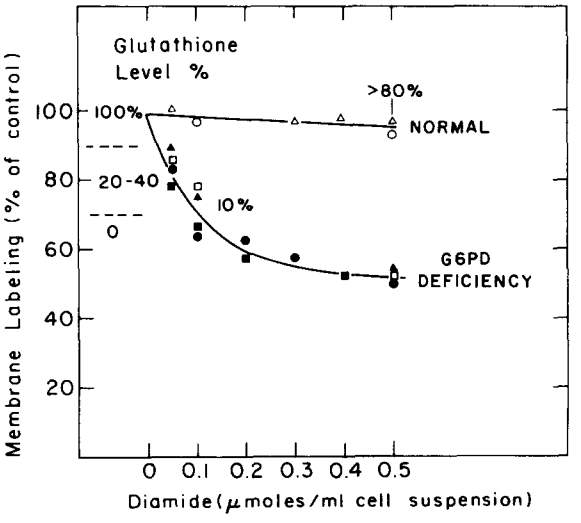

Fig. 1. Monobromobimane-labeled membranes of normal and G6PD-deficient red cells. Cells were incubated for 30 min with or without 0.05–0.5 μmol of diamide/ml 8% cell suspension with 10 mM glucose, then labeled with monobromobimane. Cell membranes were then isolated and labeling measured by absorbance and by fluorescence emission. Red cells from four individuals with glucose-6-phosphate dehydrogenase deficiency (●, □, ■, ▲) and from two normal individuals (○, △) were used.

TABLE I

REDUCTION OF MEMBRANE DISULFIDES IN NORMAL AND G6PD-DEFICIENT RED CELLS

Expt. groups (No.)	Cell type	Incubation		GSH (% of control) [a]	Membrane SH labeled with monobromobimane (% of control) [b]	
		first Diamide (μmol/ml cells)	second		Absorbance	Fluorescence
1	G6PD-deficient	6.3	–	0	59	54
		6.3	glucose	0	59	57
		6.3	DTT	93	108	108
	Normal	6.3	–	0	53	
		6.3	glucose	65	95	
		6.3	DTT	98	110	
2	G6PD-deficient	3.8	–	0	61	
		3.8	glucose	0	66	
		3.8	DTT	75	120	
	Normal	3.8	–	0	63	65
		3.8	glucose	73	100	97
3	G6PD-deficient	5	–	0	52	55
		5	glucose	0	49	
		5	DTT	95	113	116
	Normal	5	–	0	66	60
		5	glucose	87	102	98
		5	DTT	95	102	106

[a] The GSH content (DTNB analysis; Ellmann, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77), was 1.9–2.6 mM in normal red cells, and 1.4–1.8 mM in G6PD-deficient cells.

[b] The absorbance of the monobromobimane-labeled membrane proteins in the control samples was similar for the two types of cells. Membrane SH group content (DTNB analysis; loc. cit.) was 72–88 nmol/mg of membrane proteins in control samples of both types of cells.

regenerated with glucose, but were produced by dithiothreitol. Hemoglobin (Hb) was unaffected in both normal, and deficient cells: the total number of SH groups, measured by DTNB, was 6.0–6.4/Hb in control and in diamide-treated samples; 2.0 reactive SH groups/Hb were labeled by monobromobimane in both control and diamide-treated samples.

In the presence of glucose, diamide produced little change in GSH and membrane labeling in the normal cells (Fig. 1). In contrast, changes in GSH and in membrane labeling were found in G6PD-deficient cells similarly treated (Fig. 1). In samples in which 40% or more of the GSH (0.6–1.0 mM GSH) remained, monobromobimane labeling of membrane proteins was similar to that of the control. In samples with little or none of GSH remaining, a significant diminution of membrane labeling was found, reaching a minimum of about 50% of control values.

Distribution of membrane thiol groups and protein profiles

Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis.

Membrane proteins from diamide-treated G6PD-deficient cells exhibit alterations in both the distribution of the label and the stained protein bands (Fig. 2B vs. control shown in Fig. 2A): a significant diminution in labeling is observed in most bands; the profile of the Coomassie blue stained proteins shows a diminution in some of the bands (mainly 1, 2, 2.1, 4.2, and 4.5b), along with a large amount of high molecular weight proteins. The altered distribution of fluorescence and of the protein bands remain upon subsequent treatment of the cells with glucose (Fig. 2C), but treatment with dithiothreitol leads to the complete reversal of the patterns to those of the control samples (Fig. 2D).

Normal red cells treated with diamide in the absence of glucose exhibit alterations similar to those of G6PD-deficient cells. When such normal cells are subsequently treated with glucose, the membrane protein pattern returns to a large extent to that found without oxidation [7].

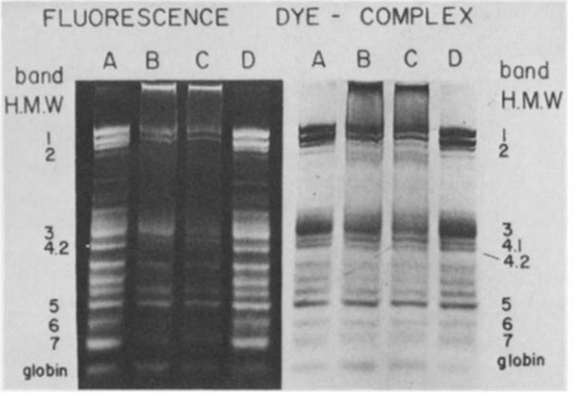

Fig. 2. SDS-polyacrylamide gel electrophoresis patterns of red cell membrane proteins isolated from G6PD-deficient red cells incubated with or without diamide (0.3 μmol/ml of 8% cell suspension) but without glucose for 30 min. Aliquots were then incubated with glucose or dithiothreitol, labeled with monobromobimane and processed. Left, fluorescence; right, Coomassie blue staining. A, control cells; B, diamide-treated cells; C, diamide-treated cells incubated with glucose; D, diamide-treated cells after subsequent treatment with dithiothreitol. H.M.W., high molecular weight proteins.

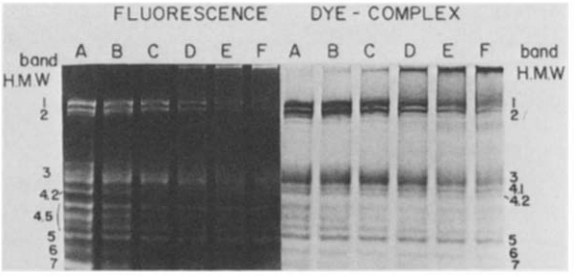

Fig. 3. SDS-polyacrylamide gel electrophoresis patterns of red cell membrane proteins isolated from G6PD-deficient red cells incubated with diamide and glucose for 30 min. Left, fluorescence; right, Coomassie blue staining. A, control cells; B–F, diamide-treated (μmol diamide/ml 8% cell suspension) cells: B, 0.05; C, 0.1; D, 0.2; E, 0.3; F, 0.5. GSH concentrations (mM) in the cell samples were as follows: A, 1.5; B, 0.5; C, 0.1; D, E and F, zero GSH. H.M.W., high molecular weight proteins.

Gel electrophoretic patterns of membrane proteins, derived from G6PD-deficient cells incubated with various amounts of diamide in the presence of glucose, are shown in Figs. 3 and 4. A slight diminution of labeling in bands 4.2 and 4.5, without changes in the protein dye-complex profile, is observed in samples in which 0.5 mM GSH is present (Fig. 3B vs. Fig. 3A (control)). With higher amounts of diamide (GSH down to less than 0.2 mM), a decrease in the fluorescence of additional bands (1, 2, 2.1, 6 and 7) is seen, along with traces of high molecular weight proteins (Fig. 3C and Fig. 4B). With still higher concentrations of diamide, significant diminution in labeling, decrease in some stained protein bands and increasing amounts of high molecular weight proteins (Figs. 3D, 3E, 3F and Fig. 4C) occur. Some proteins (e.g. band 5) are affected only slightly.

To identify the proteins forming the high molecular weight protein complexes, a two-dimensional gel electrophoresis of proteins isolated from diamide-treated G6PD-deficient cells was performed (Fig. 5). Disulfide bonds in the proteins electrophoresed in the first dimension, are cleaved before electrophoresis in the second dimension. Polypeptides separated by the first stage and unaffected by the reductive treatment appear in a diagonal line in the slab. Protein complexes, formed in the red cell membrane by interchain disulfide

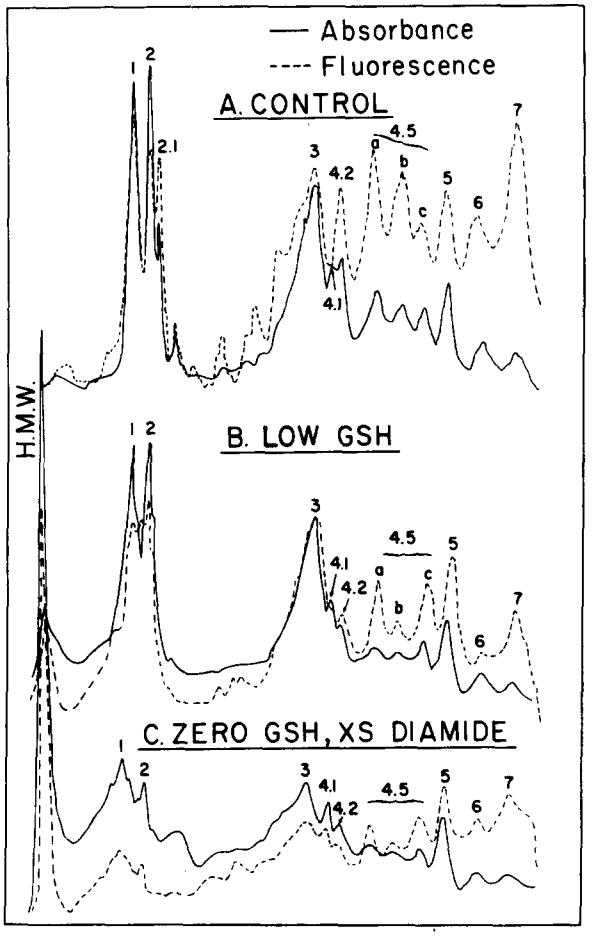

Fig. 4. Densitometric scans of protein distribution observed by either fluorescence or Coomassie blue staining on SDS-polyacrylamide gel electrophoresis. For experimental details, see Fig. 3. A, control cells; B, diamide-treated cells, 0.1 μmol/ml 8% cell suspension; C, diamide-treated cells, 0.5 μmol/ml 8% cell suspension. Fluorescence,- - - - -;protein dye complexes, ———; H.M.W, high molecular weight proteins.

formation through thiol oxidation, appear after the second electrophoresis as off-diagonal spots at levels corresponding to the membrane polypeptides from which they were formed. As shown in Fig. 5, the high molecular weight proteins, which remain at the top of the gel in a usual electrophoresis, consist mainly of spectrin, with some contribution from the proteins belonging to bands 2.1, 3, 4.2 and 4.5. In addition, a small amount of band 3 dimer protein was present in the diamide-treated cells. No involvement of band 5 protein, nor of hemoglobin, is found.

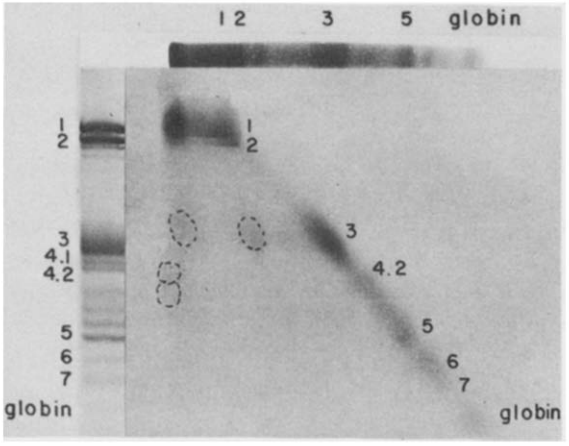

Fig. 5. Two-dimensional SDS-polyacrylamide electrophoresis of membrane proteins of G6PD-deficient red cells treated with diamide. The pattern for membrane proteins from a control is shown on the left side. The first-dimension gel of the diamide-treated cells is shown horizontally on the top of the slab gel. The agarose layer that contained 30 mM dithiothreitol, through which the proteins of the first electrophoresis pass before entering the slab gel is not shown.

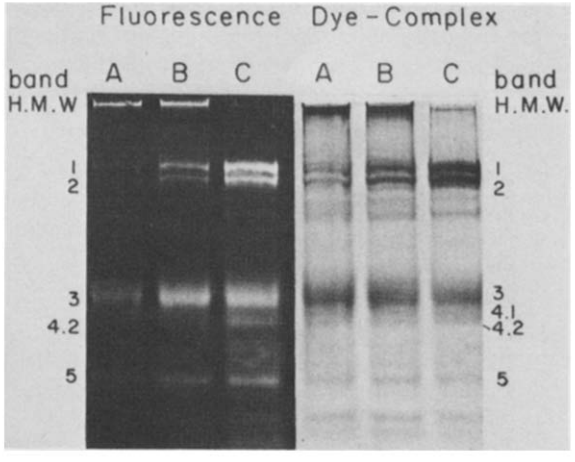

Fig. 6. SDS-polyacrylamide gel electrophoresis patterns of proteins from membranes isolated from cells treated with 1.0 μmol diamide/8% cell suspension and incubated with or without GSH or dithiothreitol. Ghost suspensions (containing 0.6 mg protein/ml) were incubated for 30 min with or without 5 mM GSH, or with 5 mM dithiothreitol, washed, and incubated with 0.6 mM monobromobimane for 15 min. Samples were then washed and processed for electrophoresis. A, membranes of diamide-treated cells incubated in buffer alone; B, membranes of diamide-treated cells incubated with GSH; C, membranes of diamide-treated cells incubated with dithiothreitol.

Patterns of membrane proteins from normal cells treated with diamide in the presence of glucose were similar to those of control cells (not shown).

Effects of GSH on the altered membrane proteins

Ghosts were prepared from diamide-treated cells, and incubated with or without NADPH, GSH, or dithiothreitol, then labeled with monobromobimane. In ghosts derived from cells treated with moderate amounts of diamide (0.3 μmol/ml 8% cell suspension), GSH led to an almost complete reversal of the diamide-induced alterations. No disulfide reduction is observed in samples treated with NADPH (not shown).

In ghosts prepared from cells treated with a very high concentration of diamide (Fig. 6A), GSH led to a partial reversal of the alterations (Fig. 6B). Treatment of these ghosts with dithiothreitol reverses completely all the diamide-induced alterations (Fig. 6C).

Discussion

In the present study, the behavior of G6PD-deficient red cell membrane proteins upon treatment with diamide has been investigated with the aid of the fluorescent labeling agent, monobromobimane. Differential sensitivity of membrane proteins to oxidation is demonstrated. Minor amounts of intrachain disulfides are noted in cells in which 60–70% of the GSH is oxidized; more oxidant leads to the formation of more intrachain as well as interchain disulfides. Some membrane protein thiols are unaffected even with large amounts of diamide, as shown by fluorescence labeling with monobromobimane, e.g. band 5 (actin).

In G6PD-deficient red cells, oxidized GSH is not regenerated [10]. In these red cells, disulfide formation in the membrane, either intrachain or interchain, is not prevented by the presence of glucose during diamide treatment, nor is reversal achieved by the subsequent addition of glucose. In the normal red cell, treatment with excess diamide in the absence of glucose leads to GSH oxidation and to membrane protein alterations similar to those found for the deficient cells. Most changes are reversed with subsequent glucose treatment [7]. If glucose is present during the diamide treatment, allowing the uninterrupted presence of some GSH, membrane changes are averted. The differences between the membranes of the normal and the deficient cell are thus related to the cellular level of GSH, and to the reductive capacity of the cell.

The high molecular weight proteins induced by diamide in G6PD-deficient cells are similar in composition to those found in vivo in G6PD-deficient individuals with chronic, active hemolysis [11,12]: in both cases, the high molecular weight proteins are composed of proteins of bands 1, 2, 2.1, and (some) 4.5, but not globin, and are completely dissociated by dithiothreitol. They differ from the high molecular weight proteins formed via γ-glutamyl peptide crosslinking (Ca^{2+}-activated transglutaminase [13,14]), as well as from the high molecular weight proteins found in splenectomized individuals [15].

Membrane damage, as evidenced by ultrastructural alterations and culminating in hemolysis, can occur without intracellular hemoglobin denaturation [3,16]. The oxidative membrane alterations reported here occur without changes in the hemoglobin thiol groups, nor is there any hemoglobin-membrane protein mixed disulfide formation. Thus, membrane damage in G6PD-deficient red cells can and does occur independently of intracellular alterations of the hemoglobin.

Loss of deformability and membrane changes are presumed to be responsible for the enhanced in vivo destruction of the G6PD-deficient red cells, with easier entrapment of the cells and enhanced recognition of the altered membranes by the sequestration system. Membrane high molecular weight aggregates may be responsible for the altered behavior of red cells [12,14,17]. Diminished red cell deformability is found in patients with chronic hemolysis [12]. High molecular weight protein formation and decreased deformability are found in diamide-treated dog red cells, the latter showing a decreased survival time when reinjected into the animal [18].

Intrachain disulfide formation may also result in altered membrane properties (e.g., changes in ion transport, complement binding, and agglutinability [19–22]). The contribution of intrachain disulfide formation to the enhanced destruction of the deficient cell remains to be studied.

The addition of GSH to ghosts prepared from

diamide-treated cells leads to a reversal of the membrane alterations (which is complete or partial, depending on the magnitude of the diamide-induced alterations), whereas the addition of NADPH does not. These results indicate that GSH is necessary for the reduction of membrane disulfides. The case of rat red cell is of interest in this regard: diamide treatment of the rat cell leads to the formation of GS-hemoglobin mixed disulfides and not GSSG. GSH cannot be regenerated, in spite of the presence of glucose-6-phosphate dehydrogenase and normal amounts of NADPH, since the mixed disulfides are not reduced in the absence of GSH [23]. Disulfides formed in the diamide-treated rat cell membrane are not reduced upon subsequent treatment with glucose under conditions in which NADPH, but not GSH, is present (unpublished results).

Thiol-protein disulfide interchange reactions are not well characterized [24,25], with questions raised about the role of GSH as a major reductant in these reactions [25]. GSH is, however, a substrate in some oxidation-reduction reactions, such as those involving insulin, cholera toxin [25,26], and glutaredoxin, a glutathione disulfide transhydrogenase [27]. Some mixed GS-disulfides cannot be reduced directly by glutathione reductase and NADPH, but are reduced by GSH with catalysis by thiol-disulfide oxidoreductase [23,24,28–30]. We have shown that GSH reduces some membrane disulfides in the absence of cytoplasmic enzymes or factors. More complete, physiological reduction may require the enzymes and factors cited.

We have presented here the first experimental evidence for GSH serving as a reductant of membrane protein disulfides, as well as confirmation of the role of GSH in averting membrane thiol oxidation. These findings are significant not only with respect to red blood cells, but also for other processes in which membrane thiol-disulfide exchange reactions are important, including opiate receptor function [31], the clustering of enkephalin receptors [32], insulin binding [33], and in the transition to and from the dormant state of bacteria [34].

Acknowledgement

This study was supported by grants from the Chief Scientist's office, Ministry of Health, Israel, and from the Commission for Basic Research, Israel Academy of Science.

References

1 Piomelli, S., (1974) in Hematology of Infancy and Childhood (Nathan, D.G. and Oski, F.A., eds.), pp. 346–377, W.B. Saunders, Philadelphia
2 Luzzato, L. and Testa, U. (1978) in Current Topics in Hematology. (Piomelli, S. and Yachnin, S., eds.), Vol. 1, pp. 1–70, Alan R. Liss, New York
3 Kosower, N.S. and Kosower, E.M. (1978) Int. Rev. Cytol. 54, 109–160
4 Kosower, E.M., Pazhenchevsky, B. and Hershkowitz, E. (1978) J. Am. Chem. Soc. 100, 6516–6518
5 Kosower, N.S., Kosower, E.M., Newton, G.L. and Ranney, H.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3382–3386
6 Kosower, N.S., Newton, G.L., Kosower, E.M. and Ranney, H.M. (1980) Biochim. Biophys. Acta 622, 201–209
7 Kosower, N.S., Kosower, E.M., Zipser, Y., Faltin, Z. and Shomrat, R. (1981) Biochim. Biophys. Acta 640, 748–759
8 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77
9 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31, 172–180
10 Kosower, N.S., Vanderhoff, G.A. and London, I.M. (1967) Blood 29, 313–319
11 Allen, D.W., Johnson, G.J., Cadman, S. and Kaplan, M.E. (1978) J. Lab. Clin. Med. 91, 321–327
12 Johnson, G.J., Allen, D.W., Cadman, S., Fairbanks, V.F., White, J.G., Lampkin, B.C. and Kaplan, M.E. (1979) N. Engl. J. Med. 301, 522–527
13 Lorand, L., Weissmann, L.B., Epel, D.L. and Bruner-Lorand, J. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4479–4481
14 Lorand, L., Siefring, G.E., Jr. and Lowe-Krentz, L. (1978) J. Supramol. Struct. 9, 427–440
15 Lux, S.E. and John, K.M. (1977) Blood 50, 625–641
16 Kosower, N.S., Song, K.R. and Kosower, E.M. (1969) Biochim. Biophys. Acta 192, 23–28
17 Fischer, T.M., Haest, C.W.M., Stohr, M., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 510, 270–282
18 Johnson, G.J., Allen, D.W., Flynn, T.P., Finkel, B. and White, J.G. (1980) J. Clin. Invest. 66, 955–961
19 Pillion, D.J., Moree, L., Rocha, H., Pashley, D.H., Mendicino, J. and Leibach, F.H. (1977) Mol. Cell. Biochem. 18, 109–115
20 Scutari, G., Ballestrin, G. and Covaz, A.L. (1980) J. Supramol. Struct. 14, 1–11
21 Kosower, N.S., Marikowsky, Y., Wertheim, B. and Danon, D. (1971) J. Lab. Clin. Med. 78, 533–545
22 Sheetz, M.P., (1980) in Genetic Disorders of Glutathione and Sulfur Amino-acid Metabolism (Schulman, J.D., moderator), Ann. Intern. Med. 93, 341–343
23 Kosower, N.S., Kosower, E.M. and Koppel, R.L. (1977) Eur. J. Biochem. 77, 529–534
24 Flohé, L. and Günzler, W.A. (1976) in Glutathione: Metabolism and Function (Arias, I.M. and Jakoby, W.B.,

eds.), Kroc Foun. Ser. Vol. 6, pp. 17–34, Raven Press, New York
25 Freedman, R.B. (1979) FEBS Lett. 97, 201–210
26 Moss, J., Stanley, S.J., Morin, J.E. and Dixon, J.E. (1980) J. Biol. Chem. 255, 11085–11087
27 Luthman, M., Eriksson, S., Holmgren, A. and Thelander, L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2158–2162
28 Mannervik, B. and Eriksson, S.A. (1974) in Glutathione (Flohé, L., Benöhr, H.C., Sies, H., Waller, H.D. and Wendel, A., eds.), pp. 120–132, Georg Thieme, Stuttgart
29 Birchmeier, W., Tuchschmid, P.E. and Winterhalter, K.H. (1973) Biochemistry 12, 3667–3672
30 Mannervik, B. and Axelsson, K. (1980) Biochem. J. 190, 125–130
31 Marzullo, G. and Hine, B. (1980) Science 208, 1171–1173
32 Hazum, E., Chang, K.J. and Cuatrecasas, P. (1979) Nature 282, 626–628
33 Jarett, L., Schweitzer, J.B. and Smith, R.M. (1980) Science 210, 1127–1128
34 Fahey, R.C. (1977) in Protein Crosslinking (Friedman, M., ed.), Part A, pp. 1–30, Plenum, New York